

**INNERVATION AND TROPHIC
SUPPORT OF MUSCLE CELLS IN TISSUE CULTURE**

**PROEFSCHRIFT TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
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**Dit proefschrift kwam tot stand in het Medisch Biologisch Laboratorium TNO onder
supervisie van Dr. W. F. Stevens en Dr. E. Meeter.**

STELLINGEN

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1. De conclusie, dat het afwijkend gedrag van gekweekte myoblasten van patienten met Duchenne dystrofie myogeen bepaald is, gaat uit van de onbewezen veronderstelling, dat de ontwikkelingsmogelijkheden van de satellietcellen niet neuronaal beïnvloed worden.

Thompson et al.: Nature, 268, 241-243, 1977.

2. De tetrodotoxine resistentie van gedeeltelijk gedenerveerde spieren, zoals die door Cangiano en Lutzemberger is waargenomen, zou even goed het gevolg kunnen zijn van een blokkade van de productie van een trofische factor als van de aanwezigheid van een degeneratieve factor.

Cangiano en Lutzemberger: Science, 196, 542-545, 1977.

3. De aanwezigheid van trofische effecten van spinale ganglia op spiercellen in weefselkweek, zoals aangetoond door Tolar, en de afwezigheid hiervan in onze culturen is een gevolg van verschil in kweekomstandigheden.

Tolar: Physiol. Bohemoslov., 23, 182, 1974.

4. De aanwezigheid van een verhoogde extra-junctionele gevoeligheid voor acetylcholine is niet noodzakelijk het gevolg van de chronische vermindering van efferente pulsactiviteit.

Howe et al.: Exp. Neurol., 56, 42-51, 1977.

5. Anderson en Cohen menen, dat voor de vorming van neuromusculaire verbindingen een directe interactie van het neuron met acetylcholine receptoren van de spiervezel essentieel is. Deze voorstelling van zaken is in zoverre onjuist, dat de blokkade van de receptorfunctie in dit verband geen consequenties heeft.

Anderson en Cohen: J. Physiol. (Lond.) 268, 757-773, 1977

6. Omdat "frequentie-vensters" verwacht kunnen worden kunnen veiligheidsnormen voor elektromagnetische golven pas worden vastgesteld, wanneer de biologische, niet-thermische, effecten van elektromagnetische golven over een uitgebreid frequentiegebied zijn onderzocht.
7. Bij de studie van de veranderingen in de biochemische parameters tijdens de celdifferentiatie wordt onvoldoende aandacht geschonken aan tevens optredende veranderingen in de samenstelling van de membraan van de cel.
8. Bij onderzoek ter bepaling van de toxiciteit van nieuwe chemische stoffen moeten ook technieken gebruikt worden, die invloeden op het zenuwstelsel kunnen aantonen.
9. De verontrusting over de neutronenbom wordt grotendeels veroorzaakt door het misverstand, dat er ooit zoiets als een atoomdrempel bestaan zou hebben.
10. Vergoeding en belastingaftrek van reiskosten maken het volbouwen van het groene hart van de Randstad aantrekkelijk.

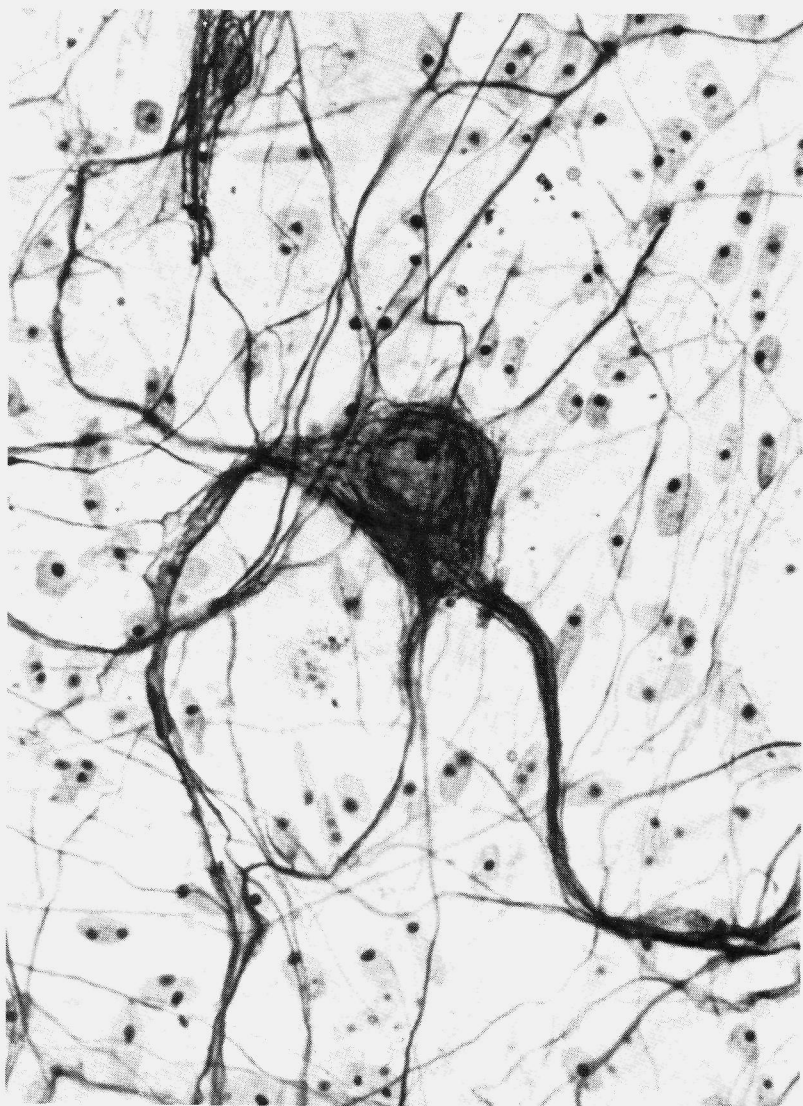
11. De door een zuigfles gevoede fabriek zal ondanks de in dit proefschrift beschreven experimenten een hersenschim blijven.

Vroman, Brieven uit Brooklyn, 1975.

J. Hooisma

Utrecht, 11 oktober 1977.

Innervation and trophic support of
muscle cells in tissue culture



Met het schrijven van een proefschrift wordt een periode van vorming afgesloten. Daarom wil ik allen, die bijgedragen hebben aan mijn opvoeding en opleiding, en alle mensen en instellingen, die het mogelijk gemaakt hebben om tot deze afsluiting te komen, bedanken door aan hen dit proefschrift op te dragen.

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INTRODUCTION

Motoneurons not only provide the nerve impulses necessary for electrical activity and contraction of skeletal muscles, but they also have long-term influences on the morphological and functional properties of the skeletal muscle fibres. The presence of such "trophic" influences becomes manifest when the axon of a motoneuron that innervates a skeletal muscle fibre is injured accidentally in humans or is severed in denervation studies in laboratory animals. It has been observed that damage to the motor nerve not only produces paralysis but also has morphological and functional consequences for the denervated muscles. Studies of the trophic support of muscle fibres by their nerves demonstrated the action of several processes that are involved in the maintenance of normal functional muscular properties. The relative importance of these processes for certain properties is still fiercely debated (Gutmann, 1976, a, b; Lömo, 1976; Gordon et al., 1976).

Several diseases that result in atrophy of the skeletal muscles are believed to result from the malfunctioning of the innervating motoneuron. Failure of the same processes that are impaired by denervation might be responsible for this malfunctioning. Knowledge of the processes that influence persistently the neuromuscular relationship is therefore not only of scientific interest but may also have great consequences for the treatment of some neurological diseases.

The trophic influences of motoneurons on skeletal muscles have so far mainly been studied by surgical or pharmacological experiments in developing and in adult animals. Although the final proof that a certain process or mechanism is involved in trophic interactions can only be obtained in

experiments in vivo; it will be clear that such experiments have a number of serious limitations. In surgical experiments other tissues may be damaged, phagocytic reactions occur and unwanted regrowth of neurons and tissues cannot always be prevented. Moreover, the paralysis may in a more general way disturb the normal physiology and behaviour of the animal. In pharmacological experiments unwanted side effects of the applied drugs may complicate the interpretation of the results. Colchicine, for instance, applied in cuffs around the motor nerve in order to block axonal flow and to study its importance for trophic interaction, has also direct effects on the membrane properties of muscle fibres, similar to those caused by denervation. Usually such experiments can only be done once in the same animal and if gradual changes are to be studied different animals are required.

The use of nerve and muscle cells in tissue culture has advantages for the study of trophic interactions between nerve and muscle cells. With this technique it is possible to cultivate neurons and muscle cells of embryos and newborn animals. Under tissue culture conditions immature nerve cells can develop to differentiated neurons with the morphological and electrophysiological properties of the mature neuron. Muscle cells fuse and form multinucleated cells which then develop cross striations and the capacity to contract (Nelson, 1975). Neurons and muscle cells can be kept in culture for many months in rather well defined media. Because cultures usually consist of monolayers of cells arranged in a one cell-layer thick sheath, observation of individual cells with phase-contrast or interference microscopy is possible without disturbance of growth or development of the cells. The morphology of one cell or a group of cells can thus be studied in culture at different stages of maturation. Moreover, electrophysiological studies under sterile conditions will in the near future enable the study of the differentiation of functional properties of individual

cells in nerve and muscle cell cultures.

Around 1970 it was demonstrated that spinal cord neurons cultured within explants do not only survive but also form functional interneuronal connections and functional contacts with fragments of muscle explanted in the same culture dish (Crain et al., 1970). Peterson and Crain (1970) and Robbins and Yonezawa (1971) observed that innervation affected the morphological development of the innervated muscle fibres in a favourable way. It thus appeared that trophic influences are exerted in tissue culture. It was at that time that the potencies of these new techniques were understood by a group of scientists headed by Dr. E. Meeter and Dr. W. F. Stevens at the Medical Biological Laboratory TNO in Rijswijk. A program in neurobiology was started taking advantage of the fact that within the laboratory a longstanding expertise was present in the study of the physiology and biochemistry of the neuromuscular junction as well as a thorough experience in tissue culture techniques. Because only a few research groups at that time had experience in the investigation of the electrical maturation of cultured neurons or in the study of the formation of functional neuromuscular junctions in mixed cultures with dissociated muscle cells the first aim of the new group was to investigate the conditions required for optimal survival and maturation of nerve and muscle cells in tissue culture, and to set up the techniques for intracellular electrophysiological measurements in cultured cells. A multidisciplinary team was formed of a physiologist (E. Meeter M. D.), a biochemist (W. F. Stevens Ph. D.), a physicist (D. W. Slaaf) and a biologist (the author). The formation of this team was made possible by the financial support from the Foundation for Medical Research FUNGO of the Netherlands Organization for the Advancement of Pure Research (ZWO) and from the Foundation Promeso and the Foundation "De Drie Lichten", who recognized the importance of the development and the use of these new techniques in The Netherlands.

At that time it seemed reasonable in a study of trophic relationships to try to prepare mixed cultures that contained mainly motoneurons from embryonal spinal cord and muscle fibres formed by fusion of embryonal myoblasts. The purpose of the first studies was therefore to investigate whether different groups of neurons and especially the ventral horn motoneurons could be recognized on morphological grounds in cultures of neurons dissociated from spinal cord material (Chapter 2). When this appeared to be impossible due to the constant morphological changes even in neurons cultured for several weeks, an alternative preparation was chosen that consisted of the co-culture of whole spinal cord explants and muscle cells. Sobkowitz and co-workers had demonstrated that the cytoarchitecture of neurons in such explants was stable during long periods in culture (Guillery et al., 1968; Sobkowitz et al., 1968) and large cells, probably motoneurons, could be recognized in the ventral horn area of the explant.

The innervation of muscle fibres in the area around the explants was investigated by intracellular electrophysiological techniques (Chapter 3). In a parallel study the innervation of skeletal muscle cells by cholinergic neurons in the ciliary ganglion of chick and rabbit embryos in culture was investigated by D. W. Slaaf (1977).

It appeared that under appropriate culture conditions trophic effects of spinal cord explants could be demonstrated. Because in the literature only qualitative descriptions of the trophic effect have been reported, it was necessary to find a quantitative method to evaluate the morphological development of muscle fibres in tissue culture. With the adopted method the effect of chick spinal cord explants on chick muscle cells was investigated. The results led to questions about trophic effects of neuronal tissues taken either from the same species or from a different species as that from which the muscle cells were derived. Moreover, the question was raised

whether innervation was needed for trophic action. Finally, it was felt necessary to investigate whether activity induced by spinal cord neurons in the muscle fibres was essential for their trophic influence on the morphology of the muscle fibres (Chapter 4).

Soon after the beginning of these studies Oh and colleagues demonstrated qualitatively that extracts of embryos (Oh et al. , 1972; Oh, 1975) and of sciatic nerves of adult animals (Oh, 1976) exert trophic influences on muscle fibres in culture. These were exciting findings because they demonstrated the existence of (a)humoral trophic factor(s) and the identification of such a factor became feasible. In the present studies the method used to quantify the morphological development of muscle fibres was employed to study the effect of extracts obtained from tissues of embryos and of adult animals. In addition to the effects on the morphology the effects of explants and extracts on the electrophysiological properties of muscle were studied (Chapter 5).

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CHAPTER I

NEUROTROPHIC INFLUENCES AFFECTING SKELETAL MUSCLE FIBRES IN VIVO AND IN TISSUE CULTURE

Neurotrophic relations between motoneurons and skeletal muscles

Introduction

In the middle of the previous century Brown-Séquard (1849), among others, drew attention to the fact that in man and animals damage to the motor nerve not only produces paralysis but also has morphological consequences for the denervated muscles. In those days the resulting atrophy was thought to be caused by the disuse of the muscle in the same way as hyperactivity led to hypertrophy. This idea was strengthened by the observation that tenotomy, which strongly reduces the mechanical activity in a muscle but leaves the innervation unimpaired, also produces a striking atrophy (see Eccles 1944). Histological observations on denervated muscle, however, suggested to Tower (1935) that the resulting atrophy was not merely caused by disuse but might partly be due to loss of some other influence of the nerve on the muscle. She noticed that the initial proliferation of nuclei following denervation did not occur after immobilization of the muscles by isolation of the innervating segment of the spinal cord.

Quite apart from the atrophy caused by denervation Philipeaux and Vulpian (1863) described abnormal phenomena in denervated muscles which had nothing to do with the ensuing paralysis. In the tongue of a dog which

had its hypoglossal nerve cut, they noticed slow contractions when the chorda tympani was stimulated. Now we understand that these contractions were caused by the acetylcholine (ACh) released by the vasomotor endings of chorda tympani axons that run to the tongue. Normally these small amounts of ACh do not affect the tongue muscle. It is very likely that they were the first to observe the hypersensitivity to acetylcholine, which develops in a denervated muscle; a phenomenon that although already essentially understood by Ginetzinsky and Shamarina (1942) in the Soviet-Union, had to wait until the studies of Axelsson and Thesleff (1959) and Miledi (1959, 1960) before it became generally accepted.

At present ample evidence is available showing that the motoneuron not only provides the nerve impulses necessary for impulse activity but also has another influence on the muscle cell which has important consequences for its maintenance and long-term functional properties. About this so-called neurotrophic effect of a motoneuron on its target muscle, much work has been done since 1960. It is still not clear, however, in which way this influence is brought about.

Since more than one neuronal mechanism might be involved in this neurotrophic action it seems wise to define, with Drachman (1974a), "neurotrophic" as "any long-term relationship in which nerve cells and target cells interact so as to influence the structure and function of either member of the pair". The present study will only deal with the "orthodromic" neurotrophic relationship, i. e. the effect of the neuron on the muscle cell.

If the adult muscle cell for its maintenance depends on neurotrophic influences, it might be expected that such influences also play an important part in the development of the embryo and during early life. This contention is borne out in the present study which deals mainly with the effects of embryonic neuronal material and embryonic muscle cells.

Neuronal influences on developing muscles

Harrison (1904) observed in tadpoles that after extirpation of the neural tube before muscle formation had started, muscle differentiation followed a normal pattern. Only the diameter of the non-innervated fibres was smaller than those of the control fibres. These findings were confirmed by many other experiments in amphibia and it thus appears that muscle differentiation in amphibia is independent of neuronal influences. Limb grafts of chick embryos explanted into the chorio-allantois membrane or in the coelomic cavity differentiated normally until 10-12 days after explantation (Hunt, 1932). However, only grafts that before that time became innervated by spinal cord neurons survived for longer periods, while those that were not innervated then started to disintegrate. It thus appeared that in later stages of differentiation of avian muscles, innervation is indispensable. This also seemed to be the case in mammals. In the rat extrafusal fibres can differentiate from the myotube stage to the myofibre stage without innervation, but when denervated in the myotube stage the process of maturation is slowed down as compared with normal muscle and in some fibres differentiation is totally arrested (Zelená, 1962). Engel and Karpati (1968) observed that type I fibres in the rat are relatively independent of innervation but the development of many type II fibres was arrested by denervation at the myotube stage. Both papers report that if a muscle is denervated before intrafusal fibres have formed such fibres will not develop. This phenomenon thus demonstrates the trophic effect of sensory nerves. The growth of muscles and the increase in diameter of fibres in muscles denervated in early life lag very much behind that of normal innervated fibres (Zelená and Hnák, 1957).

M o r p h o l o g i c a l c o n s e q u e n c e s o f d e n e r v a t i o n

Evidence for neurotrophic relations between motoneurons and muscles has traditionally been sought by studying the effect of interruption of the peripheral nerves which run from the spinal cord to the muscle. It is remarkable that although the oldest known long-term changes in muscle caused by denervation are morphological, little detailed knowledge is available about these morphological changes. The fact that in fewer years more became known about electrophysiological and biochemical alterations due to denervation is certainly caused by the circumstance that such phenomena are more easily quantified than morphological changes. Most of what is known about the subject has already been reviewed by Gutmann and Zelená in 1962. Tower (1935) distinguished three subsequent stages in the denervation atrophy of skeletal muscle: i. atrophy in the stricter sense, i. e. a reduction in diameter of the muscle fibres resulting in a loss of total volume, ii. degeneration, that is disintegration of fibres, and iii. dedifferentiation, i. e. replacement of muscle fibres by fat cells and connective tissue. In a very early stage, before the atrophy starts, small changes of muscle structure can be observed. Already two days after denervation subsarcolemmal nuclei change in shape and size. Nuclei of innervated rat fibres are elongated and become rounded after denervation and the karyoplasm becomes very light. In some species the number of nucleoli changes also. Whether there is an early proliferation of nuclei is still debated (Zelená, 1962). Later changes in number of nuclei have been ascribed to either mitosis or changes in absolute volume of the muscle fibres. There is, however, a relative increase in DNA content.

After atrophy has started, there is an increased proliferation and a fast turnover of nuclei. The degree of atrophy of denervated muscles can be studied by measurement of fibre diameters in cross sections. The weight of a muscle usually gives little information because loss of fibres is often compensated by proliferation of fat cells and to a lesser extent by replacement by connective tissue. The decrease in diameter is caused by loss of sarcoplasm and myofibrils. After a long period of denervation the myofibrils in many fibres disappear completely and only a narrow sarcolemmal sack remains which contains a chain of centrally placed nuclei. Cross striation can usually be observed as long as a fibre is not reduced to this state.

Loss of fibres starts slowly but it goes on even at the time when loss of weight and decrease of fibre diameter have stopped. Fibres are not lost in all muscles after denervation. Degeneration in the rat usually starts with vacuolization but it affects only a few fibres except in the soleus muscle where after 1 month whole groups of fibres degenerate. At later stages after denervation hyalin degeneration predominates; a short segment of fibre degenerates and cross striation is lost. The fibre endings retract and are infiltrated by other cells. Even after a long period of denervation there is no tendency to further progression of the degeneration.

Ultrastructural studies of early changes caused by denervation of frog and rat muscles were done by Pellegrino and Frazini (1963) and by Muscatello et al. (1965). They observed a relative increase in sarcotubular elements. In fast and slow muscles of the rat Engel and Stonnington (1974) studied ultrastructural effects of denervation for up to 84 days. Myofibrillar atrophy was proportional to reduction in fibre size. An absolute increase in mitochondrial mass and sarcotubular surface was observed. In a later stage the absolute mass of mitochondria decreased.

Other changes induced by denervation

With modern techniques it has become possible to distinguish a number of changes in electrical and ultrastructural properties after denervation which were hitherto unknown. In table 1 some denervation phenomena are listed. Comprehensive monographs and reviews have appeared in recent years which treat various aspects of the consequences of denervation (Gutmann, 1962; Gutmann and Hník, 1963; Guth, 1968; Drachman, 1974b; Gutmann, 1976 a and b). A general review of neurotrophic "inductive" relations of neurons was written by Harris (1975). In a review by Gordon et al. (1976) the chemosensitive properties of skeletal muscles and their relations to innervation are discussed. Lömo (1976) discussed the role of activity in the regulation of muscle properties.

Particularly during the embryonic development but also in later life the trophic state of muscles is by no means exclusively regulated by innervation. Nutrition, androgenic hormones and intrinsic genetic make up also play an important role. During the early development the fusion of myoblasts in the formation of myotubes and the appearance of ACh-receptors in the cell membrane of the not yet innervated myotube are independent of the presence of nervous material (Gutmann, 1976a). It could be that, although the presence of nerve elements is required for further development of the muscle cells, for some steps only some sort of trigger action is needed from the nerves after which their presence is no longer essential. It is not precisely known which part of the development is induced by activity brought about by the motor innervation but this is certainly a very important factor. Much less is known about the long-term effects of substances released by the axon terminal. In the following paragraph some experimental

Table 1. Properties of muscles affected by denervation

decrease of resting potential	Albuquerque et al., 1971; Redfern and Thesleff, 1971.
TTX resistant action potentials	Redfern and Thesleff, 1971.
fibrillation potentials	Salafsky et al., 1968.
increased specific membrane resistance	Albuquerque and Thesleff, 1968.
increased specific membrane capacity	Westgaard, 1975.
decreased K^+ permeability	Klaus et al., 1960; Kernan and McCarthy, 1972.
action potential becomes slower	Redfern and Thesleff, 1971.
hyperpolarizing afterpotential	Thesleff, 1974.
extra-junctional ACh hyper- sensitivity	Axelsson and Thesleff, 1959; Miledi, 1960.
decreased ACh-esterase activity	Guth et al., 1964; Crone and Freeman, 1972.
altered pharmacological properties	Beránek and Vyskočil, 1967.
disappearance of junction	Miledi and Slater, 1968.
receptivity to innervation	Elsberg, 1917.
increase of RNA	Manchester and Harris, 1968; Muchnik and Kotsias, 1975.

Table 1 continued.

faster breakdown of all proteins	see: Gutmann,1962.
DNA synthesis increased	
transient hypertrophy	Stewart and Martin,1956.
atrophy	Solandt and Magladery,1940.
reduction of fibre number	
smaller myofibrils	see: Gutmann,1962.
increased number of nuclei	

approaches are discussed that have been employed in order to give answers to these questions.

In 1970 Miledi and Slater reported that after severance of the nerve, degeneration of the end plate started later if the peripheral nerve stump was longer. Other authors found that the time of onset of the decline of the membrane resting potential and the appearance of fibrillation potentials were also delayed by the presence of a long nerve stump (Albuquerque et al., 1971; Harris and Thesleff, 1972; Card, 1977). The appearance of extra-junctional ACh-sensitivity and the loss of weight were also found to be delayed (Luco and Eyzaguirre, 1955; Gutmann, 1970). These observations were interpreted as evidence for a trophic factor being present in the motor nerve, which was longer available after denervation if a longer nerve stump was left to the muscle. Lömo (1976), however, pointed out that the phagocytic reaction in the muscle that occurs after denervation, came later and severely criticized this contention; he found that the phagocytic reaction in itself can cause hypersensitivity of the muscle fibres to ACh, even when induced in innervated muscles. He therefore expects this denervation phenomenon simply to be the result of this phagocytic reaction and has no need for a separate humoral trophic factor normally released by the nerve terminals.

Since more became known of the blocking effects of the vinca alkaloids colchicine and vinblastine on the fast axonal transport from neuron to target cell (Dahlström, 1969; Kreutzberg, 1969), these substances have been used as tools in degeneration studies. A number of authors have applied these substances locally to motor nerves (Hoffman and Thesleff, 1972; Albuquerque et al., 1972; Cangiano, 1973). They found that the vinca alkaloids produce denervation phenomena in the muscles such as decrease in resting potential, the development of TTX-resistant action potentials and the onset of atrophy, while the muscles involved were not

paralysed and the conduction of action potentials in the treated motor nerves was not blocked. It thus appeared possible to "dissect" the trophic effect of the nerve from its activity inducing effect. However, this seemingly clearcut proof of a separate trophic factor did not go unchallenged.

Cangiano and Fried (1974) confirming other findings about the development of extra-junctional ACh-sensitivity in muscle fibres which had their motor nerve treated with colchicine, found that even if there was no block of axonal flow, the extra-junctional sensitivity still developed. They thought that the latter effect might be caused by a systemic effect of the locally applied colchicine. Moreover, Lömo (1974) reported that denervated muscles in which the appearance of extra-junctional ACh-sensitivity had been prevented (or delayed) by direct electrical stimulation, developed this type of sensitivity after colchicine injection into the muscle.

Since the nerve endings in the neuromuscular junction release ACh during rest as well as during activity this transmitter itself could be the humoral factor responsible for the neurotrophic effect on the muscle. If that were so, the incomplete denervation phenomena after paralysis of a muscle by spinal isolation (Tower, 1935) could readily be understood. It is known that the toxin of *Clostridium botulinum* produces a prolonged neuromuscular paralysis by blocking the ACh release by the nerve terminals (Brooks, 1956; see also Spitzer, 1972). Harris and Miledi (1971) reported that application of botulinum toxin to muscles, although it did not completely block all resting ACh release in all muscles, changed the properties of the muscle fibres as if they had been denervated. Duchen found that local treatment of a muscle with botulinum toxin caused its motor nerve to sprout as if it had lost its target (Duchen and Strich, 1968), whereas Fex et al. (1966) observed that under such conditions the muscle accepts innervation by a foreign nerve. Clearly, after application of the toxin the nerve and the muscle behave as if they are no longer aware of each others presence.

These findings could mean that the block of ACh release by the toxin takes away the essential communication between nerve and muscle and so causes the denervation phenomena. Great care should, however, be applied in interpreting these findings as showing that ACh is the trophic substance because lack of ACh always means inactivity which in itself is known to be an important factor. Moreover, paralysis by botulinum toxin does not cause the junctional acetylcholinesterase to disappear as denervation does. Finally, it cannot be ruled out at present that the toxin might not only block the release of ACh but at the same time that of some other important factor.

In order to test the ACh-hypothesis, Drachman studied the effects of other drugs which interfere with the function of ACh at the end plate (see review Drachman, 1974b). He found that hemicholinium-3, which blocks ACh synthesis by stopping the uptake of the necessary choline in the nerve terminals, causes atrophy, degeneration and fatty replacement of the muscle fibres. Similar results, however, were obtained with prolonged curarization of muscles, a procedure which does not interfere with ACh release but paralyzes the muscle by a blockade of the ACh-receptors. Hofmann and Thesleff (1972) employed β -bungarotoxin to block ACh release by the nerve terminals and also obtained denervation phenomena. It therefore appears that whether or not ACh is released is not the primary factor in the development of denervation phenomena. These results instead appear to show that what is important is the inactivity produced by the drugs.

Among others, Lömo and Westgaard (1975, 1976) showed that direct electrical stimulation of denervated muscles brings some of their properties a good way back to normal or prevents the appearance of some denervation phenomena. Recently, Lömo (1976) has given a detailed account of experiments carried out by many authors with respect to the effects of activity as such and of the details of the pattern of activity on the morphological

electrical and biochemical properties of skeletal muscle. These findings all show an enormous effect of activity in the innervated as well as in the denervated muscle. It therefore seems impossible to come to definite conclusions about the importance of hypothetic trophic factors with the aid of experiments in vivo in which the flow of nerve impulses, ACh release in activity and during rest, and mechanical activity cannot be separated with certainty.

Neurotrophic action in tissue culture

In tissue culture it is possible to bring nerve cells and muscle cells together in all sorts of combinations and in the presence of known concentrations of drugs during well defined periods of time; while the morphological development of the cells can be followed through the microscope and electrophysiological measurements can be carried out at any time. In this way it has been possible to investigate the effects of the presence of various neurons or that of tissue extracts on the development of the - usually embryonic - muscle cells, either in the presence or absence of drugs. The effects of these factors on the electrical properties and on the distribution of ACh-receptors has been studied as well. An excellent review of most of the literature about these studies up to 1973 was published by Crain and Peterson in 1974.

Studies on the development of the morphology

Fetal muscle cells

Under suitable experimental conditions isolated vertebrate skeletal muscle fibres can develop in tissue culture from embryonic myoblasts and survive for several weeks (Shimada et al., 1967; Yaffe, 1968; Murray, 1972). One of the prerequisites for such a development is the addition of certain sera and extracts from adult or embryonic material to the artificial - i. e. synthetic - culture media like Eagle's minimum essential medium. Peterson and Crain (1970) using culture medium and substrate that according to them was optimal for maturation of spinal cord and dorsal root ganglia (DRG), observed atrophy in explants of fetal rodent muscle after three weeks in culture. In their cultures they observed the formation of myotubes and the initial appearance of cross striation. In order, however, to keep the myotubes healthy for an extended period innervation by spinal cord neurons appeared to be required. Innervated muscle fibres could be kept in culture for months. This trophic effect of innervation was very pronounced when spinal cord explants with adhering DRG were added to muscle explants that had been cultured for several weeks and that showed signs of severe atrophy. Within four days after addition of the cord explants reversal of the atrophy was observed. This influence was also exerted in a hetero-specific system consisting of rat atrophied muscle together with mouse spinal cord explants. These experiments showed that trophic influences of spinal cord neurons can be exerted in tissue culture. Peterson and Crain could make their model even more sensitive for the detection of trophic influences by selectively depressing muscle cell differentiation with cortisone, this quickened the degeneration of noninnervated muscle fibres.

Robbins and Yonezawa (1971) who also used a culture medium that had been made optimal for the development of neurons found no cross striation in myotubes obtained from fragments of fetal rat hind limb muscle. Well maintained cross striation developed in the same medium when rat spinal cord fragments were added to these muscle cultures. They found evidence that the innervation and not merely the presence of the explant was the significant factor. Their criteria for innervation included a twitch response to indirect stimulation, a 4-14-fold increase in chronaxie with curarization and the presence of epp's either evoked or spontaneous during intracellular recording from the myotubes. In contrast, direct contact of the spinal cord explant with the myotubes had adverse effects on the myotubes and made them atrophy and degenerate.

Peterson and Crain (1972) found that, in contrast to the fetal muscle fibres, adult muscle fibres when brought in tissue culture show few signs of regeneration if they do not become innervated. There appeared to be an early stage in culture during which regeneration of the adult muscle fibres could be brought about in a rather non-specific way by the presence of many types of fetal tissue e.g. lung, liver, meninges, peripheral ganglia and material of the central nervous system. Further development and maintenance, however, could be brought about only by neurons of organotypic explants of the spinal cord. Muscle fibres innervated by such explants could be maintained for periods of at least a year.

These observations were confirmed by Paul and Powell (1974) in a morphological study on the innervation of normal and dystrophic skeletal muscle fibres obtained from adult mice. When spinal cord slices from mouse embryos were added to the muscle fibres, well differentiated muscle fibres survived. In cultures of adult muscle alone, the fibres regenerated poorly. The authors did not investigate whether the surviving muscle fibres in the combined cultures were actually innervated by the spinal cord neurons.

A parallel between these experiments and those carried out in vivo with regenerating degenerated muscle fibres can be drawn. Jirmanová and Thesleff (1972) used methylbupivacaine to cause degeneration of muscles of adult rats. Regeneration up to the myotube stage appeared independent of innervation, for further development, however, innervation was needed. Thus the systems in tissue culture are good models to study the regeneration of muscle fibres and the role of innervation in this process. In future it may become possible to exclude certain cells from the culture or to add extracts of certain types of cells in order to study their specific role in the process without the interference of other factors such as for instance blood circulation.

Denervation experiments

Not only innervation studies but also denervation studies have been carried out in tissue culture. Robbins and Yonezawa (1971) found that 2-3 days after denervation of muscle cells due to the degeneration of the axon, cross striation became indistinct and the muscle cell nuclei moved from a peripheral to a central position. The presence of a spinal cord explant nearby or a functioning cord-muscle pair in the same culture failed to prevent this loss of cross striation in the denervated muscle cell. They concluded that "This evidence implies that the influence of nerve requires intimate nerve-muscle contact". Crain and Peterson (1974) mention in their review that surgical extirpation of the spinal cord explants from combined cultures with muscle fibres results in degeneration of the motor nerve fascicles and in atrophy of the muscle. In their cultures, however, atrophy occurred within 2-4 weeks after denervation. It consisted of disruption of cross striation, appearance of fusiform swellings, reduced fibre diameter of the myotubes and translocation of muscle nuclei from subsarcolemmal to more central loci. Explantation of sympathetic ganglia in such

combined cultures before the denervation of muscle cells resulted in prolonged maintenance of cross striation and postponement of atrophy beyond a period of up to 6-9 weeks after extirpation of the spinal cord explant. Upon electrophysiological investigation only a minority of the muscle fibres appeared to be functionally innervated. This investigation is a fine example of how tissue culture techniques may help to study the question whether trophic effects are exerted specifically by spinal cord neurons or whether the effects are merely dependent on the presence of neuronal material in general.

The effects of drugs

Investigations in which drugs are used to study the trophic influence of neurons in tissue culture show the obvious advantage of this method in that the drug used can only affect those tissues present in the culture, while in vivo effects of the drug on other tissues can complicate the results. Moreover, in cultures the concentration of a drug can be kept at any value during a desired period of time. Crain and Peterson (1974) reported preliminary results of studies in which adult rodent muscle explants and fetal spinal cord explants were chronically exposed to 50 µg/ml xylocaine, a local anesthetic that blocks impulse conduction. Within two weeks after the first exposition, atrophy resembling denervation atrophy occurred in the muscle fibres present in the mixed cultures. Control experiments to exclude a direct toxic influence of xylocaine on the muscle fibres were not reported. The results of these experiments should therefore be interpreted with caution, the more so because the blocking effect of xylocaine on action potentials may affect axonal flow (Blunt and Vrbová, 1975).

Chronic exposure of combined cultures of spinal cord neurons and muscle cells to curare does not appear to interfere with the formation of neuromuscular connections (Crain and Peterson, 1971; Cohen, 1972). Rodent

muscle fibres regenerated normally after innervation in a medium containing a neuromuscular blocking concentration of curare (up to 100 µg/ml). After one month in this medium the muscle morphology became abnormal and evidence of atrophy was found. This atrophy could be reversed by return to curare-free medium.

Effects mediated by extracts of neuronal tissues

Acceleration of mitotic activity on myoblast fusion has been reported by many authors who tested the effects of neuronal extracts on muscle culture (see Slater, 1976). Trophic influences of extracts of brain and spinal cord were observed in cultures of cells dissociated from fetal thigh muscle of chicks (Oh et al., 1972; Oh, 1975). These extracts not only accelerated mitotic activity and myoblast fusion but also morphological maturation of the muscle fibres. The total AChE content of the treated cultures was somewhat enhanced in comparison with non-treated cultures. The same authors found an accelerated morphological maturation and prolonged survival of muscle fibres in the presence of spinal cord explants cultured either in contact with or at some distance from the muscle cells. Later, experiments were done with extracts obtained from sciatic nerves of adult chicks (Oh, 1976). When no extract was added, atrophy in cultures of muscle cells started at 12 days in culture but addition of extract reversed atrophy within 3 days. Well differentiated fibres were found in treated cultures for up to 7 weeks, while in non-treated cultures degeneration was complete after 2-3 weeks. The effect of sciatic nerve extract was abolished by treatment of the extract with pronase, trypsin or neuramidase. Diaflo filtrates of the extract, which only contained material with a molecular weight between 10,000 and 50,000, still showed the trophic effects. The author therefore concluded that the effective factor was glycoprotein.

E f f e c t s o n e l e c t r i c a l p r o p e r t i e s

Generation of the action potential

The influence of liver and spinal cord extracts from adult mouse on cultured denervated mouse extensor digitorum longus muscle has been studied by Kuromi and Hasegawa (1975). The muscle was denervated and three days later transferred to tissue culture and kept for 3 days. The denervated muscles were cultured in medium that contained either no extract, liver extract or spinal cord extract. Action potentials elicited by direct stimulation in muscle fibres that were cultured with spinal cord extract had a mean maximal rate of rise of 593 V/sec and a mean maximal rate of fall of 190 V/sec. Action potentials of muscles cultured either without extract or with liver extract were significantly slower, they had a mean maximal rate of rise of 458 and 420 V/sec and a mean maximal rate of fall of 143 and 114 V/sec, respectively. It was concluded that a specific factor is present in spinal cord extract that increases the maximal rate of rise and fall of the action potential in previously denervated muscle fibres. This factor clearly does not influence the resting membrane potential, because no difference in this parameter was found between fibres cultured with or without extract. It is well-known that tetrodotoxin blocks the generation of action potentials in innervated muscle fibres but not in denervated fibres (Redfern and Thesleff, 1971). Kuromi and Hasegawa (1975) therefore studied the effect of tetrodotoxin on the generation of action potentials in the same experiments. The rate of rise of action potentials of muscle fibres grown in medium with spinal cord extract was now significantly slower than that in muscles cultured either without extract or with liver extract, when the action potentials were elicited in the presence of tetrodotoxin. This suggests that in the

presence of spinal cord extract, the mechanism for the generation of the action potential had returned to characteristics resembling those of the innervated state. In more recent experiments Hasegawa and Kuromi (1977) demonstrated that such changes in the action potential mechanism can not only be reversed, but can also be prevented if the denervated muscle is placed in organ culture at once after denervation in medium containing spinal cord extract.

These are very interesting findings, the more so because recently Cangiano and Lutzemberger (1977) suggested that the resistance to tetrodotoxin is caused by products of nerve degeneration. They found that after partial denervation of the extensor digitorum longus muscle of the adult rat in vivo, muscle fibres in which mepp's could be recorded still developed tetrodotoxin resistant action potentials. The experiments of Hasegawa and Kuromi showed, however, that there is a factor present in the spinal cord that is able to reverse the effect of such degeneration products. An alternative explanation might be, that after partial denervation the motoneurons are affected in such a way that the production of a trophic factor is blocked.

Passive electrical properties

Engelhardt and co-workers (1976) studied the influence of innervation by neurons present in fragments of the ventral half of the spinal cord of chick embryos on skeletal muscle cells in cultures obtained by dissociation of skeletal muscles of chick embryos. They found that the specific membrane resistances (R_m) of muscle fibres cultured with or without neurons were $1.1 \text{ k}\Omega\text{cm}^2$ and $3.3 \text{ k}\Omega\text{cm}^2$, respectively, while no difference in specific membrane capacity was found. Consequently, the input resistance, the space constant and the time constant of the muscle fibres that were cultured with neurons were lower than of those cultured without neurons, because the dimensions of the muscle fibres were approximately the same. These

results thus demonstrated a neuronal influence in tissue culture that changes the properties of muscle fibres in the same way as observed in denervation experiments in vivo. In later experiments with the same kind of cultures Engelhardt et al. (1977) observed that the frequency distribution of the specific membrane resistances of fibres cultured without neurons was bimodal, while that of muscle fibres cultured with neurons was unimodal. They suggest that the bimodal distribution could be due to the presence of slow and fast fibre types in their non-innervated cultures, while due to innervation the proportion of slow fibres in the cultures was decreased.

Effects on the localization of acetylcholine receptors

Innervation and activity

Kano and Shimada (1971) were the first to report localized sensitivity to ACh on muscle fibres in cultures of dissociated muscle cells together with spinal cord explants of chick embryos. In non-innervated muscle fibres the sensitivity to ACh was evenly distributed over the entire surface whereas in mixed cultures highly localized areas of increased sensitivity were observed at sites of neuromuscular contact. The sensitivity to ACh of muscle membrane areas remote from these sites was very low. The iontophoretic pulses used in these experiments were very long and the time between a pulse and the onset of the response was usually also long, signalling a rather large distance from pipette to cell membrane. Moreover, the muscle fibres investigated were embedded in a sheath of cells. The results reported may therefore be complicated by errors due to diffusion of ACh (cf. Nelson, 1975).

At about the same time Harris et al. (1971) reported that increased local

sensitivity to ACh developed in muscle fibres formed by a continuous myogenic cell line L-6 when co-cultured with neuroblastoma cells. No signs of transmission were found, however, in such cultures. This increased local ACh-sensitivity was found at areas of the muscle membrane where a neuroblastoma cell had become firmly attached to a muscle fibre. There was no increase in sensitivity at sites where such contacts were more loose and easily separable. The localized sensitivity was 2-25 times higher than the background sensitivity. The sensitivity to ACh of muscle fibres cultured without neuroblastoma cells was uniformly distributed over the entire surface.

In contrast to these findings are the observations of Sytkowski and co-workers (Vogel et al., 1972; Sytkowski et al., 1973). They used autoradiography with radioactive α -bungarotoxin to investigate the distribution of ACh-receptors on muscle fibres in cultures of dissociated muscles of chick embryos and in cultures obtained from the myogenic cell lines L-6 and L-8 derived from the rat. Surprisingly, they found in non-innervated muscle fibres clusters ("hot spots") of toxin binding sites, somewhat comparable with those found in rat diaphragm end plates. The overall binding of toxin in the background was a factor 10 lower. It appears, therefore, that localization of ACh-receptors can occur in the absence of innervation.

Fischbach and Cohen (1973) found similar hot spots in primary cultures of chick muscle cells. They used cultures of dissociated pectoral muscles together with neurons dissociated from the spinal cord of chick embryos. With their method of iontophoretical application of ACh and intracellular recording of the membrane potential they obtained a spatial resolution of 10 μ m. In non-innervated muscle fibres hot spots were found with an ACh-sensitivity 2-10 times higher than that of the surrounding muscle membrane. Spots with a higher sensitivity of the same magnitude, i. e. 2-10 times higher than the background sensitivity, were found in 10 out of 18 innervated muscle fibres. Only one or a few areas with such a higher sensitivity were

present on each innervated muscle fibre. Generally, neurites were present near these spots of high sensitivity. The average background sensitivity in innervated and in non-innervated fibres was equal and reasonably high, and this remained so during at least 25 days. Later Fischbach et al. (1976) reported results from experiments in which they had been able to localize the sites of transmitter release by extracellular stimulation of nerve processes on muscle fibres, while the conduction of action potentials had been blocked by tetrodotoxin. At these sites of ACh release relative peaks of sensitivity to ACh were found on areas that had a diameter of approximately 5–10 μm . They also observed that the mere presence of nerve processes did not increase the sensitivity above the average background level. These results can be interpreted in two ways: i. the localized sensitivity is induced by the transmitter releasing nerve process or ii. the nerve process seeks out an already existing hot spot to make a synapse with.

Recent results from our own experiments (Stevens et al., in preparation) make the first hypothesis the most likely. In non-innervated muscle fibres in cultures from leg muscles of chick embryos used by the present authors no hot spots are present. In mixed cultures, however, with explants of embryonic mouse spinal cord areas with a high density of ACh-receptors were demonstrated by labeling with radioactive α -bungarotoxin. Spots with a high sensitivity to ACh were also demonstrated in mixed cultures with embryonic chick ciliary ganglia and the same muscle fibres as used in the foregoing experiment (Slaaf et al., in preparation).

Activity of muscle fibres is an important factor in the determination of the extra-junctional sensitivity to ACh in vivo (Lömo and Westgaard, 1976). In tissue culture activity of the muscle fibres influences the sensitivity to ACh. When cultured chick muscle fibres were electrically stimulated and their mean sensitivity to ACh was compared with that of muscle fibres that had been grown in medium continuously containing tetrodotoxin in order

to prevent muscle contraction, it appeared that the sensitivity of stimulated muscle fibres was considerably lower. The geometric mean sensitivity of stimulated fibres was 11.8 mV/nC and that of immobilized fibres was 456 mV/nC. Hot spots were scarce in stimulated fibres, but generally present in fibres from tetrodotoxin treated cultures (Cohen and Fischbach, 1973). Shainberg and Burnstein (1976) used radioactive labeling of ACh receptors of chick muscle fibres in culture with α -bungarotoxin to study the effect of contractile activity on ACh-receptor synthesis. They demonstrated that in muscle fibres in which activity was blocked by tetrodotoxin the receptor synthesis was much faster than in non-treated fibres. Moreover, the synthesis of ACh-receptors in muscle fibres that had not been treated with TTX, was faster than in muscle fibres that underwent electrical stimulation. The higher sensitivity of inactive muscle fibres was thus due to a higher rate of receptor synthesis.

Purves and Sakmann (1974) observed an influence of activity on the extra-junctional sensitivity in previously denervated rat diaphragm that had been maintained in organ culture. A marked reduction of extra-junctional sensitivity was obtained after one week by direct electrical stimulation of the muscle fibres in a stimulus pattern corresponding to the natural breathing rhythm. Continuous stimulation caused only a small reduction of the extra-junctional sensitivity.

Influence of ACh on the localization of ACh sensitivity

Steinbach et al. (1973) have studied the role of ACh in the formation of areas with a high density of ACh-receptors that are brought about in their cultures by neuroblastoma cells in contact with rat muscle fibres from clone L-6. They used α -neurotoxin to block ACh-receptors and N-hydroxyethyl-4-(1-naphtylvinyl)-pyridinium bromide to inhibit the synthesis of ACh during the culture period. Under these conditions, without release of ACh

and with blocked ACh-receptors areas with a higher density of ACh-receptors were formed in a normal way. They conclude that other mechanisms than those that are transmitter mediated must be involved.

AChE activity and localization

AChE is localized and active in cultures of adult rodent muscle and fetal spinal cord (Crain et al., 1970; Peterson and Crain, 1972). Histochemically small areas of AChE activity could be shown and its activity in cultures older than three weeks was detected by studying the effect of cholinesterase inhibitors e.g. eserine, on neuromuscular transmission. It appeared that the reversal to normal transmission after a blockade by d-tubocurarine was accelerated by these compounds.

Fischbach et al. (1974) on the other hand found electrophysiologically that AChE was not active in their cultures of dissociated muscles and spinal cord obtained from chick embryos. Histochemically AChE was demonstrated in innervated fibres and in non-innervated ones, in both cases the enzyme was localized in certain areas. Electronmicroscopy revealed that most of the AChE seen in lightmicroscopy was situated beneath the surface of the muscle cell membrane. The different results obtained by the various authors might be attributed to the use of different species and different neurons either in explants or as dissociated cells. In the experiments described there was also a difference in age of the cultures studied. Koenig et al. (1976) also reported histochemical localization of AChE at end plate-like structures in cultures of spinal cord fragments and muscle cells of chick embryos. Moreover, after three weeks in culture the presence of a specific isoenzyme 16S AChE was demonstrated. The authors reported that 16S AChE was found in innervated diaphragm muscles *in vivo*, but not

in denervated muscles.

The effects of tissue extracts on the appearance of AChE in dissociated muscle cells was mentioned previously in this chapter were the experiments of Oh and colleagues were discussed. In contrast to their results showing that extracts of embryos increase the amount of AChE, are the findings of Harvey and Dryden (1974). They found no evidence for a trophic action of neurons or extracts containing neuronal material on the amount or on the localization of muscle AChE in their cultures of dissociated embryonic chick muscle. The appearance of locations high in AChE was a common phenomenon in their older cultures.

In newt muscles in organ culture loss of AChE activity caused by previous muscle denervation was delayed by the presence of neuronal tissues or extracts from these tissues (Lentz, 1974). The presence of one dorsal root ganglion already caused a considerable decrease of the loss of AChE from the denervated neuromuscular junction. This effect lasted for two weeks. Surprisingly, explantation of two dorsal root ganglia had no effect. Spinal cord explants were less effective. Extracts of dorsal root ganglia, spinal cord and brain of the newt had some positive effect. Extracts from rat brain had no influence on this decrease, thus the factor involved may be species specific.

Walker and Wilson (1975) measured the activity of muscle AChE and AChE released into the culture medium in cultures of dissociated muscles of chick embryos. Cultures that had been electrically stimulated were compared with unstimulated cultures. It appeared that stimulation decreased the amounts of AChE in the muscle fibres and the amounts released in the cultures as compared with those measured in unstimulated cultures. The difference between these two types of cultures became even larger if the unstimulated cultures were grown in a medium containing tetrodotoxin which stopped all contractile activity in these cultures. The same authors

(Wilson and Walker, citation in Walker and Wilson, 1975) observed that during maturation in situ neuronal activity decreases AChE activity everywhere in the muscle fibres of chicks except at the neuromuscular junction. Thus it may be that a factor is released by the axon terminal that prevents the loss of AChE at that part of the muscle membrane where the neuromuscular junction is formed.

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CHAPTER II

THE MORPHOLOGICAL DEVELOPMENT OF NEURONS OBTAINED BY DISSOCIATION OF THE SPINAL CORD OF MOUSE EMBRYOS

Introduction

Tissue culture permits a combined study of the *morphological and electro-physiological* events which take place during the development of neuronal excitability, the formation of interneuronal synapses, and the development of functional connections between neurons and other target cells. Studies of mixed cultures of neurons and muscle cells have greatly contributed to the knowledge about the development of the neuromuscular junction (Fischbach et al., 1974). Ideally, for such studies the neurons should be clearly visible and their identity as *motoneurons* should be established beyond doubt.

Presently two methods are in use to obtain spinal cord neurons in culture. Either spinal cord material is dissociated and the resulting cell suspension is plated and cultured, or whole slices of the spinal cord are explanted and cultured. The cultures of dissociated neurons offer the advantage that the density of the neurons in culture can be chosen such that good visibility is obtained. The *major disadvantage* is, however, that there is no method available to distinguish *motoneurons* from other neuronal cells. Since Vera and Luco (1967) and Landmesser (1972) have shown that *in vivo*, vagus fibres can innervate skeletal muscle, a neuron cannot be assumed to be a *motoneuron* solely on the basis that it innervates muscle cells.

The spinal cord explant, as shown by Sobkowicz et al. (1968) preserves its cytoarchitecture in tissue culture for many weeks. It therefore offers the possibility to identify cells which are likely to be motoneurons in culture on the basis of their position. However, the visibility in the explants is usually rather limited which seriously hampers the study of the morphology of the cells and totally precludes the tracing of their processes. So far electrophysiological methods have been of little help in distinguishing various types of spinal neurons in tissue culture, although differences have been found between spinal cord neurons and those from the dorsal root ganglion (Peacock et al., 1973; see also Nelson, 1975).

In the present investigation the morphology was studied of spinal cord cells during their development in tissue culture. The aim was to see whether and when morphological properties might become visible which would allow classification of the neurons in different groups. A long-term study was made of individual cells which were regularly inspected and photographed from approximately 2 weeks until 7 weeks in culture.

Materials and methods

Tissue culture

Mice in the 13th day of pregnancy were sacrificed by cervical dislocation and the uteri were removed under sterile conditions. Spinal cords and dorsal root ganglia were dissected under a low power stereo microscope in calcium and magnesium-free Tyrode's solution in a laminar flow cabinet. Care was taken to remove all dorsal root ganglia adhering to the spinal cord when cultures of spinal cord cells were made. Usually 6 spinal cords or 80 to 140 dorsal root ganglia were prepared in one session. For the dissection of the ventral horn from the spinal cord the latter was placed in

a drop of medium with its ventral surface pointing downwards.

Attempts to keep spinal cords in good condition for many hours have not been succesful but embryos have been stored with good results in Tyrode's solution at 4°C for periods of up to 5 h.

Two methods were used for the dissociation of the neuronal material, one purely mechanical (A) and the other with the additional use of trypsin (B). For method A the neuronal material was minced with a pair of scissors in a drop of culture medium and subsequently dissociated in 1 ml medium by batchwise trituration through fire polished Pasteur pipettes. For method B the tissues were minced and subsequently incubated for 15 min at 37°C in calcium and magnesium-free Tyrode's solution containing 0.05% trypsin. The trypsin solution was then sucked off and the tissues were washed with 1 ml fetal calf serum (Flow, Scotland). Dissociation was completed by batchwise trituration as in method A. In both procedures the cell suspension obtained was made up to an initial plating density of $15 \cdot 10^4$ cells/cm².

The cells were cultured on "Cellfinders" (Micropure, Driebergen, The Netherlands), i. e. glass plates provided with a printed grid marked with letters to facilitate the location of a particular neuron. To begin with, a cellfinder was coated with collagen and wetted with a few drops of medium. Subsequently 0.65 ml of the cell suspension was uniformly spread over the surface. The cellfinders were placed in Petri dishes and stored overnight in a mixture of air and 5% CO₂ at 37°C. The next day medium was added to a final volume of 3 ml. To all culture media used for dissociation and culture of dorsal root ganglia, nerve growth factor (NGF, Wellcome) was added to a concentration of 1 biological unit/ml.

After dissociation by method A the cells were cultured in a modified "minimum essential medium" (MEM) (see chapter 3) supplemented with 3.2% glucose and 10% heat-inactivated horse serum. During the first 8 days in culture 10% fetal calf serum was added (Godfrey personal communication).

In order to prevent the neurons from being overgrown by non-neuronal cells the medium contained fluorodeoxyuridine (FUdR) and uridine in concentrations of 20 and 50 $\mu\text{g/ml}$, respectively, during days 8–12 in culture. FUdR was added in order to inhibit DNA synthesis in dividing cells, uridine was added to minimize the effect of FUdR on RNA synthesis.

Following dissociation by method B, the cells were grown in 1415 medium (Healy and Parker, 1966), to which 10% fetal calf serum and 10% horse serum was added during the whole culture period. To control the growth of non-neuronal cells the folic acid antagonist aminopterin (0.26 $\mu\text{g/ml}$) was added to the medium at day 3 for 48 hours.

Morphological measurements

Every 2 – 3 days photographs were made of individual neurons through an inverted phase contrast microscope (Zeiss) at a magnification of 400x. At first it was thought necessary to study the development of free lying neurons as well as of neurons situated in groups. Soon it appeared, however, that only the free neurons could regularly be retrieved with certainty and thus the investigation of groups was discontinued.

The surface area of the body of the neurons studied was estimated by measuring their longest axis (a) and the diameter perpendicular to that axis (b). Assuming that the body of neuron usually had a shape somewhere between that of an ellipsoid body and a flat ellipse, the surface area was calculated as being $3/4 \pi ab$, as proposed by Nelson et al. (1971).

Results

Two methods were employed for the cultivation of dissociated neuronal material. Essentially, method A consisted of mechanical dissociation

followed by treatment with FUdR to inhibit proliferation of non-neuronal cells, whereas in method B additional use was made of trypsin, and aminopterin was used instead of FUdR. For all investigations together 150 cultures were made with method A and 200 with method B. Cell survival, as determined by trypanblue exclusion by living cells, was 65 to 75% with either method. Both methods yielded healthy neurons surviving at least 50 days. There was no difference in the survival of the neurons during cultivation. No systematic comparison was made between the neurons cultured according the 2 methods but the impression was that method B favoured the survival of dorsal root ganglion neurons in culture.

Development of the cultures

By dissociation of spinal cord material a suspension of rounded cells was obtained with a maximum diameter of 20 μ m. A few cells carried a short process, presumably a cut off neurite. Fifteen min after plating of the suspension, cells were seen with thin, threadlike filaments extending into the medium or adhering to the collagen. A number of cells firmly settled and flattened on the collagen within one hour. Other cells, probably neurons, kept their rounded shape and sometimes formed aggregates of up to 100 cells within 8 h. This aggregation could be reduced by using as little growth medium as possible during this period. During the same period most rounded cells formed growth cones (figure 1a). After 2 days in culture the rounded cells had formed an extensive network of processes. At that stage the nuclei in these cells were not visible but the cells distinguished themselves as neurons by staining brightly with methylene blue. After about 9 days in culture the neurons

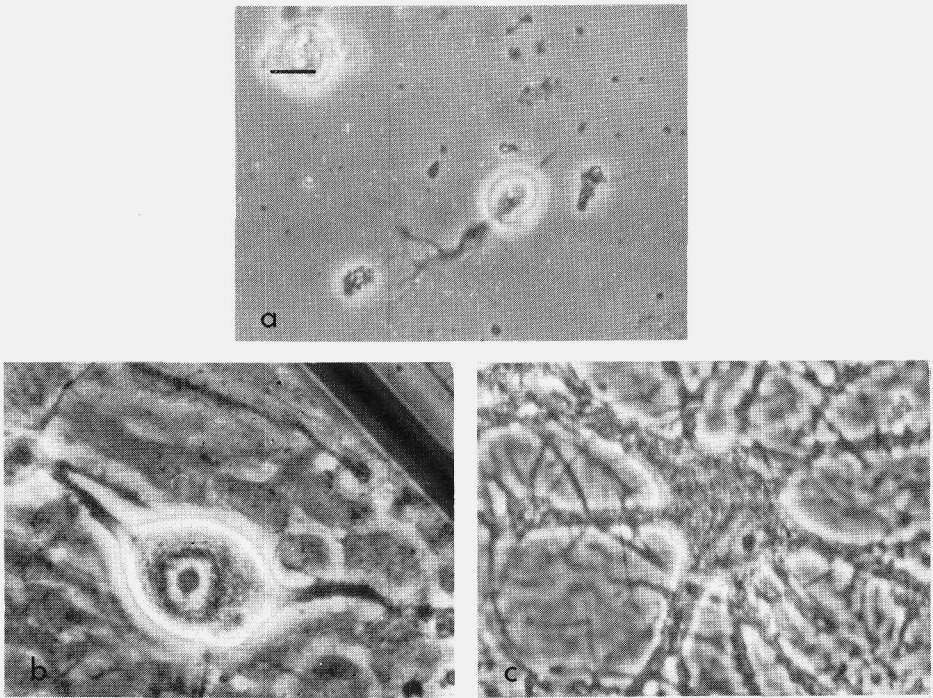


Fig. 1. Neurons in tissue culture; a, neuron 8 hours after plating, extending its growth cone; b, fully developed neuron from a dorsal root ganglion; c, idem from the spinal cord. All photographs have the same magnification, calibration bar 10 μ m.

had visible, excentric nuclei and showed marked differences in shape, some having a rounded form while others were much more flattened. The former ones were situated on top of the monolayer of non-neural cells which had formed by that time whereas the flat neurons were embedded in this monolayer. At that stage the neurites had started forming bundles. After 3 weeks in culture the monolayer of non-neuronal cells had thickened and the majority of the nuclei in the neurons had taken a central position. From that time onwards the over-all appearance of the cultures showed no further change. During the various stages of development no myelination of neurites was observed with light microscopy. Degeneration became manifest by the development of coarse granules within the cell, the withdrawal of

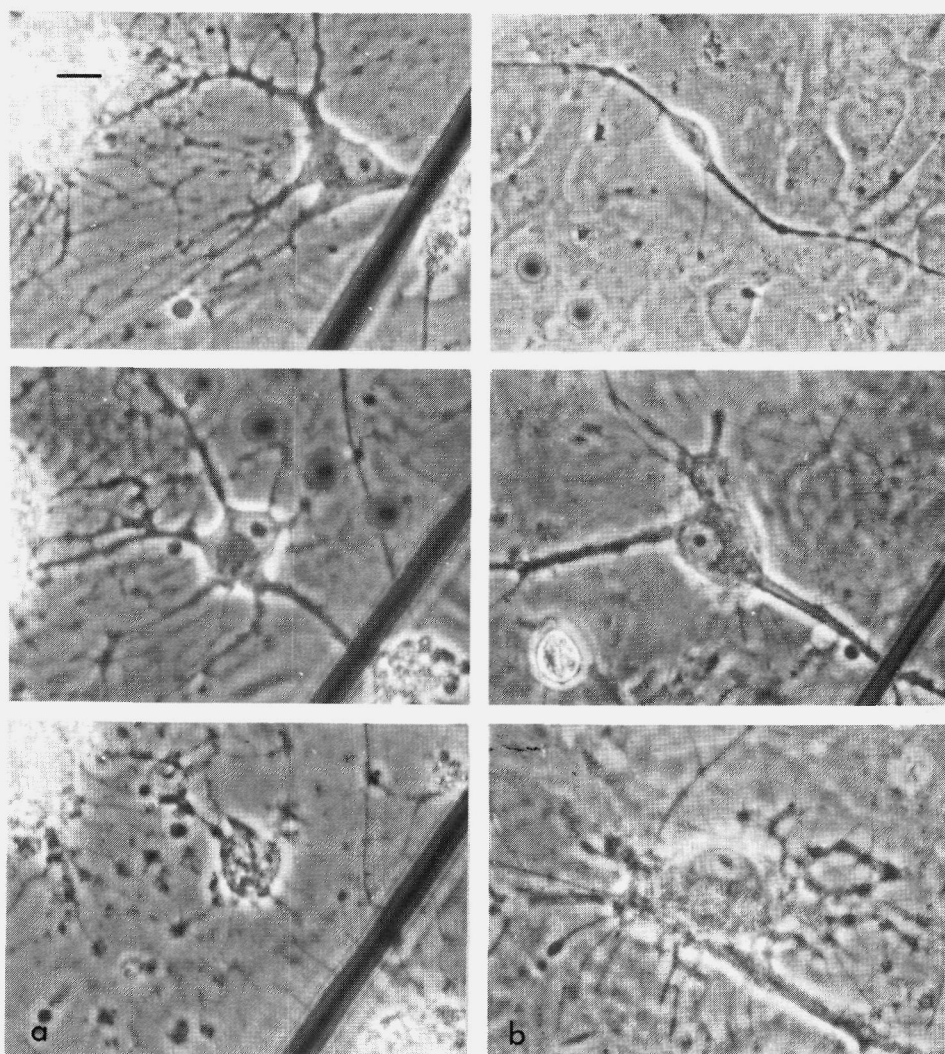


Fig.2. Spinal cord neurons in culture; vertical row a shows various phases of degeneration at 2 day intervals, row b - taken at 4 day intervals - shows growth and branching of a neuron, calibration bar 25 μ m.

the neurites and by the fact that the nucleus and the cell wall became almost invisible (figure 2a).

The fates of individual spinal cord neurons

For a detailed study of the morphological development of the spinal cord neurons in the dissociated cultures 48 single neurons from 12 separate cell cultures obtained with method B, were located on cellfinders and photographed every 2 - 3 days. The first picture was usually taken after about 2 weeks of culture because in preliminary studies it had been observed that by that time the neurons had to some extent stabilized as to shape and place, and had "decided" whether to remain single or to join an aggregate. Although the localization of neurons was not completely stable, migration was never so extensive in a 2 day interval that retrieval of a healthy neuron became ambiguous. Probably, the displacement of neurons, which could be as much as 40 μ m in two days, was largely caused by movement of the sheath of non-neuronal cells because usually the position of the neurons relative to one another remained approximately the same and the neurites never became twisted but kept essentially the same arrangement with respect to the perikaryon from which they originated.

Permanent and non permanent neurons

The development of 27 neurons, henceforth called "permanent" neurons could be followed up to the end of the experiment at 48 days after the initiation of the culture. The other 21 neurons disappeared, either suddenly within a 2 day interval or after a more prolonged period of degeneration. When a neuron died in the beginning of the experiment, a new one was chosen for further investigation in order not to end up with too small a number. As a result, the onset of the observations was different for the various neurons.

In order to see whether the moment of disappearance of the non-permanent neurons was evenly distributed over the total observation period, the fates

of these 21 neurons were compared. As graphically demonstrated in figure 3, 18 of them died before the 30th day in culture whereas only 3 lived beyond that day. Obviously, from the whole population of 48 neurons, 90% of

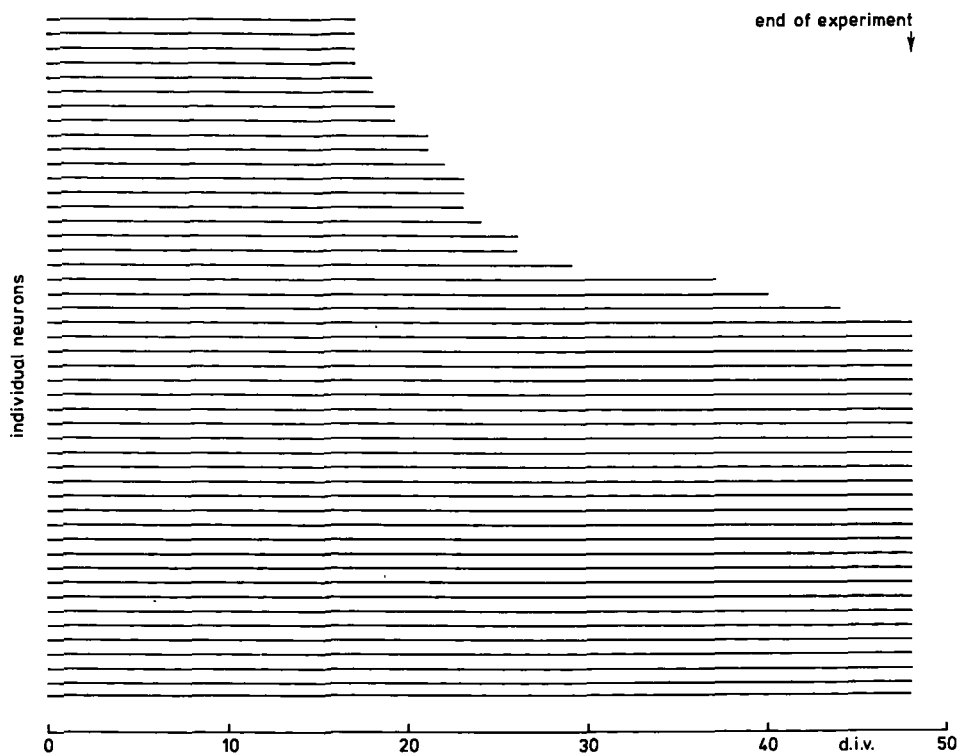


Fig. 3. Survival of spinal cord neurons in culture. Each horizontal line represents the lifespan of an individual neuron in culture. All neurons represented had already survived for 2 weeks in culture.

those that survived day 30 also lived until the end of the experiment. In other words the moment of disappearance of a neuron is not evenly distributed over the whole period between 2 and 7 weeks in culture but clearcut subgroups can be distinguished: those who die before day 30 and those who live much longer.

In order to decide whether visible differences existed between neurons belonging to the 2 groups, a comparison was made as to surface area and

number of neurites visible. In the hope of comparing neurons at relevant moments in their life, the 17 non-permanent neurons that died within the first 4 weeks were assessed at the last observation day on which no signs of degeneration were seen. For comparison 17 permanent neurons were chosen randomly and assessed on matched days in culture. The surface areas of the non-permanent and the permanent neurons were 660 ± 320 and $930 \pm 400 \mu\text{m}^2$, respectively (mean $\pm$ S.D.). These values were significantly different at $P_2 < 0.05$ (Wilcoxon rank-correlation test). The number of visible neurites in both groups did not differ much, being 3.0 ± 1.4 (mean $\pm$ S.D., range 0-6) and 2.5 ± 0.8 (range 0-5) in the non-permanent and permanent neurons respectively.

The death of part of the total population was not due to unnoticed technical shortcomings in particular cultures, because of the 12 separate cultures (Petri dishes) 10 had non-permanent cells (1 to 3) and the only culture responsible for 3 non-permanent neurons contributed a normal (3) number of permanent cells for the study. It thus appears that the 2 categories of neurons can be distinguished on the basis of survival and surface area.

Differences between permanent neurons

The permanent neurons were evaluated with respect to changes in surface area and shape of the perikaryon. In figure 4 these 27 neurons are arranged in the order of the length of the period during which they still showed changes in surface area. For each neuron this period of changes is represented by a horizontal line which starts at the onset of observations. The dashed, solid and dotted lines represent the periods of increase, stability and decrease of the surface area, respectively. The lines start at different days. This is caused by the fact that, as already mentioned, when a neuron died another one from the same culture was often chosen for further study.

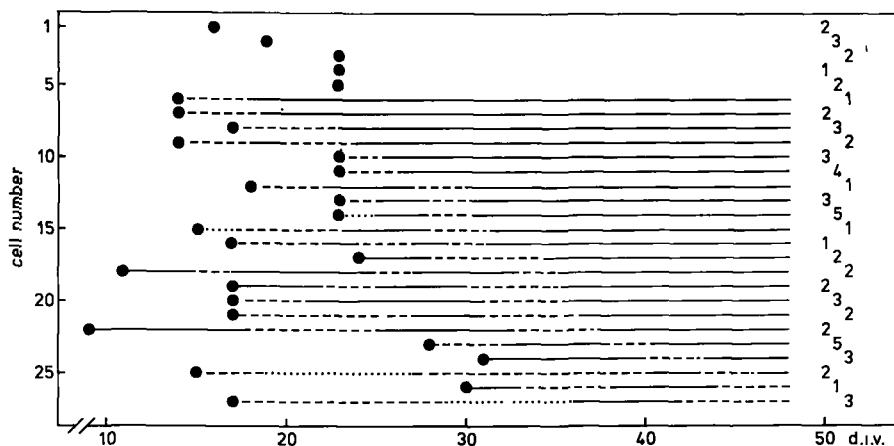


Fig. 4. Duration of changes in surface area of spinal cord neurons in culture. Horizontal interrupted and dotted lines represent the periods during which changes were observed in 27 neurons that survived until the end of the experiments, that is 48 days. Increase in surface area -----, decrease Periods of stability are represented by solid lines. Column of figures on the right side: final number of visible neurites. The first day of observation is indicated by a dot.

If the group of 27 neurons contained major subgroups which differed in their moment of reaching the final size, the end points of the lines in figure 4 would be non-uniformly distributed over the total observation period. The actual distribution of the end points, however, is fairly even and allows no classification of the neurons.

In figure 5 the ultimate surface area of each neuron was plotted against the length of the period needed to reach it. It appeared that whereas a group of 18 neurons sooner or later ended up with a surface area between 500 and $1500 \mu\text{m}^2$ a group of 8 neurons distinguished themselves by stabilizing at values above $2000 \mu\text{m}^2$. Since large and small neurons were found side by side in the same cultures, differences in treatment could not be responsible for this difference in size. Statistical analysis showed, however, that the whole group had a normal distribution (Kolmogorov normality test), thus the surface areas of both groups did not differ at the end of the

experiment. In order to check whether the 8 largest neurons at 48 days were already relative large cells at an earlier stage, their properties have been compared with those of other neurons at day 30, that is at a time, when 90%

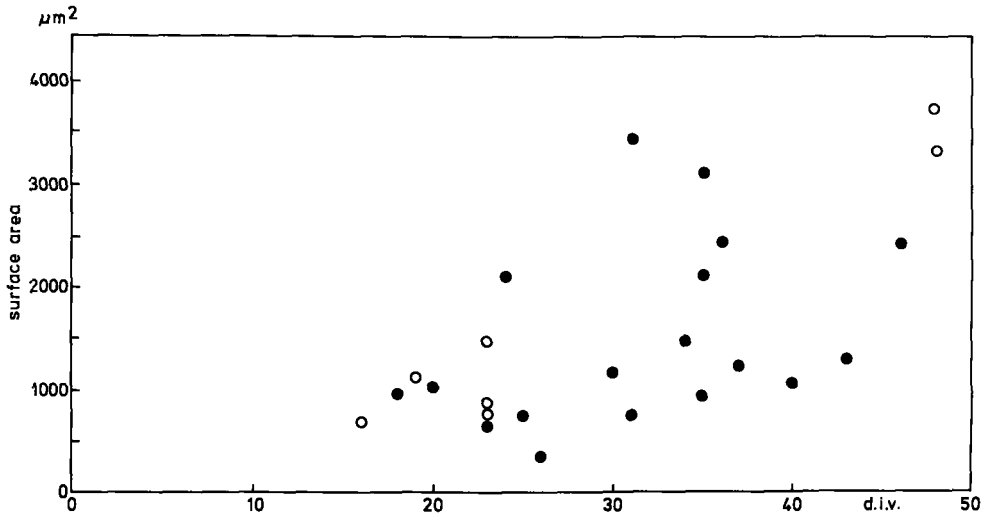


Fig. 5. Final surface area of permanent neurons. Open circles left indicate that changes in surface area may have stopped earlier. Open circles right indicate that changes in surface area might have continued.

of the non-permanent neurons had disappeared. A comparison showed, however, that 3 of the 8 large permanent neurons at that time still had a surface area well within the range of that of the small ones, and therefore prediction of which neuron will become large is not possible at that stage. Moreover, it appeared that on day 20 all future large permanent neurons had surface areas within the range of the other permanent neurons. It thus appears that the future large ones rather suddenly increased their surface area (figure 2b). In fact this sudden increase occurred in 5 cells following day 20 and in 3 after day 30. Four of these neurons at least doubled their surface area within a week. The neurite numbers at the end of the experiment were compared for the small and the large permanent neurons, being 2.5 ± 1.3 (mean $\pm$ S.D.,

range 1-3) and 2.0 ± 0.68 (range 1-5), respectively. This difference is not statistically significant. Due to limited visibility of thin neurites, neurite counts must show large differences to be of importance.

In the permanent neurons changes in shape were regularly noted and the moment was recorded when the shape became more or less fixed. Contrary to expectation, no correlation existed between the time of stabilization of shape and the time of stabilization of either the surface area or the neurite number. Some neurons stopped changing their shape a week or more before the surface area stabilized whereas others went on reshaping themselves long after that moment. Attempts to group the neurons on the basis of shape, changes in shape or neurite number failed. So far only survival time and surface area proved helpful in this respect.

Dissociated neurons from dissected ventral horns and dorsal root ganglia
The experiments described so far showed that considerable changes take place in the morphological parameters of spinal cord neurons during their growth in tissue culture, and that it was not possible to identify motoneurons. Similar studies were therefore undertaken in which, instead of the whole spinal cord, the ventral horns and the dorsal root ganglia (DRG's) were isolated and dissociated. This was done in the hope that in the cultures so obtained larger differences might exist between the selected neuron populations and identification of motoneurons or other specific neurons might be easier.

It appeared that DRG neurons had rather characteristic morphological properties which distinguished them from other neurons. The surface area of DRG neurons is usually larger and they have less neurites than spinal cord neurons. The nucleus is always clearly visible and the DRG neurons show a pronounced halo when viewed through phase-contrast optics (figure 1 b and c). These observations confirmed those of Peacock et al. (1973),

who were able to distinguish DRG neurons from spinal cord neurons in mixed cultures. In the present experiments, however, it was impossible to distinguish "ventral horn neurons" from "spinal cord neurons".

D i s c u s s i o n

The development of neurons obtained by dissociation of the spinal cord material of 13 day old mouse embryos was investigated in tissue culture. The shape, the number of neurites and the surface area of the neuron were studied each 2 - 3 days during 48 days. On the basis of survival two classes of neurons could be distinguished: I non-permanent neurons that die or degenerate during the first 30 days and II permanent neurons that remain healthy for at least 48 days. There was a significant difference in the surface area between both classes of neurons in the period up to 30 days in culture, but not in the number of neurites. The location of the cell nucleus was not used as a parameter, because its location in the perikaryon varies continuously as Pomerat (1953) has demonstrated with the aid of time-lapse microcinematography of neurons in tissue culture. The differences between the two classes are not based on variations in culture conditions because neurons belonging to both groups were generally found in the same culture dish. Bird and James (1973) and Peacock (personal communication) also observed considerable degeneration in cultures of chick spinal cord neurons and mouse spinal cord neurons, that could not be attributed to the procedure used to dissociate the spinal cord tissue. The absence of myelin is probably the consequence of the use of inhibitors of mitosis which kill or inhibit the proliferating glial cells.

Some of the permanent neurons showed a considerable increase in surface area late in the period of observation. Nevertheless, the permanent

neurons could not be fitted in groups according to their size at the end of the period of observation. It was also impossible to distinguish groups in an earlier stage, according to their shape, surface area or neurite number.

These experiments demonstrated that the cell shape and the number of neurites of the cultured neurons change considerably and that these parameters are not suitable to distinguish different types of cultured spinal cord neurons. One neuron for instance had two neurites when the observations started and it had three at the end of the experiment, but in between at some stage it had one and at another stage five neurites. It is possible, as Peacock et al. (1973) have demonstrated and as has been confirmed in the present study to distinguish cultured spinal cord and dorsal root ganglion neurons on the basis of the parameters used in this study.

There appear to be two groups of spinal cord neurons, as judged to their survival in culture. It is known that many types of neurons in vivo cannot survive if their target cells have been destroyed or removed. Embryonic motoneurons (Prestige, 1967), cranial motoneurons (Cowan and Wenger, 1967) and sympathetic neurons (Dibner and Black, 1975) for instance fail to mature and usually die when their target cells have been removed. For many neurons in tissue culture no target cells are present in cultures of spinal cord cells. This might be the cause of their large scale degeneration. The question can be raised whether synaps formation with other spinal cord neurons would be sufficient to prevent degeneration. From electrophysiological measurements it is known that spinal cord neurons mature electrically in culture and form functional synaptic connections (see Nelson, 1975). It could well be that survival in culture depends on the success of finding a receptive target cell. In that case permanent neurons should participate in a neuronal network where non-permanent neurons failed to acquire synaptic relationships with other neurons in the culture. It would be interesting to know whether more neurons would survive if at some stage muscle

cells were added to the culture. An indication that muscle cells affect cultured spinal cord neurons comes from studies of Giller et al. (1973). They demonstrated an increase in choline acetyltransferase activity in mixed cultures of spinal cord neurons and muscle cells as compared with enzyme activity in cultures of spinal cord neurons alone.

The results described here demonstrate that a classification of spinal cord neurons up to 48 days in culture based solely on differences in shape, number of neurites and surface area is impossible. The possibility that large permanent neurons in the culture are motoneurons is still open. These cells stain with histological methods for the detection of acetylcholinesterase, however, this esterase is present in many cultured neurons. By intracellular recording and assay for the choline acetyltransferase in mixed cultures of spinal cord neurons with skeletal muscle cells it might become possible to identify cholinergic neurons which are able to innervate these muscle cells.

S u m m a r y

1. The morphology of neurons dissociated from spinal cord material and from dorsal root ganglia of mouse embryos was studied during 48 days in tissue culture. The shape, the number of neurites and the surface area of the perikaryon of the neurons were used as parameters.
2. Two classes of neurons were found: i non-permanent neurons that died usually before 30 days in culture and ii permanent neurons that survived at least till the end of the experiment.
3. The surface area of non-permanent neurons was smaller than that of permanent neurons. The group of permanent neurons could not be subdivided in different classes.
4. Dorsal root ganglion neurons and spinal cord neurons were different in

surface area and number of neurites.

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CHAPTER III

THE INNERVATION OF CHICK SKELETAL MUSCLE FIBRES BY MOUSE SPINAL CORD NEURONS IN TISSUE CULTURE

Introduction

The formation of neuromuscular junctions between spinal cord neurons and muscle fibres in tissue culture has been demonstrated with the aid of histological and electrophysiological techniques. Heterospecific innervation of mammalian i. e. rodent and human tissues has been observed (Crain et al., 1970) and recently Obata (1977) demonstrated that neuromuscular junctions can form between rat and chick tissues. Also neurons of the sympathetic ganglia (Nurse and O'Lague, 1975) and the parasympathetic ciliary ganglion (Hooisma et al., 1975a; Betz, 1976) innervate skeletal muscle cells in tissue culture (for reviews, see Shimada and Fischman, 1973; Nelson, 1975).

In a series of experiments regarding trophic influences of neurons in tissue culture on muscle fibres, carried out by the present author, the aim was to study also the influence of neurons in heterospecific combinations. The present paper reports the formation of junctions between mouse spinal cord neurons and chick skeletal muscle fibres. The studies have been carried out in a system of spinal cord explants of mouse embryos and chick muscle cells. This system was chosen because: i the junctions formed by neurons grown in explants develop to a more mature stage than those formed by neurons that are cultured as single cells (Shimada and Fischman, 1966; Bornstein et al., 1968; James and Tresman, 1968, 1969; Shimada et al.,

1969; Kano and Shimada, 1971; Pappas et al., 1971; Fischbach, 1970, 1972; Bird and James, 1974), ii the visibility of individual muscle fibres and consequently the possibility to study the morphological appearance of muscle fibres is very good in cultures of dissociated cells treated with the mitotic inhibitor arabinosylfuranosylcytosine (ara-C) to prevent overgrowth by non-muscle cells (Fischbach, 1972).

The neuromuscular junctions were functionally characterized with the aid of intracellular electrophysiological measurements. It appeared that mouse spinal cord neurons form neuromuscular junctions with chick muscle fibres and the observations justified their use as a model to study neurotrophic relations between heterospecific tissues.

Materials and methods

Tissue culture

Mixed cultures of chick muscle cells and mouse spinal cord explants were used. Cultures of chick striated muscle cells were obtained by trypsin dissociation of leg muscles from 11 day old chick embryos. The cell suspension was plated at a density of $5 \cdot 10^4$ cells/cm² on collagen coated "Cellfinders" (Micropure, Driebergen, The Netherlands) and cultured in 1.8 ml medium in 60 mm Petridishes. After two days in culture 6-10 explants of spinal cord from 14 day old mouse embryos were added. Pregnant mice were killed by cervical dislocation and the embryos were taken out under sterile conditions. The spinal cords were dissected and the dorsal root ganglia were removed in Tyrode's solution under a stereo microscope in a laminar flow cabinet. Slices were cut as thin as possible with the aid of razor blades. The slices were allowed to settle on the muscle layer in a thin film of medium during 8 hours in a moist chamber. After attachment 1.8 ml medium containing

10^{-5} M arabinosylfuranosylcytosine (ara-C) to prevent cell proliferation was added for two days (Fischbach, 1972).

Culture medium and drugs

The culture medium consisted of 70 parts Eagle's minimum essential medium (MEM) in Earle's balanced salt solution (10.96 g Gibco MEM powder in 1 liter distilled water), 10 parts NaCl solution (8.07 g/l), 15 parts horse serum (Flow, Scotland) and 5 parts freshly prepared chick embryo extract. The medium was supplemented with L-glutamine (200 mg/l), biotin (0.007 mg/l), ferric nitrate (0.7 mg/l), NaHCO_3 (1.71 g/l), glucose (0.7 g/l), penicillin (7000 U/l) and streptomycin (7 mg/l). The osmolarity was adjusted to a final value of 320 mOsm. The cultures were maintained during the first two days at 37°C and afterwards at 35°C always at pH 7.2 and in a water-saturated 95% air - 5% CO_2 atmosphere. The medium was changed every 2 days. Tetrodotoxin (TTX, Calbiochem) was dissolved in distilled water and quantities to be used in one experiment were freeze-dried at once and stored in vacuo. Freeze-dried TTX and d-tubocurarine (Nourypharma) were dissolved just before use in Tyrode's solution.

Electrophysiological measurements

The age of the cultures investigated ranged between one and two weeks, except the age of those used in experiments with physostigmine that were about three weeks old. For a measurement a culture was taken from the culture dish and placed in an open perfusion chamber on the stage of a Zeiss phase-contrast inverted microscope. The prewarmed Tyrode's bathing solution was kept at 37°C . The Tyrode's bathing solution contained 1.9 and 0.5 mM Ca^{2+} and Mg^{2+} , respectively. In some experiments the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio was either 20-fold increased or 40-fold decreased.

This was accomplished by making Tyrode's solution containing either 3.8 mM Ca^{2+} and 0.05 mM Mg^{2+} or 0.5 mM Ca^{2+} and 5 mM Mg^{2+} , respectively.

Muscle fibres were impaled with single 3 M KCl filled microelectrodes, with a resistance between 10–20 $\text{M}\Omega$. Microelectrodes were pulled from glass capillaries containing a glassfibre (Clark, Electromedical Instruments) and they were filled just before use with a syringe. Muscle fibres were impaled with the aid of a piezo-electric driving device (Rikmenspoel and Lindemann, 1971). Stable recordings could routinely be obtained lasting up to three hours. Extracellular stimulating electrodes filled with Tyrode's solution had resistances of 3–10 $\text{M}\Omega$. Stimulating currents of about 10 μA and a duration of 2 msec were used. Registration of intracellular potentials was done via a WPI-model M4-A electrometer. The end plate potentials were displayed by a Tektronix 5103N storage oscilloscope with 5A22N differential amplifiers and stored for further analysis by a Hewlett-Packard 3960 instrumentation recorder. After completion of the electrophysiological measurements cultures were routinely stained either with haematoxiline-eosine or by silver impregnation according to a modification of the Bielschovsky method (Sevier and Munger, 1965).

R e s u l t s

Morphology of cultured muscle fibres

Mononucleated muscle cells (myoblasts) started to proliferate in these cultures of chick muscle cells two hours after plating. After two days many myoblasts had fused to form muscle fibres (figure 6). The term "muscle fibre" is used throughout this paper for all multinuclear muscle cells irrespective of their stage of development. The muscle fibres were faintly cross striated at the fourth day in culture and cross striation became clearly visible from

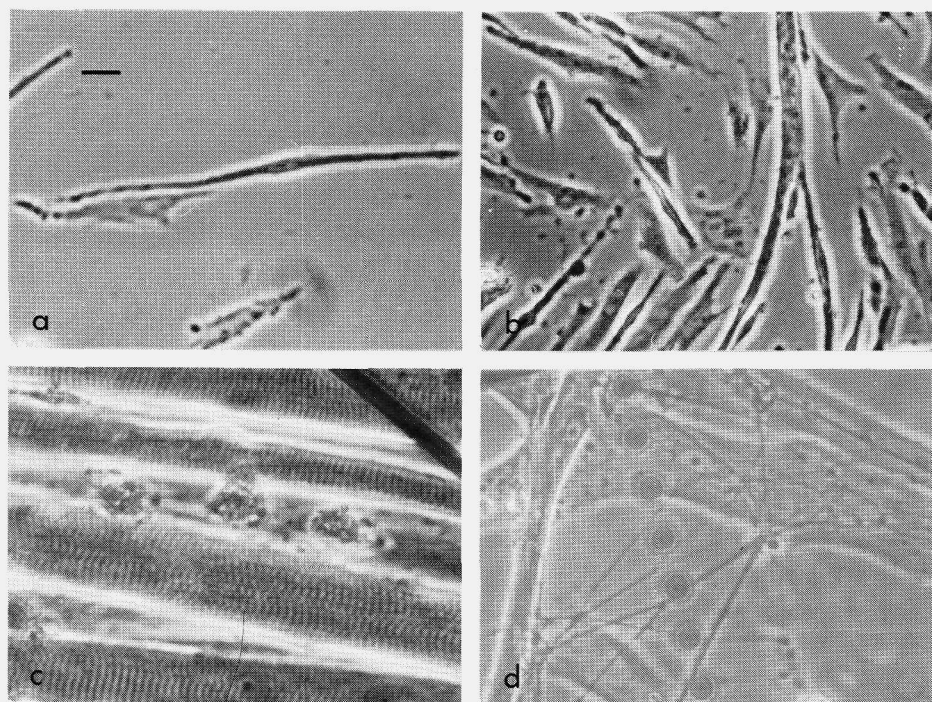


Fig. 6. Development of myoblasts in culture; a, mononucleated myoblast and some fibroblasts; b, fusion of myoblasts; c, myotube-stage with cross striation; d, myotubes innervated by spinal cord explant (explant not shown).

All photographs have the same magnification, calibration bar 25 μ m.

this time onwards. In spite of the ara-C treatment a confluent monolayer had formed after 8-10 days in culture. Vacuoles began to appear shortly afterwards and some muscle fibres were lost. Usually the loss of muscle fibres progressed very slowly or even seemed to stop after four weeks in culture. At that time the number of muscle fibres had stabilized more or less and most cultures contained cross striated, thick muscle fibres embedded in a monolayer of fibroblasts.

Mixed cultures with spinal cord explants

Slices cut from the spinal cord of mouse embryos were explanted on top of the muscle cells that had been cultured for two days. The explants attached within eight hours and started to form processes within 12 hours. Some of these processes were seen to be in contact with muscle fibres already one day after explantation (figure 6d). The muscle fibres near an explant were usually contacted by many neurites, however, in unstained cultures no specialized synaptic structures were observed.

Notwithstanding the treatment with ara-C on day 3 and 4, after 8 days in culture explants became surrounded by a sheath of proliferating cells, several cell layers thick, that was usually devoid of muscle fibres. In the area surrounding that sheath, muscle fibres were present. During cultivation the explants gradually thinned and in many of the neurons the nuclei and sometimes also the cytoplasm became visible after 15-18 days. Within the explants myelin was formed and many peripheral axons became surrounded by a myelin sheath that showed interruptions resembling nodes of Ranvier.

Silver impregnations of cultures older than three weeks showed bulbous swellings of neurites (figure 7). Peterson and Crain (1970) described similar specializations in their mixed cultures and thought they might be end plate structures. In our cultures, however, these swellings were found on muscle fibres as well as in areas that seemed devoid of any cell.

Sluggish contractions of the muscle fibres, lasting up to 5 sec, were observed from day 5 onwards in cultures without spinal cord explants. When the muscle fibres became older the contractions became faster. In the presence of spinal cord explants, however, muscle fibres situated in the vicinity of an explant already showed fast contractions after 4 days in culture, i. e. 2 days after the explants had been added. This suggests

that these muscle fibres had become innervated at that time.

Electrophysiology

Muscle fibres in mixed cultures

The average membrane resting potential of chick muscle fibres in culture was 75.1 ± 10.2 ($\pm$ S.D., $n=104$). This value did not change during the period from 3 to 25 days in culture (Hooisma, in preparation). Short lasting, small transient depolarizations were found in muscle fibres contacted by neurites. They were never encountered in muscle fibres grown in cultures without explants or in fibres situated at a great distance of any explant. In young muscle fibres only very slow depolarizations were observed, that accompanied the slow contractions described in the foregoing section. These slow depolarizations, so-called plateau potentials (Kano et al., 1972), had a rise-time of about 100 msec and sometimes lasted up to 5 sec, with a plateau at -30 to -40 mV during 3 sec.

The short lasting, small depolarizations in fibres near an explant were observed from 2 days until at least 4 weeks after the addition of the explant. They were probably a mixture of both miniature end plate potentials (mepp's) and end plate potentials (epp's). They could be blocked reversibly by perfusion of the bath with Tyrode's solution containing $1-2 \mu\text{g/ml}$ d-tubocurarine. Apparently, some kind of junction had been formed between the neurite and the muscle fibre where acetylcholine was released. The end plate depolarizations (mepp's + epp's) recorded from one muscle were usually very different in rise-time and time of half-decay. Epp's with the same amplitude showed sometimes an almost continuous range of times of rise and times of half-decay (figure 8). This large variation in time course of the end plate potentials was probably due to the presence of several junctions

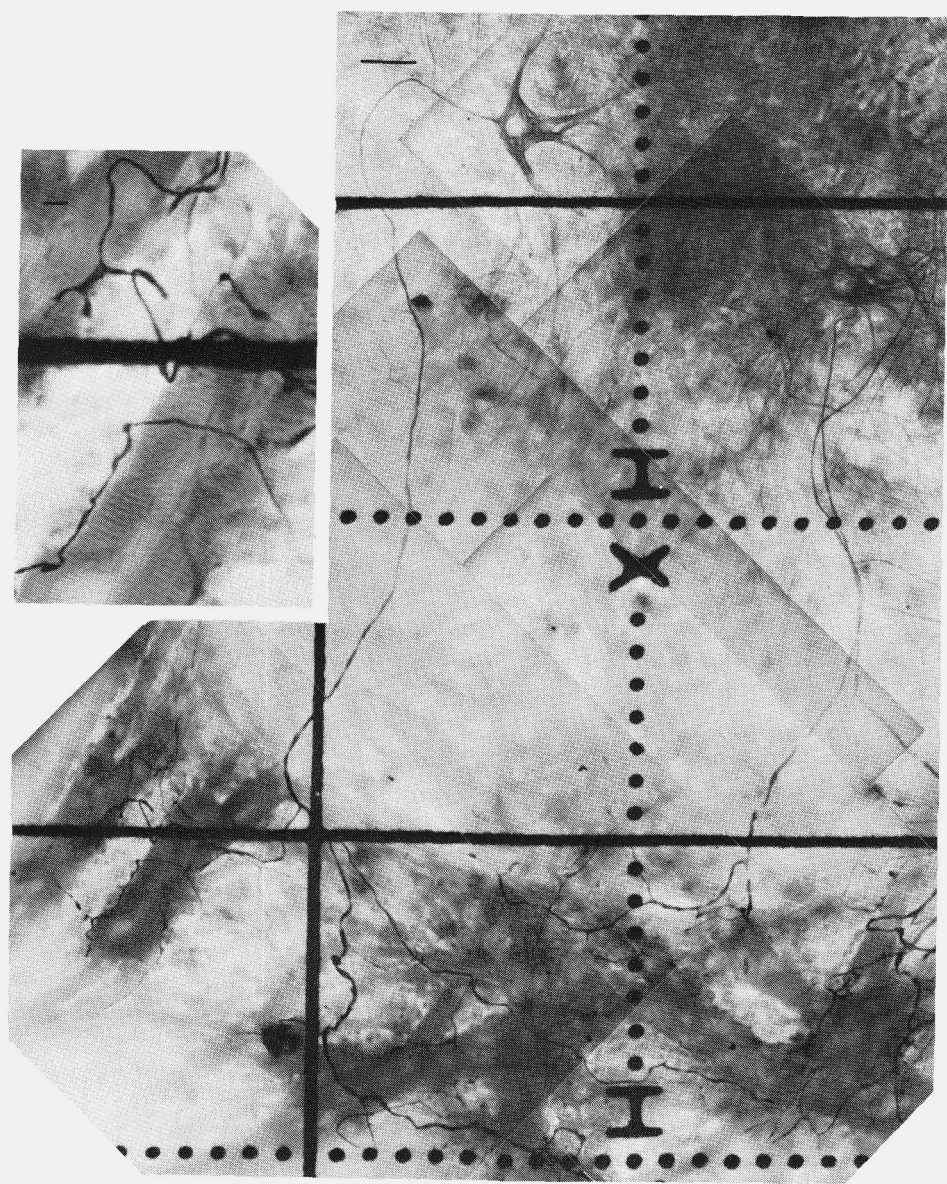


Fig.7. Ventral horn neurons in a spinal cord explant (top) in contact with myotubes (bottom). Silver impregnation, calibration bar 50 μ m, calibration bar in insert 10 μ m.

on the impaled muscle fibre, located at various distances from the tip of the microelectrode. Most muscle fibres in the vicinity of the explant seemed to be multiply innervated. Seldom were muscle fibres encountered with one or only a few distinct types of end plate depolarizations. The minimum times of rise and of half-decay were about 2-4 and 2-10 msec, respectively.

The amplitudes ranged from noise level, 0.2 mV to 40 mV. The epp's occasionally triggered active membrane potentials like plateau potentials and action potentials. Surprisingly not all epp's did so even if they were generated in a muscle fibre with a normal resting membrane potential and had an amplitude as large as 40 mV. A correlation between the age of the culture and the occurrence of triggered active potentials was not found. The amplitude distribution of the end plate depolarizations was either skewed to the right in multiply innervated muscle fibres or multimodal. The end plate potentials occurred either at random or in clusters, that could last up to 3 sec. If epp clusters were recorded in a muscle fibre they usually had the same duration. The frequency of the potentials in such a cluster was often so high that a sustained depolarization of the membrane potential resulted. No muscle fibres were observed in which epp clusters were present together with randomly occurring mepp's or epp's. Muscle fibres with either clusters or randomly occurring potentials could be found in the vicinity of the same explant.

Effect of tetrodotoxin

In order to test whether at the newly formed neuromuscular junctions end plate potentials were generated by action potentials, 1 µg/ml tetrodotoxin (TTX) was added to the Tyrode's solution in 6 experiments. TTX blocks the conduction of action potentials and thus abolishes the spike evoked end plate potentials. After addition of TTX the larger depolarizations were blocked reversibly, leaving the smaller depolarizations intact (figure 9).

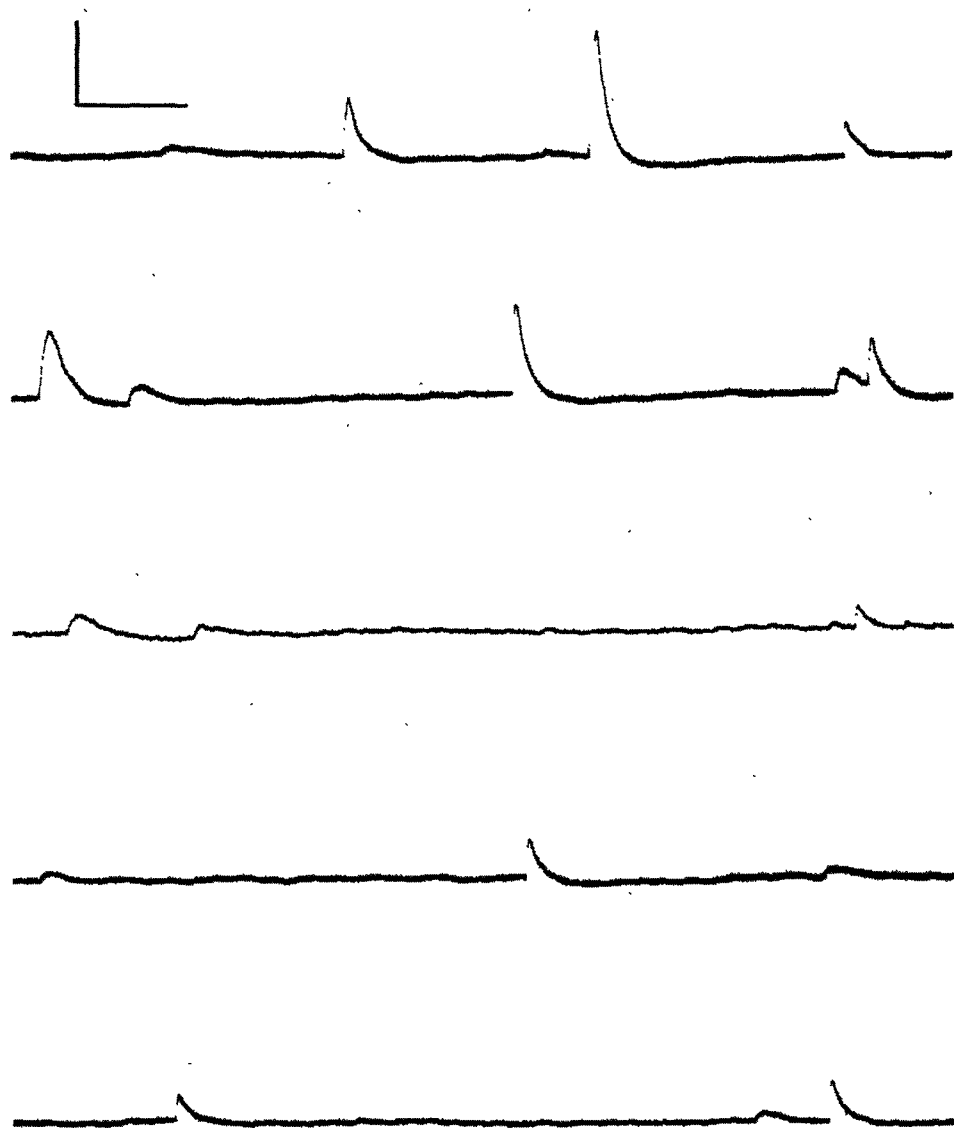


Fig. 8. Epp's recorded in one muscle fibre. Epp's with similar amplitudes have different rise-times and times of half decay which demonstrates multiple innervation.
Horizontal calibration is 100 msec; vertical calibration is 3 mV.

These smaller depolarizations had a unimodal skewed amplitude distribution.

It was therefore concluded that mepp's as well as epp's were generated at the newly formed junctions. During TTX application the number of small end

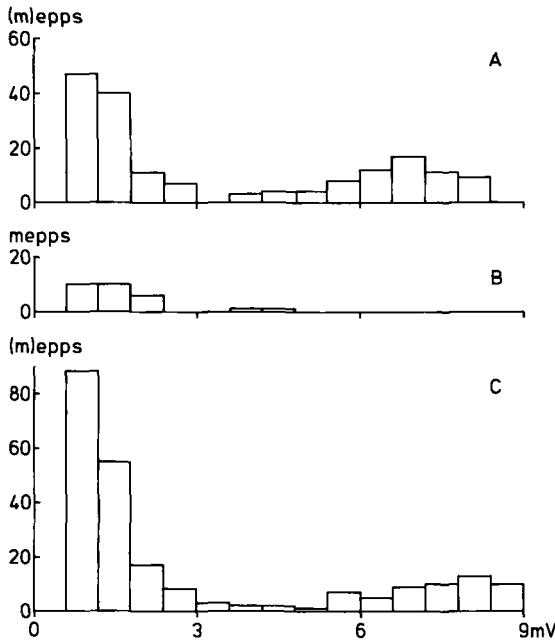


Fig. 9. Amplitude histograms of end plate depolarizations ((m)epp's) recorded intracellularly in an innervated muscle fibre during two minute periods. A: in Tyrode's solution. B: in Tyrode's solution containing 1 μ g/ml tetrodotoxin. C: in Tyrode's solution after wash-out of tetrodotoxin.

plate potentials was less than during perfusion with normal Tyrode's solution. The amplitude of the mepp's generated during TTX application was less than 5 mV, and their frequency ranged from 0.3 to 30 per minute. Time interval histograms of these mepp's could be fitted by a negative logarithmic distribution which demonstrated that they were distributed randomly in time.

Extracellular stimulation of the neurite

Conduction of action potentials in neurites that made contact with muscle

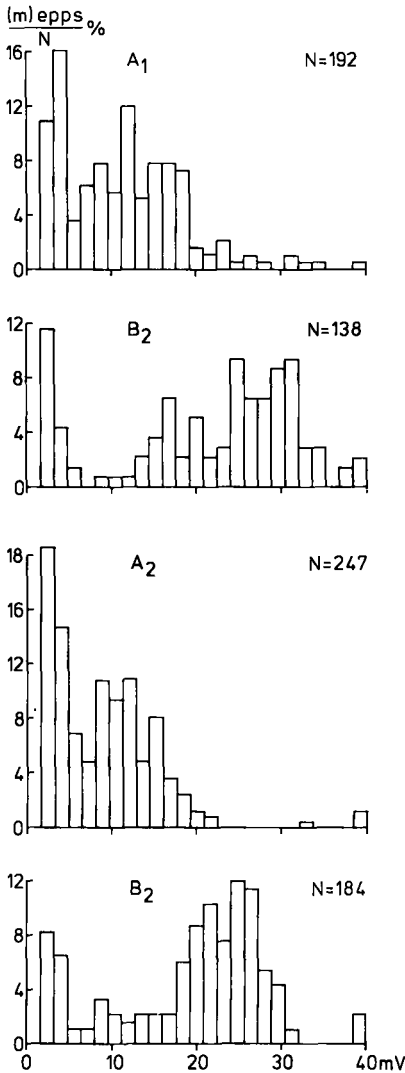


Fig. 10. Normalized amplitude histograms of (m)epp's, all recorded in one muscle fibre during two minute periods. A: in Tyrode's solution. B: in Tyrode's solution with a 20-fold increased $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio.

fibres was also demonstrated in 5 experiments by extracellular stimulation of the neurites. Stimulation lead to the generation of end plate potentials that had the same size as those occurring spontaneously in the fibre, and caused synchronous contraction in the innervated muscle fibre. In order to be successful, stimuli to myelinated neurites had to be applied at the interruptions of the myelin sheath. At other sites even very high stimulus intensities had no effect.

Effect of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio

A 20-fold increase of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio in the bath fluid causes a considerable increase in amplitude of the epp's in isolated nerve-muscle preparations (see Hubbard, 1973). In 6 experiments in muscle fibres innervated in culture, the influence of an increased $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio was studied. The Ca^{2+} concentration was raised 2-fold and the Mg^{2+} concentration was lowered 10-fold.

In 2 muscle fibres an increase in the number of large end plate potentials was observed. Many of the epp's became larger than the largest ones recorded in normal Tyrode's solution. In these 2 experiments the overall frequency of the end plate depolarizations was somewhat lower in the altered solution. The amplitude histograms of one of these experiments are shown in figure 10. In the other 4 muscle fibres no such changes took place. Instead, the frequency of the potentials increased considerably and a small decrease in amplitude of the largest epp's was observed (figure 11). In one of these 4

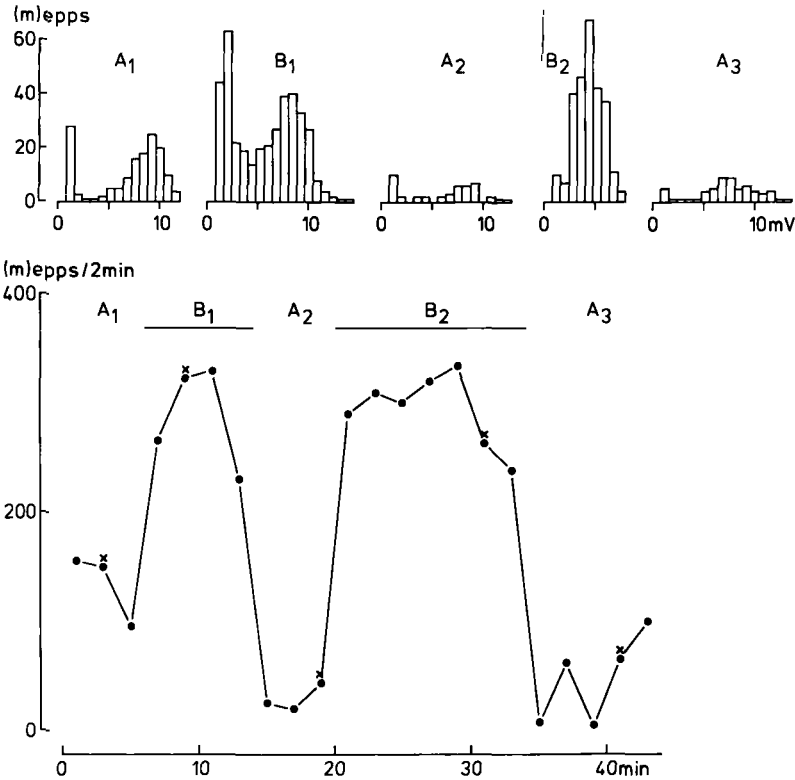


Fig. 11. Number of end plate depolarizations ((m)epp's) recorded during two minute periods in a muscle fibre in normal Tyrode's solution (A) and in a solution with a 20-fold increased $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio (B). The amplitude histograms of (m)epp's were recorded at times marked by the asterisks.

experiments a muscle fibre was impaled in which the end plate depolarizations occurred in clusters. The frequency of these clusters increased when the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio was raised (see figure 12). No end plate depolarizations were observed in the periods between the clusters. It was impossible to detect whether more depolarizations were generated during a cluster in the presence of the raised $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio because of the high frequency

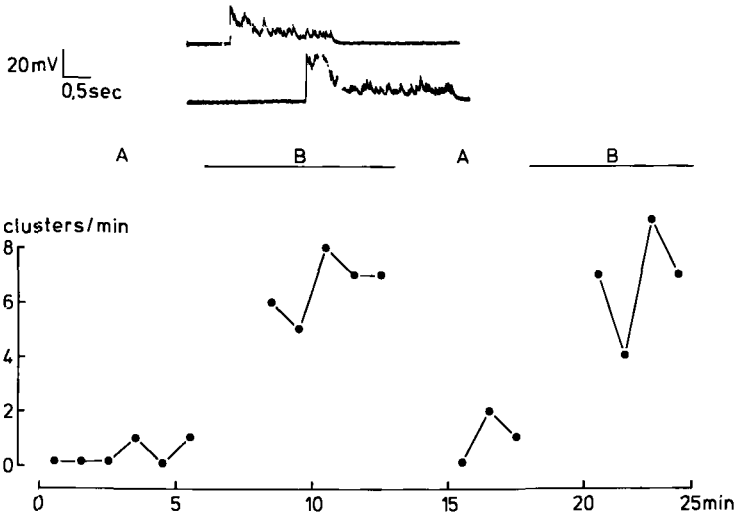


Fig. 12. Number of high frequency clusters (shown in upper traces) recorded during one minute periods in one muscle fibre in Tyrode's solution (A) and in Tyrode's solution with a 20-fold increased $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio (B).

of the depolarizations; their amplitudes were not changed. Thus in only 2 of the 6 muscle fibres the epp generating system reacted as it does in vivo. In order to investigate whether this anomalous behaviour was due to effects on the epp's or on the mepp's, the influence of the increased $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio was studied in the presence of TTX (1 $\mu\text{g}/\text{ml}$). Since this toxin blocks the conduction of impulses in the neurites, only mepp's will remain. The results of a typical experiment are given in figure 13. It appeared that in the presence of TTX there was no effect of a raised $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio on the

potentials.

In isolated nerve-muscle preparations a decrease in the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio causes a diminution of the amplitude of the epp's and a small decrease in amplitude and frequency of the mepp's (see Hubbard, 1973). In 7 experiments

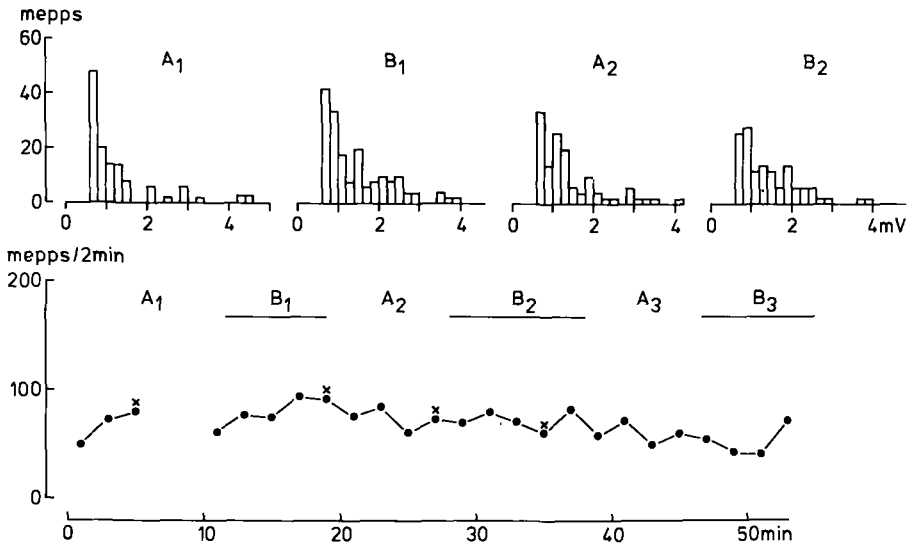
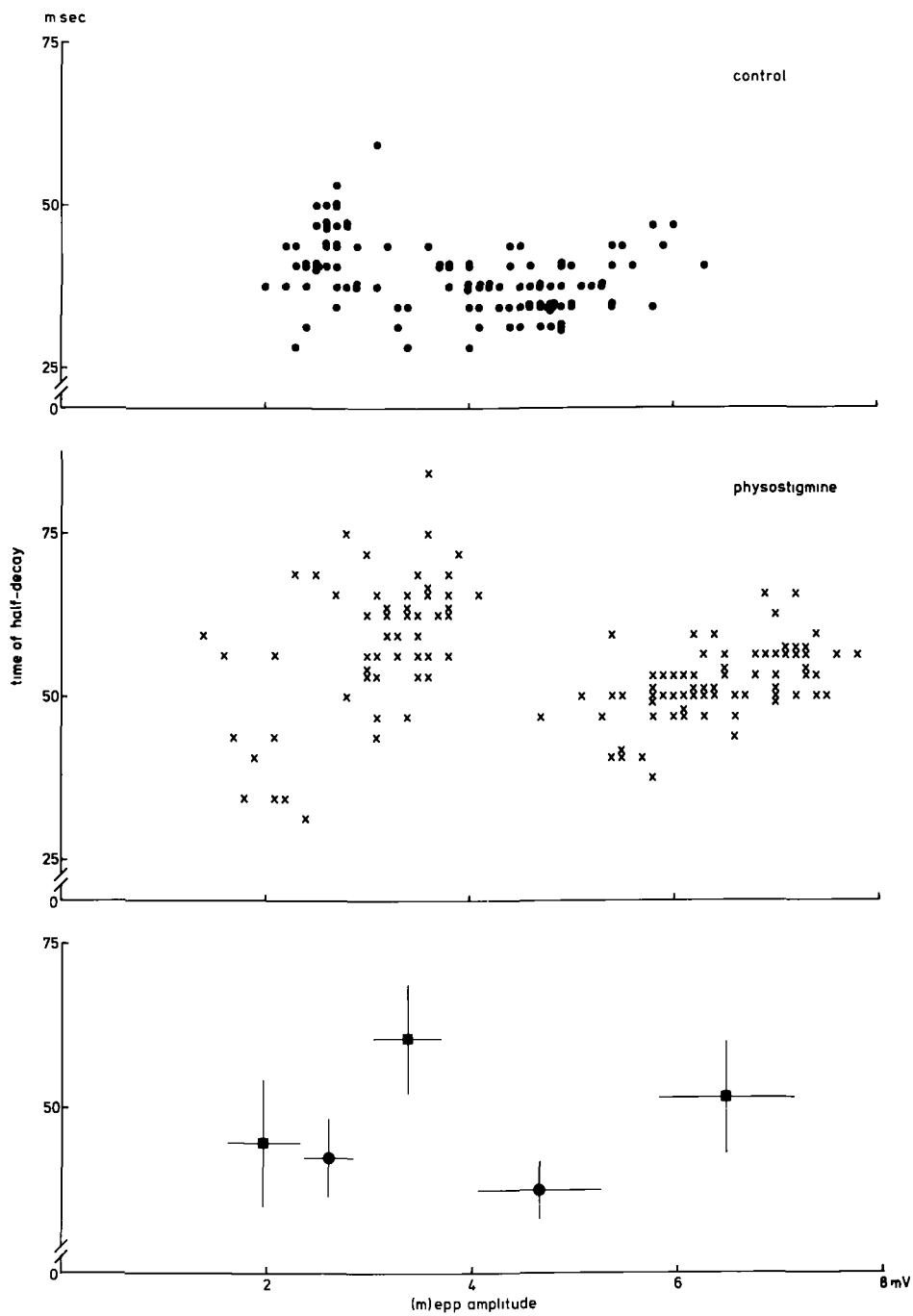


Fig. 13. Number of mepp's recorded during two minute periods in one muscle fibre in Tyrode's solution containing 1 $\mu\text{g}/\text{ml}$ tetrodotoxin (A) and in Tyrode's solution with a 20 times higher $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio also containing this toxin (B). The amplitude histograms of the mepp's were recorded at times marked by the asterisks.

in which the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio was lowered 40-fold, similar effects were noticed in innervated muscle fibres in culture. The amplitude of the largest epp's decreased and the frequency of the smaller potentials became lower than that of depolarizations of the same amplitude under control conditions. In 2 muscle fibres which in normal Tyrode's solution produced at least 75 depolarizations per period of two minutes, the end plate depolarizations were completely abolished in the altered bath solution.



Effect of physostigmine

In 4 experiments the effects of physostigmine (1 $\mu\text{g}/\text{ml}$) was studied in cultures of 19 to 22 days. In 3 experiments the frequencies of the end plate depolarizations were very high and the epp's occasionally triggered action potentials. Physostigmine reversibly increased the number of epp's that triggered action potentials. In some muscle fibres the frequency of action potentials more than doubled. Because of the very high frequency of the epp's it was often impossible to find enough individual epp's to measure their time of half-decay with reasonable accuracy. In one experiment this was possible and the data obtained are shown in figure 14. In this experiment only two classes of end plate depolarizations were recorded. During the physostigmine application a third class became apparent. The amplitude and the time of half-decay of the end plate depolarizations changed sufficiently to determine the effect of physostigmine on these parameters. Under control conditions the mean half-decay time was 40 msec, which was rather long. After addition of physostigmine the amplitude increased 30% and the time of half-decay 40%. In younger cultures no effect of physostigmine was found.

Fig. 14. Time of half decay of end plate depolarizations ((m)epp's) recorded in an innervated muscle fibre, in Tyrode's solution upper plot and in Tyrode's solution containing 1 $\mu\text{g}/\text{ml}$ physostigmine middle plot. Lower plot represents mean $\pm$ SD of grouped points in upper (■) and middle (●) plot. The depolarizations represented in the control plot were recorded before and after the period during which physostigmine had been added.

Discussion

The results of the present experiments showed that neuromuscular transmission occurs in mixed cultures of explanted embryonic mouse spinal cord neurons and embryonic chick muscle fibres. Examples of heterospecific innervation of muscle fibres have also been reported by other authors. Peterson and Crain (1970) obtained innervation in combinations of different mammalian tissues and Stevens et al. (1974) and Obata (1977) reported about combinations of mammalian and avian material.

In the muscle fibres presently studied the mepp's and epp's generated at the neuromuscular junctions were reversibly blocked by d-tubocurarine (1-2 $\mu\text{g/ml}$) in a concentration sufficient to block all end plate depolarizations in culture (Crain et al., 1970; Fischbach, 1972). Multiple innervation appeared to be a rule rather than an exception regardless of whether explanted fragments of neuronal tissue or dissociated neurons were presented; a phenomenon also observed by other authors (Robbins and Yonezawa, 1971; Fischbach, 1972). The time to peak and the time of half-decay of the end plate depolarizations were rather long compared with those reported from adult animals in vivo (Fatt and Katz, 1951; Boyd and Martin, 1956) but were within the range reported by Fischbach (1972) for epp's in mixed cultures of embryonic dissociated spinal cord neurons and muscle cells of the chick. In the presently studied muscle fibres, sometimes even fairly large epp's did not trigger active potentials. It seems unlikely that this was due to damage to the muscle fibres caused by the impalement, because the presence of the tip of the microelectrode in the fibre usually caused no marked changes in the membrane resting potential, even in experiments that lasted more than one hour. It could be that the chick muscle fibres

in culture to some extent resembled avian slow tonic fibres, in which propagated action potentials do not occur and electrical activity is spread by electrotonic conductance (Peachy, 1961).

It was observed that in most muscle fibres spontaneous end plate depolarizations occurred in a random order whereas in some fibres the epp's came in regularly appearing clusters. In the muscle fibres with random depolarizations TTX abolished the largest potentials, showing that these were induced by neuronal activity. TTX, however, also reduced the number of the small-amplitude depolarizations. In such multiply innervated muscle fibres, those epp's that are generated at a distance from the tip of the microelectrode are recorded with a smaller amplitude and a less steep rise and fall than epp's originating from a site nearby. Consequently, the disappearance of part of the small-amplitude potentials after TTX application might well be caused by abolishment of distantly generated epp's. However, evidence is available that in some types of muscle fibres increased epp activity is accompanied by an increase in the number of mepp's. Miledi and Thies (1971) found that following indirect tetanic stimulation of the frog sartorius the mepp frequency was 16 times higher than before. Fischbach (1972) observed in mixed tissue cultures a 100-fold increase of the mepp frequency after one or two epp's had been elicited. In fact, some junctions he studied only generated mepp's following an epp. Thus it might well be that reduction of the number of epp's to zero by TTX also reduced the number of mepp's. It is likely that in at least two of the present experiments this mechanism has been in operation because in those instances, prior to TTX application no epp's were conducted to the microelectrode from a distance because they all had a steep rise and fall, and still TTX lowered the number of small-amplitude potentials. A third mechanism which might lead to an overall reduction in the frequency of the end plate depolarizations could be a reduced synaptic transmission in neuronal networks in the spinal cord

explant, resulting in less nerve impulses.

It seems plausible that the results of the experiments in which the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio was lowered in the bath fluid, can be explained along the same lines. A large fall in this ionic ratio is known to reduce the quantum content of the epp to such an extent that the majority of the oncoming nerve impulses fail to elicit an epp (Del Castillo and Katz, 1956; Fischbach, 1972). In the cultured muscle fibres this may lead to a reduced output of mepp's which follow in the wake of epp's.

An increase in amplitude and frequency of the large end plate depolarizations, observed in some muscle fibres after the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio had been raised, was expected. A concomitant increase in frequency of the small-amplitude potentials could be caused by a recruitment of distant epp's, more mepp's following in the wake of epp's and an increased activity of interconnected neurons. In some fibres the control frequency was already rather high and was still increased by the altered ionic composition of the bath fluid. In such cases an increase in amplitude of the large epp's was not always seen. This may have been due to a shortage of ACh vesicles in the still primitive junctions. It is known that the quantum content of epp's generated in newly formed junctions in tissue culture as well as in vivo is low compared with that in adult preparations (Fischbach, 1972; Dennis and Ort, 1976).

In those muscle fibres which produced clusters of end plate depolarization no depolarizations were seen during the intervals between the clusters. The cluster-type activity was neuronal in origin because: i it never occurred in non-innervated fibres, ii d-tubocurarine always blocked cluster activity, and iii, most important, TTX abolished cluster activity. Crain and co-workers (see Crain, 1976) extracellularly recorded more or less regularly occurring bursts of activity in cultured spinal cord explants and in muscle fibres which were contacted by the neurites growing from such explants. They also

observed intensified burst-type activity after addition of strychnine to the bath, which was thought to cause block of inhibitory pathways in the neuronal networks which presumably had developed in the cultures. In preliminary experiments (Hooisma et al., 1975b) intracellular recording from large neurons in explants of ventral horn of the embryonic mouse spinal cord, demonstrated rather regularly appearing extended depolarizations (1-25 sec) that seemed to be due to excitatory activity originating from other neurons in the explant. These sustained depolarizations often triggered action potentials in the impaled neuron. The present results suggest that in the mouse spinal cord explants some neurons innervating muscle fibres, become interconnected in a network which generates burst-like activity. With regard to the finding that multiple innervation is the rule in such cultures, the absence of epp's between the clusters of epp's in the muscle fibres may have to be explained by assuming that junctions from neurons not participating in the burst activity are either suppressed in their activity or removed altogether. If such a developmental process indeed occurs in the cultures this would indicate that in some instances the junctions are well on the way to maturity.

Morphologically, little can be seen with phase optics of a possible specialization of the junctions, and maturity could not be judged in this way. Myelination indicates a certain maturation of the neurites. In silver impregnated preparations bulbous swellings, presumably end plate structures, were observed. Such swellings, however, not only occurred in relation to muscle fibres but also in parts of the cultures where no cells seemed to be available to form a synapse with.

The idea that the junctions developed to a fairly mature state is supported by the finding that functional acetylcholinesterase was present after three weeks. Hitherto the presence of this enzyme was electrophysiologically only demonstrated via the anti-curare action of eserine

(Crain, 1966; Crain et al., 1970). A further indication of maturation of the junctions presently studied is given by the results of studies on the distribution of acetylcholine (ACh) sensitivity on muscle fibres. Under the conditions prevailing in the present experiments, the ACh sensitivity on non-innervated fibres remains evenly distributed along the fibre. On innervated fibres, however, small areas of about 15 μ m in diameter with a 6-fold higher sensitivity develop in about a week (Slaaf et al., in preparation).

The experiments described have shown that mouse spinal cord neurons successfully innervate chick skeletal muscle fibres in tissue culture. The fact that the junctions formed had many physiological features in common with junctions *in vivo* and with junctions formed between homospecific tissues in culture, seems to justify the use of this combination as a model for the study of innervation and trophic support of cultured muscle fibres.

S u m m a r y

1. Electrophysiological and pharmacological properties of neuromuscular junctions formed between mouse spinal cord explants and chick skeletal muscle fibres in tissue culture have been investigated.
2. Functional neuromuscular junctions were formed within two days after addition of a spinal cord explant.
3. The newly formed junctions were sensitive to d-tubocurarine.
4. AChE was present at junctions in cultures older than 19 days.
5. Action potentials were conducted from the explant to the muscle fibres. They could be blocked by tetrodotoxin.
6. In some muscle fibres spontaneous epp's occurred at random, in others they appeared in fixed patterns of clusters.
7. Increase of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio of the perfusion fluid

increased either the amplitude or the frequency of the epp's, but did not affect the mepp's. Decrease of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio decreased the amplitude of the epp's and the frequency of the mepp's.

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CHAPTER IV

TROPHIC SUPPORT OF CULTURED MUSCLE FIBRES BY NEURONAL EXPLANTS

Introduction

Development of skeletal muscle is affected by neuronal trophic factors (Guth, 1968). Muscles in non-innervated limb grafts of chick embryos do not differentiate beyond a certain stage and atrophy of the muscles follows if innervation is not established (Eastlick, 1943). Trophic factors are not only required for the development of muscles but also for the maintenance of their structure and function (Gutmann, 1976). Neurons grown in tissue culture can exert trophic influences on muscle fibres. Morphological and electrophysiological properties of the muscle fibres are affected by the presence of spinal cord explants (Crain and Peterson, 1974; Engelhardt et al., 1976). Muscle fibres in explants of adult rodents do not differentiate unless they become innervated by spinal cord neurons (Peterson and Crain, 1972) and innervated muscle explants that have developed in culture atrophy after denervation (Robbins and Yonezawa, 1971). Many different neurons can survive and differentiate in tissue culture (Nelson, 1975). Neurons from the spinal cord, the ciliary ganglion and the thoracic sympathetic ganglion have been shown to innervate skeletal muscle fibres (Shimada and Fischman, 1973; Hooisma et al., 1975; Betz, 1976; Nurse and O'Lague, 1975). The ability to exert a trophic effect on muscle cells might either be common to all neurons, or only to those that can innervate these cells.

The activity induced in muscle by neuronal action potentials plays an important role in the trophic regulation of muscle properties (Lömo, 1976), but also hitherto unidentified trophic substances seem to be important (Drachman, 1976). Tissue culture offers unique opportunities to study the role of activity and trophic factors, because i activity can be blocked pharmacologically during long periods without interference with other parts of the organism, ii the morphological parameters of the same muscle fibres can be assessed at suitable intervals, iii with electrophysiological techniques the electrical parameters of these fibres can be measured at any moment during their development.

In the experiments presented in this paper the influence on the morphology and the survival of cultured chick muscle fibres exerted by spinal cord explants was compared with that exercised by neurons in cortex slices, dorsal root ganglia, sympathetic ganglia or ciliary ganglia obtained from mouse and chick embryos. The effect of innervation and neuronal activity (epp's) on the morphological development of muscle fibres was studied in co-cultures of muscle cells and spinal cord explants.

Materials and methods

Tissue culture

Muscle cells of chick embryos were cultured either alone or in mixed cultures together with neuronal tissues. The muscle cells were obtained from leg muscles of 11 day old embryos by trypsin dissociation (Harvey and Dryden, 1974). The suspension of cells was plated at a density of $5 \cdot 10^4$ cells per cm^2 on collagen coated "Cellfinder" object glasses (Micropure, Driebergen, The Netherlands) and cultured in 1.8 ml medium (see below) in 60 mm Petri dishes. In mixed cultures neuronal tissues of various origins

were added to muscle cells that had been in culture for 2 days. Explants of spinal cord were obtained either from 7 day old chick embryos or from 14 day old mouse embryos. To obtain such explants the cord was dissected and freed of dorsal root ganglia (DRG's); meningeal tissue was not removed. Subsequently, transverse slices were cut from the spinal cord, as thin as possible with the aid of two razor blades. To make cortex explants a thin slice, cut from the surface of the cerebrum, was fragmented in Tyrode's solution with a fine pair of scissors.

Dorsal root ganglia were usually taken from the lumbar segment although some thoracic DRG's may have been included. The 7 day old chick embryo was used as a source for ciliary ganglia and thoracic sympathetic ganglia. Usually 8 to 10 explants or ganglia were explanted per culture dish, with the exception of the ciliary ganglia which were always used in a quantity of 4 per dish. All neuronal material was dissected under a low-power stereo microscope in a sterile laminar flow cabinet. The explants or ganglia were allowed to settle on the layer of muscle cells while covered with a thin film of medium during 8 h in a moist chamber, after which period 1.8 ml medium was added.

The culture medium consisted of 70 parts Eagle's minimum essential medium in Earle's balanced salt solution (10.96 g Gibco powder in 1 liter distilled water), 10 parts NaCl solution (8.07 g/l), 15 parts horse serum (Flow, Scotland). The minimal essential medium was supplemented with L-glutamine (200 mg/l), biotin (0.007 mg/l), ferric nitrate (0.7 mg/l), NaHCO_3 (1.71 g/l), glucose (0.7 g/l), penicillin (7000 U/l) and streptomycin (7 mg/l). Osmolarity of the complete medium was adjusted to a final value of 320 mOsm. The medium was equilibrated against a water-saturated atmosphere of 95% air and 5% CO_2 . During the first 2 days 5 parts of freshly prepared chick embryo extract were added. Unless stated otherwise this extract was omitted during the remainder of the culture period and 5 parts Tyrode's

90

solution were added instead. The temperature was kept at 37°C during the first 2 days and at 35°C during the rest of the period. The medium was changed every 2 days. During day 3 and 4 arabinofuranosylcytosine (ara-C) was added to the medium in a concentration of 10^{-5} M in order to reduce proliferation of dividing cells. The embryo extract was made as follows, 20 chicken embryos of 10 to 11 days gestational age were washed in cold Tyrode's solution, transferred to a 10 ml injection syringe and pressed through the nozzle (Record type). The material was then shaken together with an equal volume of culture medium and centrifuged for 2 h at 18,000 g and 4°C. The supernatant was used, the sediment and the upper layer of lipid material were discarded.

Morphological evaluation

In preliminary experiments it appeared that for the evaluation of the morphological condition of muscle fibres 4 parameters were important: i the amount of cross striation, ii the apparent thickness of the fibres judged from the phase boundaries as seen through phase-contrast optics, iii the number of fibres with vacuoles, and iv the total number of fibres per unit area. For each of these parameters the condition of a culture was scored according to a five point scale from 0 to 4, with 4 for the best condition.

In order to score the total number of muscle fibres a culture was inspected through a 2.5x objective, and a phase-ring II was employed between light source and culture in order to obtain a kind of dark-field illumination; the whole culture was scanned. To score the other parameters the culture was viewed through a 40x phase-contrast objective. Homogeneous cultures, without a neuronal explant, were scanned 3 times over the whole length of the object glass (25 mm). In a culture containing explants the so-called "relevant areas" around the explants (see Results) were scanned. Table 2 presents a brief description of the various criteria employed for the scoring

Table 2. Criteria for scoring the morphological properties of muscle fibres.

score	cross striation	apparent thickness
0	no striation	fibres thin like fibroblasts
1	a few fibres faintly striated	a few are clearly thicker
2	more fibres, more clearly str.	no fibres like fibroblasts
3	good striation in many fibres	some with pronounced phase boundaries
4	most fibres show good striation	many with marked phase boundaries

score	number of vacuoles	total number of fibres
0	no fibres without vacuoles	hardly any fibres present
1	a few without vacuoles	18-20 per mm ²
2	about 50% with vacuoles	better than "1" but no full culture
3	fibres with vac ^s . easy to find	a full culture
4	fibres with vacuoles rare	fibres densely packed

of the morphological properties of the muscle fibres. The term "muscle fibre" is used for all multinuclear muscle cells irrespective of their stage of development. Within a series of cultures a high degree of similarity between the individual cultures was obtained. Each condition was generally tested in at least 3 culture dishes (triplo's) and their scores were averaged. During the culture period the morphology was usually evaluated 2 or 3 times, the first time on day 5 in culture. The morphological condition of the muscle fibres in each culture dish was scored by an investigator who was unaware of the treatment of the culture. Different experimental conditions have always been studied in at least 3 independent series of cultures.

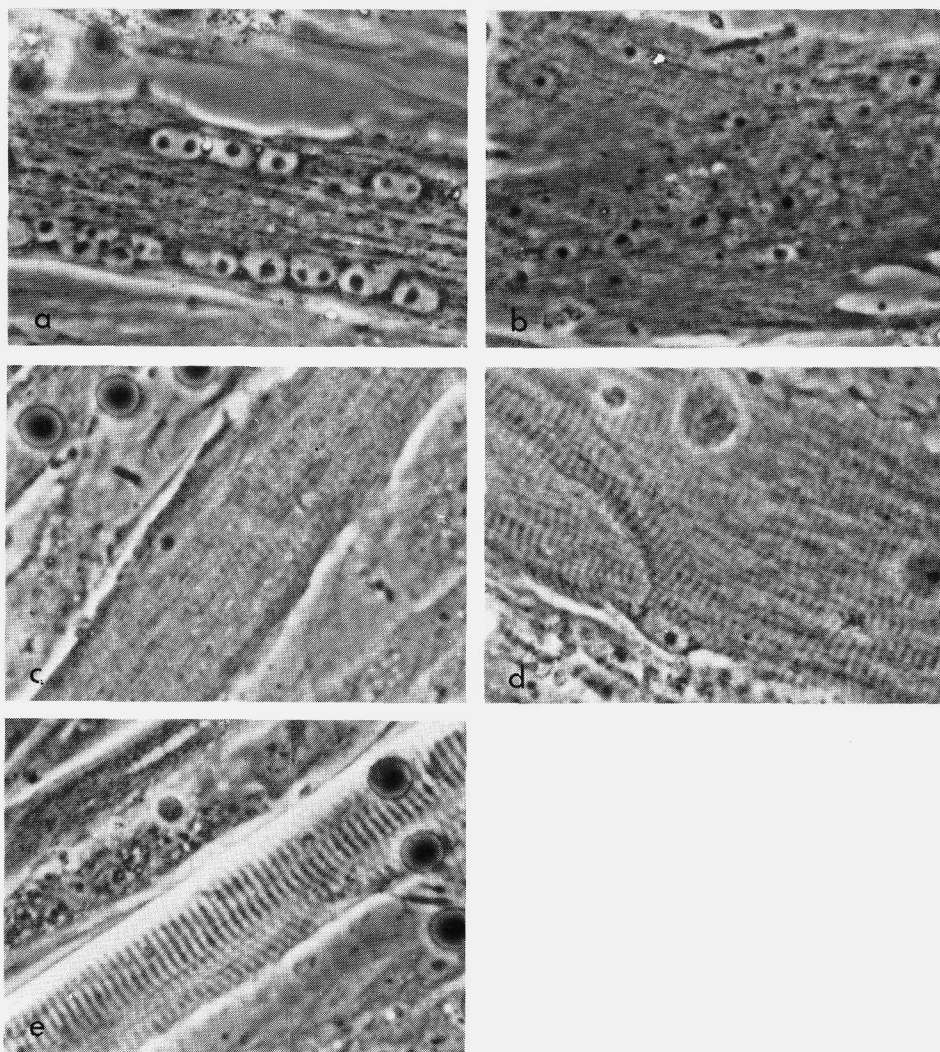


Fig. 15. Cross striation of cultured muscle fibres. Photographs of muscle fibres to illustrate the amount of cross striation in fibres with ratings from a-e.

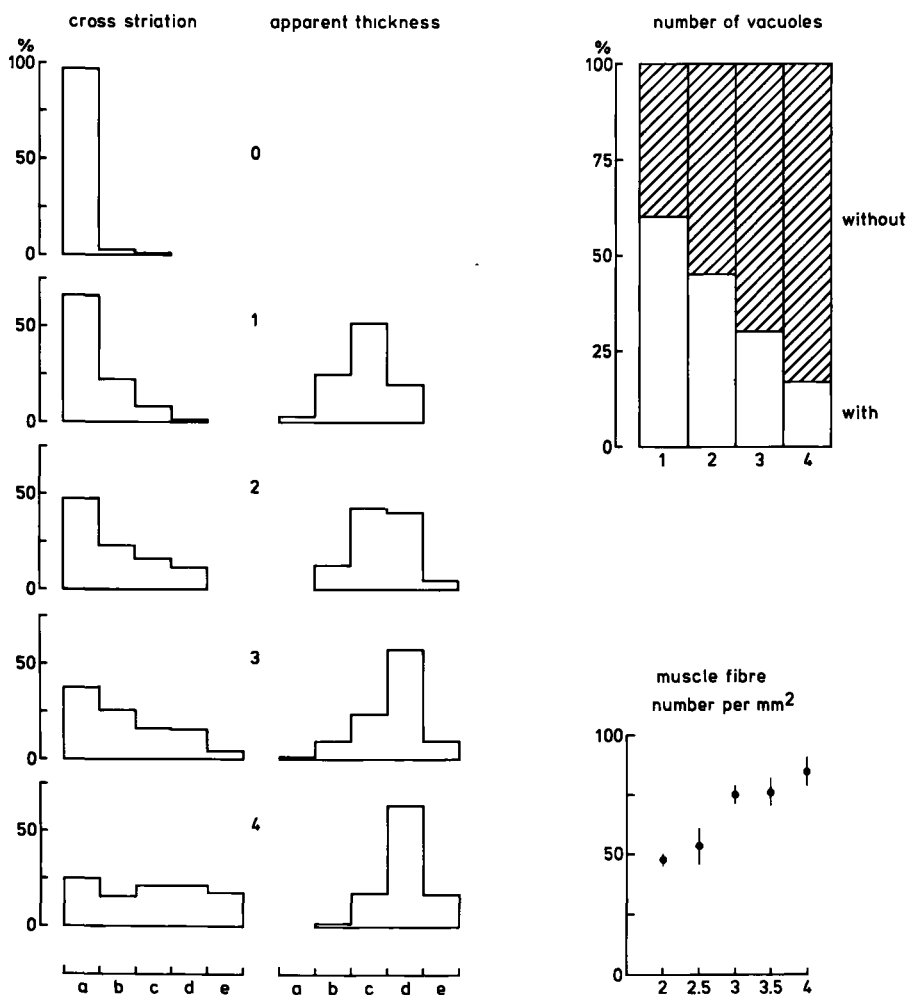


Fig. 17. Morphological properties of 2732 muscle fibres in 40 different cultures.

Left column: Cross striation in the 40 cultures was rated 0-4. Cross striation of the individual muscle fibres in these cultures was rated from a-e. The data on individual muscle fibres for cultures with the same score were pooled. For each "whole culture" rating 0-4 the degree of cross striation for individual muscle fibres (a-e) has been plotted against the percentage of fibres with that degree of cross striation.

Second column: Apparent thickness in individual fibres in cultures rated from 1-4. Cultures with rating 0 for apparent thickness were not found among the 40 cultures evaluated, and they were scarce in all subsequent experiments.

The morphological evaluation was practised and tested on cultures of muscle cells in various stages of development or degeneration. It was investigated to what degree two observers came to the same result. Both observers made independent estimates of the same set of 76 cultures. The mean differences between their results, expressed in score units $\pm$ SEM were: cross striation -0.01 ± 0.06 , apparent thickness 0.26 ± 0.09 , absence of vacuoles -0.01 ± 0.09 , total number 0.08 ± 0.06 . These results show that, although for thickness the difference was statistically significant, no systematic differences were obtained which could jeopardize subsequent results.

When it thus had been demonstrated that the scoring method was independent of the observer it was investigated what the observers were scoring. Forty cultures were independently and "blindly" scored as described, and subsequently in each culture 12 squares of $400 \times 400 \mu\text{m}$ were randomly chosen for detailed evaluation. In each square the total number of muscle fibres and branches of fibres was counted as well as the number of vacuolated fibres. Moreover, each fibre was evaluated as to amount of cross striation and apparent thickness. For the latter two properties each individual fibre or branch was rated according to a five point scale running from a to e with a for the lowest rating. Figures 15 and 16 present photographs of typical muscle fibres as encountered in this study and rated as described, whereas figure 17 presents graphs in which the scores for whole cultures are compared with the counts and ratings of individual fibres in these cultures. In cultures scoring 0 for cross striation it appeared that practically all individual fibres were devoid of cross striation whereas at score 4,

Right upper graph: Percentage of individual muscle fibres with and without vacuoles in cultures rated from 1-4.

Right lower graph: Number of muscle fibres (mean $\pm$ S. E. M.) per mm^2 in cultures with different scores. Cultures with scores lower than 2 were not found among the 40 cultures evaluated.

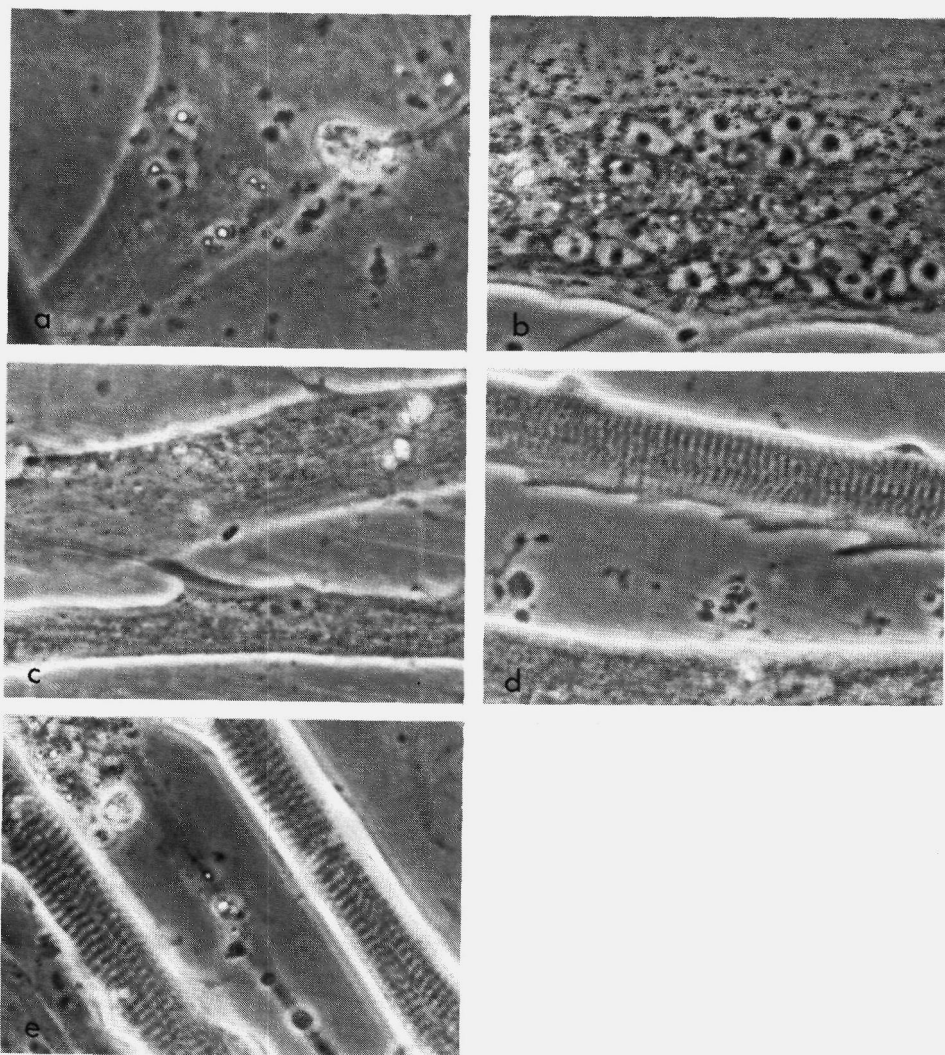


Fig. 16. The apparent thickness of cultured muscle fibres as judged from cell boundaries observed with phase-contrast optics. Photographs to illustrate the ratings a-e given to individual muscle fibres.

more than 30% of the fibres were in the two best categories. For apparent thickness the differences were less clearcut. The counts for vacuolated fibres and for total number showed a very satisfactory correlation with the respective scores of the cultures as a whole.

For each parameter the homogeneity of the variances within the triplo's, over the whole of each experiment was tested with Bartlett's test (see Snedecor, 1956). Since no heterogeneity was found, the variances were pooled. With these pooled variances, of which the values are shown in the figures, a Student's t-test was carried out for each pair of triplo's. Based on the null hypothesis that differences found between paired triplo's were due to chance alone, these t-tests were not inter-dependent and could therefore be combined according to the method of Stouffer (de Jonge, 1963). From each t-value and its number of degrees of freedom, the deviation (z) from the standard normal distribution was calculated (Wijnans, unpublished). These z-values were combined, with due regard to their sign, by summing them and dividing by the square root of the number of z's. This combined z-value was compared with the z-value for the two-tailed level of significance of 95%.

Electrophysiological measurements

For electrophysiological measurements a culture was placed in a temperature controlled bath at 37°C , that was constantly perfused with prewarmed Tyrode's solution previously gassed with 95% air and 5% CO_2 . Membrane potentials were measured with 3 M KCl filled microelectrodes (10–20 $\text{M}\Omega$) in muscle fibres that had been in culture for 5 to 25 days. The electrodes were connected to a negative capacitance electrometer amplifier with a gain of 10x. Resting potentials were recorded on a Heathkit penwriter. End plate potentials were displayed by a Tektronix 5103N storage oscilloscope with 5A22N differential amplifiers and stored for further analysis by a

Hewlett-Packard 3960 instrumentation recorder. Data from cells that did not show a stable resting potential for at least 2 min were discarded.

R e s u l t s

Morphological differentiation – effect of embryo extract

In cultures of muscle cells the fusion of the mononucleated cells (myoblasts) started during the first 24 hours of cultivation. In medium that contained embryo extract throughout the whole cultivation period, many multinucleated cells had nuclei lying in the centre of the cell on day 3. The first faintly visible cross striation appeared on day 4 and was pronounced and abundantly present on day 5. Notwithstanding the presence in the medium of ara-C during the days 3 and 4, the dividing cells between the muscle fibres grew to confluency in 8 to 10 days. At that time most muscle fibres had branches, were several millimeters long and many thick ones had hypolemmal nuclei. After 10 to 12 days vacuoles in the muscle fibres became conspicuous and fibres began to disappear.

In cultures which were left without embryo extract after the first 2 days, the muscle fibres developed only a faint cross striation or none at all. In such cultures fewer muscle fibres developed, vacuolization began as early as around day 5 and deterioration progressed more rapidly. If embryo extract was left out altogether the results were still worse; only a few muscle fibres developed and these disappeared at a very early stage.

For the testing of the effect of neuronal explants on the morphology of muscle fibres, the fibres were given time to develop during the first 2 days in the presence of embryo extract. It appeared to be essential to withdraw this extract when the fibres were brought in contact with the explants. If the explant provided adequate support, the morphology of the muscle fibres

would be maintained or further developed, if not the fibres would gradually deteriorate like those in the control culture. In pilot experiments it had been observed that if this schedule was employed the presence of a spinal cord explant had a favourable effect on the muscle fibres, but such an effect was only occasionally noticed if embryo extract was present throughout the whole culture period.

Muscle fibres together with chick spinal cord

Thin slices of spinal cord from 7 day old chick embryos were explanted on 2 day old cultures of muscle cells. Within the next 48 h muscle fibres lying near an explant became innervated and began to exhibit fast spontaneous contractions which were clearly different from the much more infrequent and sluggish spontaneous contractions of non-innervated fibres. In three 6 day old mixed cultures 54 muscle fibres that visibly seemed to be contacted by nerve processes were examined electrophysiologically; 49 of these fibres showed spontaneous end plate potentials (epp's).. Such epp's could be blocked by the addition of d-tubocurarine (d-TC) in a concentration of 1 µg/ml, demonstrating that cholinergic innervation had taken place.

In the mixed cultures many muscle fibres situated in the vicinity of a spinal cord explant became cross striated after 4 days in culture. Distant fibres closely resembled those grown in cultures without explant; they had no cross striation, were thinner in appearance and became vacuolated at an early stage. Explants in cultures older than 8 days became surrounded by an area containing proliferated cells and almost devoid of muscle fibres. This area was surrounded by a 200–400 µm wide band of well differentiated muscle fibres, which were clearly innervated. The muscle fibres in this area seemed to be under the influence of the explant. Henceforth this area, concentric with the explant, will be called the "relevant area".

From day 5 onwards the morphological properties of the muscle fibres in

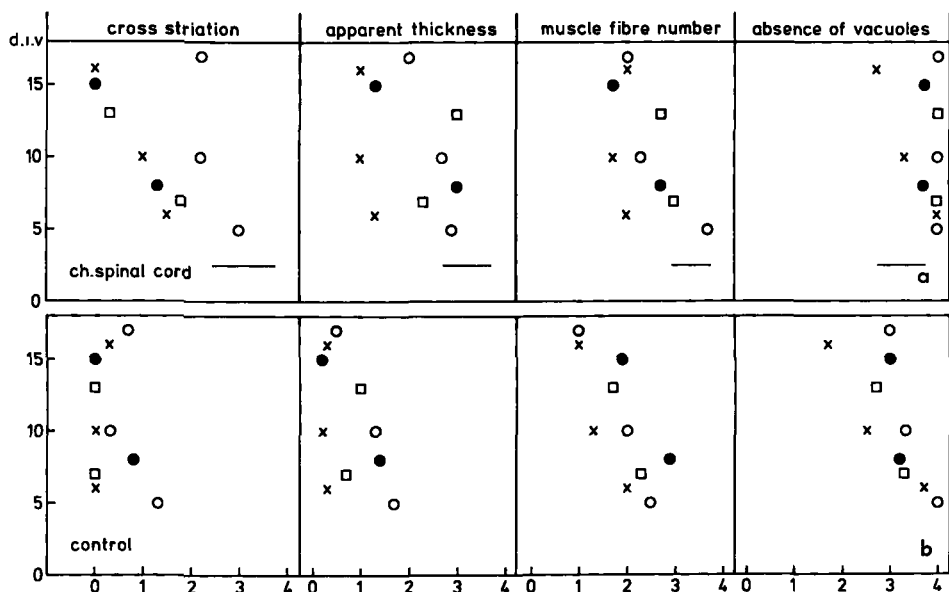


Fig. 18. The influence of spinal cord explants of chick (ch) embryos on the morphology of muscle fibres. Muscle cells were cultured for 48 hours in the presence of embryo extract, thereafter medium without embryo extract was used. In the experimental cultures spinal cord explants were added after 48 hours. Abscissa: mean score of the various morphological properties of the muscle fibres. Ordinate: days in vitro (d.i.v.).

a. morphological properties of muscle fibres in mixed cultures of spinal cord and skeletal muscle cells.

b. morphological properties of fibres in control cultures without neuronal explants.

The symbols: O, x, □ represent mean ratings each of 3 cultures; ● : mean ratings of 4 cultures. bar = 2 S. E. M. of all experimental and control cultures.

the relevant areas of such (experimental) cultures with chick spinal cord explants were scored and compared with those in cultures without explants (controls). The results are graphically presented in figure 18 (a and b), plotted against time in culture. Figure 19a shows the differences between the scores of experimental cultures and those of the corresponding controls. These differences were calculated by subtracting the mean score of the control cultures found on one particular day from that of the experimental

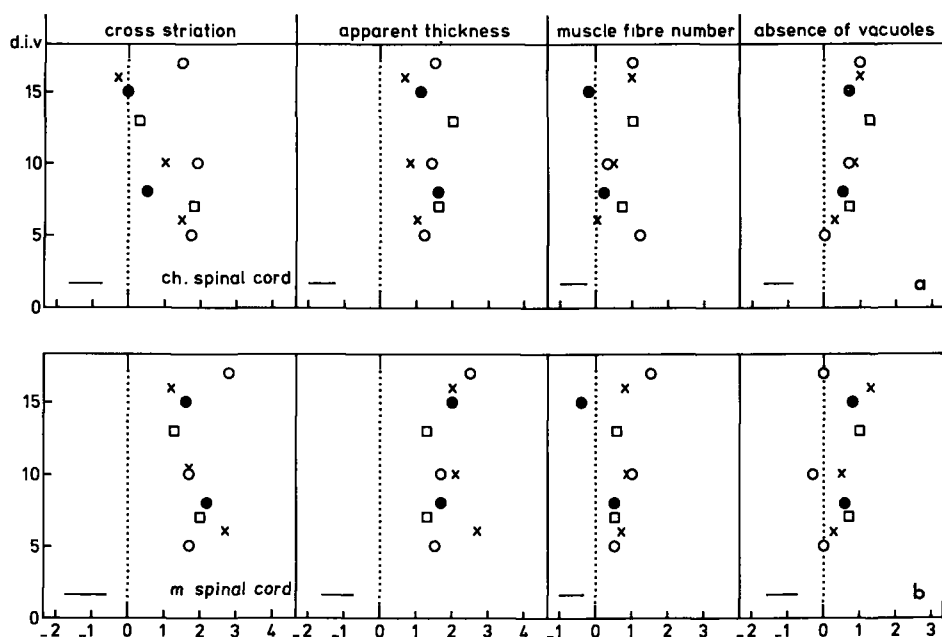


Fig. 19. a. The relative influence of spinal cord explants of chick (ch) embryos on the morphology of muscle fibres. Abscissa: differences of the values presented in fig.18 (differences of the mean score for mixed cultures and for the corresponding test cultures both rated on the same day in culture). Ordinate: days in vitro. All subsequent figures up to figure 22 are composed in the same way.

b. Influence of mouse spinal cord. Differences between the mean scores for experimental cultures and controls (without explants) on the same day are plotted against time in culture.

Symbols: O, x, □ : mean of 3 cultures; ● : 4 cultures. bar = 2 S. E. M.

cultures. A markedly favourable influence of the explants on cross striation and apparent thickness was found whereas the effect on the other two parameters, although positive, was less pronounced.

In order to compare the effect of the spinal cord explants on the morphological parameters with the effect of other types of explants, it was of advantage to express such effects by single figures of merit. Such figures must, however, be handled with care since the evaluations of the influences of

Table 3. Figures of merit of cultures of chick skeletal muscle fibres supplied with different neuronal explants. Figures marked ' different from zero at a statistical level of $P_2 < 0.05$.

	innervation ratio	cross striation	apparent thickness	muscle fibre number	absence of vacuoles
<u>chick</u>					
spinal cord	90%	1.2'	1.4'	0.5'	0.6'
ciliary ganglion	85%	0.2	0.8'	0.3'	0.7'
sympathetic ganglion	30%	-0.4	-0.2	0.1	0.0
<u>mouse</u>					
spinal cord	90%	2.1'	1.6'	0.8'	0.6'
		1.9'	1.8'	0.7'	0.4'
cortex	0	0.9'	0.7'	0.3'	0.4'
dorsal root ganglion	0	0.2	0.3	0.2	0.7'

the different explants could usually not be based on comparisons with one and the same series of control cultures, moreover, the experiments were spread in time over one year. Figures of merit were calculated by averaging the differences found between experimental and control scores obtained for a certain parameter with a certain type of explant. Since the durations of the culture periods used for the evaluation of the effects were unequal for the various types of explants the figures of merit, as presented in table 3, were exclusively calculated from the scores obtained between day 5 and day 14 in culture; a period which all cultures had in common.

Innervation by other chick neurons

Besides spinal cord neurons, several other types of neurons are able to form cholinergic neuromuscular junctions with chick muscle fibres in tissue culture. Nurse and O'Laque (1975) described innervation by sympathetic neurons and Hooisma et al. (1975) and Betz (1976) observed innervation by ciliary ganglia. A comparative study was therefore made of the ability of such explants to innervate and to exert a trophic influence on muscle fibres. It was already known that under the present experimental conditions ciliary ganglia are very effective in innervating muscle fibres. In the relevant areas surrounding such explants the majority of the muscle fibres seemed

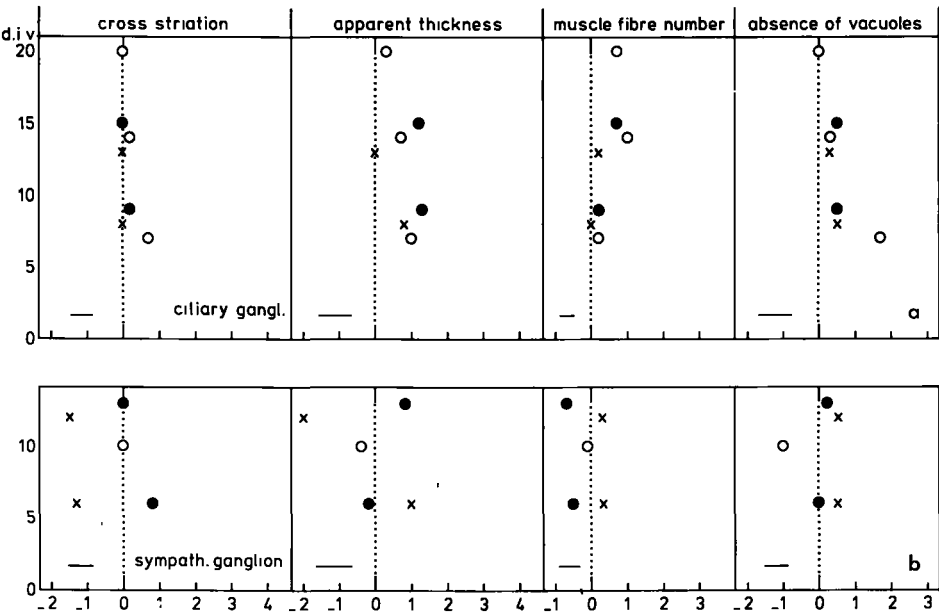
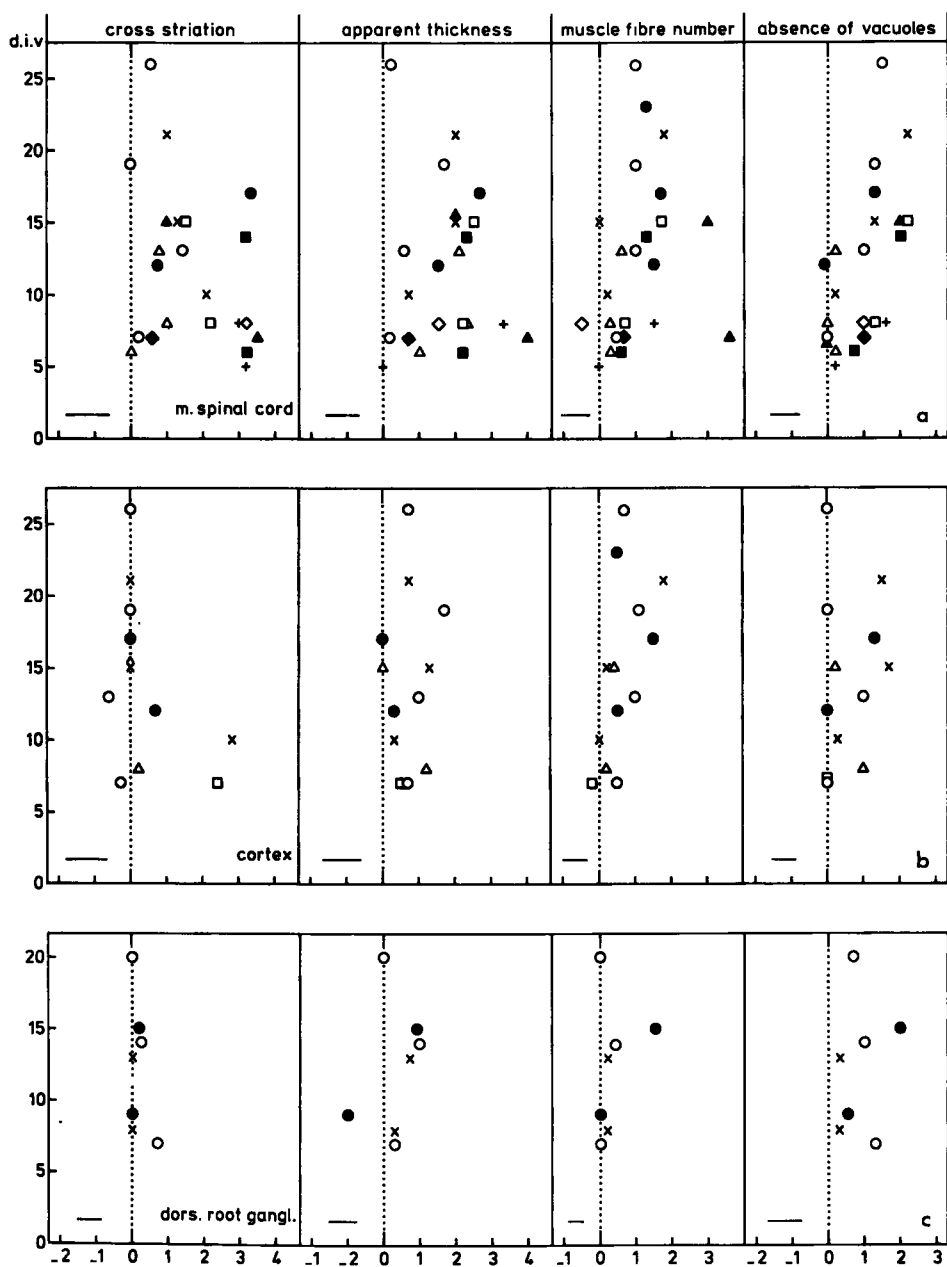


Fig.20. The influence of ciliary ganglia and sympathetic ganglia of chick embryos on the morphology of cultured muscle fibres. Differences between experimental and control cultures are plotted as explained in fig. 19a.

- a. ciliary ganglia (○ , ● : mean of 3 cultures; x : mean of 2 cultures),
b. sympathetic ganglia (○ , ● : mean of 4 cultures; x : mean of 2 cultures). bar = 2 S. E. M.



to be contacted by nerve processes. When examined electrophysiologically, epp's were encountered in 85% of such fibres. In mixed cultures with sympathetic ganglia approximately the same amount of fibres seemed to be contacted by nerve processes, but from a sample of 35 of such muscle fibres in 3 cultures only 30% showed spontaneous epp's.

In a series of 3 experiments, comprising a total of 8 experimental and 8 control cultures, the influence of chick ciliary ganglia on the morphology of the muscle fibres was assessed. In a similar series of 3 experiments (10 experimental and 10 control cultures) the influence of chick thoracic sympathetic ganglia was tested. The results were graphically presented in figure 20a and b, respectively, in a manner analogous to that employed in figure 19a. The averaged differences between experimental and control scores, presented in table 3, showed that the ciliary ganglia had a small but statistically significant positive influence on 3 of the 4 parameters whereas the sympathetic ganglia had no effect.

Influence of mouse neurons

It is known that in tissue culture muscle fibres of one species accept innervation by neurons of a foreign species (Crain et al., 1970; Peterson and Crain, 1970). It was therefore interesting to investigate the trophic effects of neurons from other species. In figure 21a the results are shown of a series of 10 experiments with mouse spinal cord explants, totalling 39 exper-

Fig. 21. The influence of spinal cord explants, cortex slices and dorsal root ganglia of mouse embryos on the morphology of cultured muscle fibres. Differences between experimental and control cultures are plotted as explained in fig. 19a.

a. mouse spinal cord explants. (Δ : mean of 4 cultures; $\circ$, $\bullet$, $\times$, $\square$, $+$, $\triangle$, $\diamond$: mean of 3 cultures; $\blacksquare$, $\blacklozenge$: mean of 2 cultures),

b. cortex (all points: mean of 3 cultures),

c. dorsal root ganglia (all points: mean of 3 cultures). bar = 2 S. E. M.

imental and 39 control cultures. These explants appeared to have a marked positive effect on all four morphological parameters, see also table 3, line 4. In order to investigate whether chick and mouse spinal cord explants were equally effective in enhancing the morphological development of chick muscle fibres, a direct comparison was made between chick and mouse explants in a series of 4 experiments comprising 13 experimental cultures with chick explants, 13 with mouse explants and 13 controls. The results are given in figure 19a and b, for chick and mouse, respectively, and are numerically presented in table 3, line 1 and 5. It appeared that on cross striation and thickness the positive influence of the mouse explant was even greater than that of the chick explant.

Explants from mouse cerebral cortex and also mouse dorsal root ganglia (DRG's) showed abundant outgrowth of nerve processes in tissue culture, and in mixed cultures with muscle fibres these processes inevitably touched muscle fibres. Such explants, however, did not form functional neuromuscular junctions. In view of the fact that Peterson and Crain (1970), Tolar (1974) and Crain and Peterson (1974) under certain experimental conditions observed favourable effects of the presence of DRG neurons on the morphology of muscle fibres, mouse cerebral cortex and DRG's were tested on this point. From the results presented in figure 21b and c and in table 3, lines 6 and 7, respectively, it appears that on some parameters these explants had statistically significant but very small effects, which were however larger than those of the chick sympathetic ganglia (table 3, line 3).

Innervation and resting membrane potential

The resting membrane potential of mammalian skeletal muscle fibres *in vivo* falls rapidly after denervation (Albuquerque and Thesleff, 1968) but no such change occurs in frog muscles (Nicholls, 1956). Cullen et al. (1975) reported a fall of about 17 mV in the (fast) posterior latissimus dorsi muscle

in young chicken but saw no fall in the (slow) anterior latissimus dorsi. In the study of the trophic effects of explants on muscle fibres in culture it would be very helpful to have, besides the morphological parameters, an easily measurable electrical parameter to quantify trophic action. It was therefore investigated whether innervation by spinal cord neurons might have an effect on the resting potential of chick muscle fibre in culture. Since it had been found that explants of mouse spinal cord had an even more pronounced positive influence on the morphology of chick muscle fibres than chick spinal cord explants, the study was carried out with spinal cord explants from 14 day old mouse embryos.

The resting potential was measured in a total of 254 muscle fibres that had been in culture for 5 to 17 days. Four different types of fibres were compared: i those innervated ($n=36$) and ii non-innervated ($n=57$) both in the absence of embryo extract and iii those innervated ($n=45$) and iv non-innervated ($n=116$) in the continuous presence of embryo extract. The resting potentials ($\pm$ S.D.) were 79.7 ± 9.1 ; 79.2 ± 8.9 ; 76.9 ± 9.3 and 75.5 ± 10.5 , respectively. Whether or not a fibre was innervated was decided according to the presence or absence of spontaneous epp's during a 2 min period of intracellular recording, it may thus be that a few innervated fibres have been wrongly categorized as non-innervated. The results obtained demonstrated no effect of age in culture upon the resting potential. Clearly under the experimental conditions used the resting potential was not altered by innervation.

Cross striation and innervation

The present experiments demonstrated a positive effect of the presence of spinal cord explants on the morphology of the muscle fibres. The results, however, give no answer to the question whether the trophic effects were caused by a factor which simply diffuses from the neurons to the muscle

Table 4. The amount of cross striation and number of epp's in chick cultured muscle fibres innervated by mouse spinal cord neurons.

Cross striation					
a	b+c	d	e		
18	3	2	1	0	
18	2	3	3	< 0.03	
13	4	10	13	< 0.25	epp frequency
4	5	9	8	> 0.25	

fibres or whether innervation itself or a very close contact between nerve processes as occurs in innervation, was essential. As mentioned earlier, many fibres in the relevant area surrounding a spinal cord explant showed signs of innervation, like fast contractions and epp's. This area also contained the muscle fibres which received trophic support from the explant. It was therefore investigated whether a correlation existed between the average frequency of spontaneous epp's in a fibre and the quality of its cross striation.

In a series of 8 cultures of 10 to 12 days, 116 muscle fibres with various qualifications for cross striation, all situated in the relevant areas surrounding mouse spinal cord explants, were examined electrophysiologically. Before a fibre was impaled its cross striation was rated (a - e). From the results shown in table 4, it appeared that of those fibres with good cross

striation (d and e) 82% showed the two highest epp frequencies, and only 18% had few if any epp's. Conversely, of the fibres with poor cross striation 61% had few if any epp's and 39% showed epp's at high frequencies. The rank correlation test of Spearman (see Snedecor, 1956) demonstrated a positive correlation between the quality of cross striation and the epp frequency at the 95% level of significance. The results, however, also show that in some cases poor cross striation may go together with a high epp frequency.

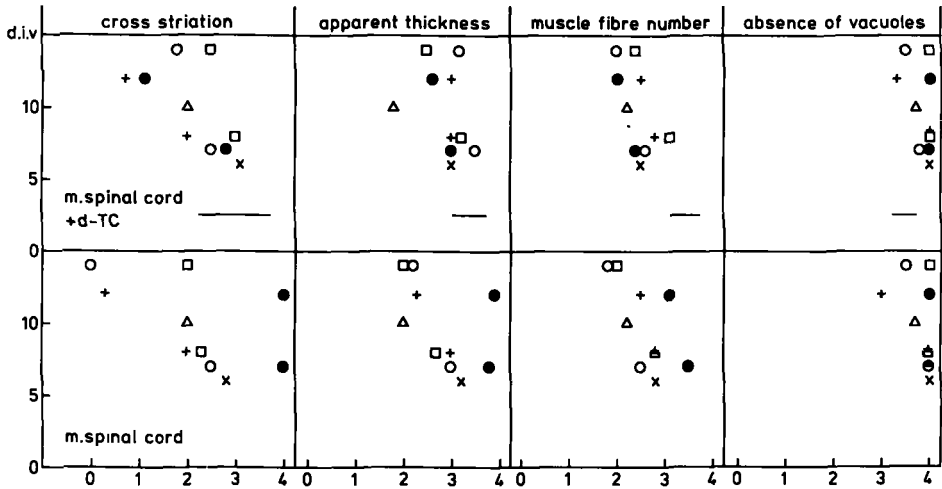


Fig. 22. Effect of continuous blockade of impulse transmission by d-tubocurarine (d-TC) in mixed cultures with mouse (m) spinal cord explants. For experimental cultures medium with d-TC was used. Control cultures were grown in normal culture medium.
 Symbols: ○ , ● , x , □ : mean of 4 cultures; test cultures with 1 μg/ml d-TC; Δ , + : mean of 3 cultures with 5 μg/ml d-TC; bar = 2 S. E. M.

Morphology and activity

Since innervated muscle fibres showed more electrical and mechanical activity than non-innervated ones, the question was raised whether the activity might be responsible for their better morphology. As stated above it was known that epp activity - and thus contractions induced by impulses coming

from neurons - could be blocked by d-TC in a concentration of 1 $\mu\text{g/ml}$. Crain and Peterson (1971), Pappas et al. (1971) and Cohen (1972) reported that the presence of d-TC does not interfere with the innervation of muscle fibres by spinal cord neurons in culture. In preliminary experiments the present authors found that d-TC (5 $\mu\text{g/ml}$) did not interfere with the innervation of chick muscle fibres by mouse spinal cord explants when present during the days 2 to 9.

In four separate experiments, each comprising 4 experimental and 4 control cultures, the influence was studied of mouse spinal cord explants on the morphology of chick muscle fibres either in the continuous presence or in the absence of d-TC at a concentration of 1 $\mu\text{g/ml}$. Two more experiments of similar kind, each comprising 3 experimental and 3 control cultures, were done with a five times higher concentration of d-TC. In all experiments the d-TC was present throughout the period when the explant was present in the culture, i. e. from day 2 until day 14. In these, as in all other, experiments the medium was replaced every 2 days by fresh medium containing d-TC. The results, graphically represented in figure 22, demonstrated that the presence of d-TC did not affect the morphological development of the muscle fibres. The absence of an effect of d-TC was not due to rapid break down of this substance in the medium because medium that had been on such cultures for 2 days still produced a reversible block of all fast contractions of innervated muscle fibres in cultures that had not been treated with d-TC. Acquired tolerance to d-TC cannot have played a role either, since fast contractions were continuously absent in the d-TC treated cultures but rapidly reappeared after washing with d-TC-free medium.

D i s c u s s i o n

Trophic effects of various neuronal tissues

In the present study the influence of various neuronal explants on the development and survival of chick muscle fibres in culture was studied. Four morphological parameters were used to estimate the overall stage of development or degeneration of the muscle fibres in the whole culture. Comparison of such estimates of a number of cultures with the condition of individual muscle fibres in these cultures demonstrated that these estimates were reliable and that different estimates were based on clearcut differences of individual muscle fibres.

Degeneration in culture occurred very rapidly if the embryo extract was omitted from the culture medium. For the study of trophic support of muscle cells this rapid degeneration was advantageous, because as a result the effect of an explant could be assessed within two weeks. The presence of spinal cord explants of chick embryos in mixed cultures with muscle fibres increased the amount of cross striation and the apparent thickness in an area surrounding the explant. In that relevant area also more fibres were present and their survival was prolonged, a phenomenon probably correlated to the observed delay in the appearance of vacuoles. Neither ciliary ganglia nor thoracic sympathetic ganglia affected the amount of cross striation, the ciliary ganglia had a moderate effect on apparent thickness and on vacuolization of the muscle fibres. The trophic effect of spinal cord explants from mouse embryos was even more pronounced for each of the four parameters than that of the homospecific chick spinal cord. Slices of mouse cortex had a considerable effect on cross striation and apparent thickness. Co-cultivation of muscle cells with dorsal root ganglia resulted in a delay of

vacuolization. The other parameters were not affected by these explants. It can be concluded that from all tissues studied spinal cord explants had the strongest influence on the morphology.

These data on the trophic effect of spinal cord explants confirm the observations of Crain and Peterson (1970), Robbins and Yonezawa (1971) and Oh et al. (1972). These authors found that the maturation and survival of cultured muscle fibres was enhanced by the presence of spinal cord explants. The present obtained negative results from co-cultivation with dorsal root ganglia do not confirm the observations of Tolar (1974), who found that rat muscle fibres in culture differentiated and could be maintained for months if they were cultured in a small amount of medium together with dorsal root ganglia. He assumed that the ganglion produced trophic substances with a high molecular weight which reached the muscle fibres by diffusion. In our studies sympathetic ganglia had no effect but Crain and Peterson (1974) reported that denervation atrophy after extirpation of the spinal cord explants from a mixed culture could be delayed by previous explantation of sympathetic ganglia in that culture.

Innervation had no effect on the resting membrane potential of muscle fibres grown in medium with or without extract. These findings are in agreement with those of Engelhardt et al. (1976) and Obata (1977).

There appeared to be no species specificity for the exertion of a trophic influence on chick muscle fibres. In fact, mouse spinal cord explants were even more effective than those of the chick embryo. Peterson and Crain (1970) have demonstrated that mouse spinal cord can influence rat muscle fibres in culture and vice versa.

Role of muscle activity in the trophic effect

It might be argued that the effect of spinal cord explants is regulated by activity induced in the muscle fibre by the neuron. This appears to be

the case with many properties of muscle fibres in vivo (Lömo, 1976). The results of the experiments in which d-tubocurarine was continuously present make such an explanation unlikely for the effect on morphology. The presence of d-tubocurarine had no effect on the trophic support exerted by the spinal cord explants. It is concluded that the muscle activity elicited at the neuromuscular junction is not required for the trophic effect so that, as far as the neuromuscular junction is involved in this phenomenon, it does not mediate the trophic effect by the action of ACh at the postsynaptic ACh receptors.

Functional innervation and trophic support by spinal cord explants

If electrophysiological function of the neuromuscular junction is no prerequisite for the trophic support, the effect might be mediated by a neuronal humoral factor acting on the muscle fibre. This raises the question whether functional neuromuscular connections are required for the trophic support by a humoral factor or that the mere presence of neuronal material in the vicinity is sufficient to provide the humoral factor by diffusion. It was observed that the effect of spinal cord explants was only exerted on muscle fibres that were situated in the relevant area around an explant. A correlation between cross striation and functional innervation has been demonstrated. It appeared that better cross striation developed in fibres in which a higher frequency of epp's was generated. Since muscle fibres in these cultures were usually multiply innervated (Hooisma et al., 1977) a high frequency of epp's could be caused by a large number of synapses or by synapses that generate epp's at a high frequency. The number of synapses per muscle fibre was rather high and therefore it was not possible to distinguish between these alternatives. It thus cannot be decided whether a large amount of cross striation was due to secretion of small amounts of the active factor at many synapses or to release of large amounts at a few

junctions with a great activity. It is concluded that in co-cultures of muscle cells and spinal cord explants the effect on cross striation is related to the presence of functional neuromuscular contacts; although the junctions do not have to generate end plate potentials.

With a different experimental approach Robbins and Yonezawa (1971) reached the same conclusion. They found that in cultured muscle fibres previously innervated by spinal cord neurons cross striation disappeared and the localization of the nuclei changed after denervation. Neither the mere presence of spinal cord explants in the vicinity nor that of other functioning neuromuscular junctions near the denervated muscle fibres prevented the changes due to denervation.

Trophic support by other neuronal tissues

Ciliary and sympathetic neurons form both cholinergic synapses with muscle fibres in culture (Hooisma et al., 1975; Betz, 1976; Nurse and O'Laigue, 1975), but the former had only a moderate effect, whereas the latter had no effect at all. Especially the effects on cross striation were quite different from that of spinal cord explants. It thus appears that the existence of functional neuromuscular junctions per se is not sufficient. One might argue that in the various co-cultures differences exist in the efficiency of innervation, for example in the total number of synapses around an explant, the multiplicity of innervation, or the frequency and the amplitude of the epp's generated in the muscle fibres. The efficiencies of innervation by spinal cord and ciliary ganglion, however, are in the same range (table 3), therefore in this way the difference between spinal cord explants and ciliary ganglia cannot be explained. The efficiency of the sympathetic ganglion is about one third of that of the spinal cord explant. It is difficult to see how this result could explain the total absence of a trophic effect, particularly if compared with the results obtained with cortex and dorsal root

ganglia which had clearcut effects on 2 and 1 parameters, respectively. These tissues contain no neurons that are able to innervate muscle fibres in tissue culture (Obata, 1977; see also table 3), which shows that in the absence of innervation a trophic, albeit moderate, effect can be exerted.

It could be that factors with favourable effects on muscle cells are present in many neuronal tissues. In this context the possible effect of diffusion of such factors from the neuronal perikaryon to the muscle fibres again turns up. However cortex as well as DRG's generated an extensive network of nerve processes and many nerve endings were in close contact with muscle fibres. Therefore also in this case long-distance diffusion is an unnecessary assumption to explain the results. A similar influence by diffusion has been proposed for neuroblastoma cells which contact cultured muscle cells. Such neuroblastoma cells do not form functional neuromuscular junctions but nevertheless influence the distribution of the ACh receptors in the muscle fibre membrane (Harris et al., 1971).

The present experiments have demonstrated that in tissue culture spinal cord neurons influence the development and the survival of muscle fibres. Some other neuronal tissues affect these fibres in a more or less similar way, although usually to a smaller extent. The influence is positively correlated with innervation but independent of functional neuromuscular transmission. The data suggest a role for a humoral agent which is transferred in the neuromuscular contacts.

S u m m a r y

1. The influence of various neuronal explants on the morphology and the survival of chick muscle fibres was studied.
2. Spinal cord explants of chick and mouse embryos had a favourable effect

on the morphology and on the survival.

3. Other chick and mouse neuronal tissues had either a moderate or no effect.
4. The amount of cross striation in a muscle fibre innervated by neurons in mouse spinal cord explants was correlated with the frequency of spontaneous end plate depolarizations in that muscle fibre.
5. d-Tubocurarine continuously present in the growth medium of mixed cultures of mouse spinal cord explants and chick muscle fibres did not diminish the effect of the explants.
6. Innervation by spinal cord neurons did not influence the resting membrane potential.
7. A role of a humoral trophic agent released at the neuromuscular junction is suggested.

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CHAPTER V

THE ABILITY OF EXTRACTS TO SUPPORT THE MORPHOLOGY AND ELECTROPHYSIOLOGICAL PROPERTIES OF CHICK MUSCLE FIBRES IN TISSUE CULTURE

Introduction

Motoneurons not only provide the nerve impulses necessary for impulse activity and contraction of skeletal muscles, but they also have other influences on the muscles which have important consequences for the long-term functional properties of the muscles. It is still not clear how these trophic (Drachman, 1976) influences are brought about, however it seems likely that more than one neuronal mechanism is involved (Gutmann, 1976; Lömo, 1976). One of these mechanisms may be the effect of hitherto unknown humoral factors that are secreted by the nerve terminal at the neuromuscular junction (Ochs, 1974). These regulating factors are supposed to be transported from the motoneuron cell body to the axon terminal by axonal transport, because if this transport is blocked by application of colchicine or vinblastine, denervation phenomena develop in the innervated muscle. For instance, extra-junctional hypersensitivity to acetylcholine develops as it does in denervated muscles (Albuquerque et al., 1972).

Recently evidence was presented that trophic factors are present in motor axons. Oh (1976) found that an extract obtained from sciatic nerve enhanced the development and survival of skeletal muscle fibres in tissue culture. Extracts of embryonic tissues appeared to have a comparable effect (Oh et al.,

1972; Oh, 1975). Electrophysiological properties of muscle fibres in organ culture were affected by extracts obtained from the spinal cord of adult mouse (Kuromi and Hasegawa, 1975).

In the foregoing chapter the effect of various types of neurons on the morphology of cultured muscle fibres was described. A method has been developed to estimate quantitatively morphological properties of muscle fibres. In the present paper this method is applied in investigations to study the effect of extracts of embryonic and adult chicks on the development and survival of chick muscle fibres in tissue culture. Furthermore, the resting membrane potential, the sensitivity to acetylcholine and the input resistance of muscle fibres cultured in the presence of different extracts were measured.

M a t e r i a l s a n d m e t h o d s

Methods of tissue culture

Suspensions of muscle cells obtained by trypsin dissociation of leg muscles of 11 day old chick embryos, were plated on collagen coated "Cellfinder" object glasses (Micropure, Driebergen, The Netherlands). The culture method and the medium have been described in detail in chapter 4. The media contained 5% embryo extract during the first 2 days in culture. On subsequent days the embryo extract was either replaced by an equal volume of the extract to be tested (experimental cultures) or left out altogether (control cultures), unless stated otherwise.

Preparation of extracts

Extract of embryos was prepared under sterile conditions. Twenty chicken embryos of 10 to 11 days gestational age were washed in cold Tyrode's

solution, transferred to a 10 ml syringe and pressed through the nozzle (Record type). After addition of an equal volume of Eagle's minimum essential medium (MEM) the extract was centrifuged for 2 hours at 18,000 g and 4°C. Extracts from various parts of the embryonic and adult chick were prepared and tested. Because the adult tissues were tougher than the embryonic material, they were minced and osmotically shocked by addition of two parts distilled water to one part of tissue fragments. After that the homogenate was centrifuged at 2000 g for 15 min and subsequently at 18000 g and 4°C for 2 hours. Extract from adult chicks could not be prepared under sterile conditions it was therefore sterilized by filtration through a 0.22 µm membrane filter. The protein content was measured according to Lowry et al. (1951). Eagle's MEM was added to adjust the protein concentration to the desired level. The osmolarity was adjusted to 320 mOsm by adding NaCl or distilled water. All extracts were frozen immediately after preparation and stored in small portions at -20°C for no longer than 4 weeks.

For some experiments embryo extract was heated, dialysed or "conditioned". Heating was done in a waterbath either at 56° or at 70°C during 30 min, after which denaturated material was removed by centrifugation at 2000 g for 15 min. Dialysis was carried out with 15 ml extract in Visking dialysing membrane for 3 days against 500 ml Tyrode's solution changed daily. Conditioning was done as follows, medium containing 1.5% brain extract was used for two days on muscle cells in culture and subsequently collected. This conditioned medium was used immediately.

Morphological evaluation

Four parameters have been employed in the assessment of the influence of tissue extracts on the differentiation and survival of muscle fibres in culture. These parameters were: i the amount of cross striation, ii the apparent thickness of the fibres as judged through the phase-contrast microscope,

iii the number of fibres with vacuoles, iv the total number of fibres. The methods used for the study of the morphological parameters and the statistical evaluation have been described in chapter 4.

Electrophysiological measurements

For electrophysiological measurements a Cellfinder object glass with a culture on it was placed in a temperature controlled bath at 37°C that was constantly perfused with prewarmed Tyrode's solution previously equilibrated against 95% air and 5% CO_2 . The membrane potentials of muscle fibres were measured with the aid of microelectrodes filled with 3 M KCl ($10\text{--}20\text{ M}\Omega$). For further details of the instrumentation see chapter 3. The space constant of these cultured muscle fibres is at least $400\text{ }\mu\text{m}$ (Slaaf, unpublished). For the measurement of the input resistance muscle fibres were therefore selected that had a reasonably constant diameter over a length of $600\text{ }\mu\text{m}$ from the end of the muscle fibre and that did not branch within $600\text{ }\mu\text{m}$ from this end. Two intracellular electrodes were placed at less than $50\text{ }\mu\text{m}$ from this end of the fibre and approximately $20\text{ }\mu\text{m}$ apart. Depolarizing and hyperpolarizing current pulses lasting 400 msec were delivered through one of the microelectrodes by a Grass S88 stimulator via a Grass SIU 5 stimulus isolation unit. The displacement of the membrane potential was measured at the end of the current pulse and plotted against the current strength. The input resistance was calculated from the slope of the graph at the hyperpolarizing part.

The sensitivity of the muscle fibres to acetylcholine (ACh) was assessed by iontophoretic application of ACh. Pipettes for iontophoresis were drawn from glass capillaries with a sealed-in glass fibre (Clark, Electromedical Instruments, England). The micropipettes were filled by means of a hypodermic needle with a fresh solution of 2 M ACh-chloride just before use ($60\text{--}80\text{ M}\Omega$). The same pipette was always used if on the same day the

ACh-sensitivity of muscle fibres in different cultures was compared. Current through the ACh-pipette was supplied by a push-pull current source via chlorided silver wires. The ACh-pipette was lowered into the bathing solution in the neighbourhood of a muscle fibre from which a stable resting potential was recorded. A braking current of 5 nA was applied to the ACh-pipette. Usually this current was sufficient to prevent leakage of ACh from the pipette. If, however, the membrane potential decreased upon advancement of the ACh-pipette, the pipette was discarded. Test pulses were delivered and the braking current was gradually reduced to a level at which just no sign of continuous ACh efflux was present. The ACh-sensitivity of a muscle fibre was usually measured at three sites about 20 μm apart, according to a method described by Kuffler and Yoshikamy (1975). The tip of the pipette was advanced to the muscle fibre membrane and current pulses were delivered that resulted in a depolarization of the membrane resting potential of approximately 2 mV. Subsequently the distance of the pipette to the membrane was adjusted to obtain a maximum response. At the distance that gave the highest response a series of current pulses with different intensities was applied and the depolarizations induced were plotted against the current intensity. The sensitivity was calculated in mV/nC from the slope of the graph. Responses to a pulse of ACh that had a latency greater than 5 msec were discarded. For further details see Slaaf (1977).

R e s u l t s

Embryo extract and the morphology of cultured muscle cells

In order to obtain quantitative information about the effect of various extracts on the morphological properties of cultured muscle fibres, 4 parameters were used to evaluate the condition of the muscle fibres. The method

Table 5. Effect of undiluted extracts on cultured muscle cells.

The mean of the differences between the scores in experimental and control cultures. In the experimental cultures medium made with undiluted extracts was used. Marked values were significant to at least $P_2 < 0.05$.

type of extract	cross striation	apparent thickness	muscle fibre number	absence of vacuoles
embryo	2.9'	2.7'	1.3'	0.6'
brain	2.6'	2.3'	1.2'	0.5'
body	2.4'	1.8'	1.1'	0.6'

used to score these parameters of the muscle fibres has been described in detail in chapter 4. The scores found for each parameter in three or more experimental cultures have been compared with those found in an equal number of control cultures. The morphological condition of the muscle fibres was usually evaluated three times during the whole culture period.

The fusion of mononucleated muscle cells (myoblasts) in culture started within 24 hours and many multinucleated muscle fibres were present after 48 hours. During the first 2 days all cultures were grown in medium containing embryo extract in order to start the experiments with cultures that contained a large number of muscle fibres. Subsequently, the extract was omitted in the control cultures and extracts to be investigated were added

to the media of the experimental cultures. The treatment of the cultures with ara-C during the third and fourth day delayed further cell proliferation.

In a first set of experiments muscle fibres grown in the continuous presence of embryo extract were compared with fibres devoid of embryo extract after

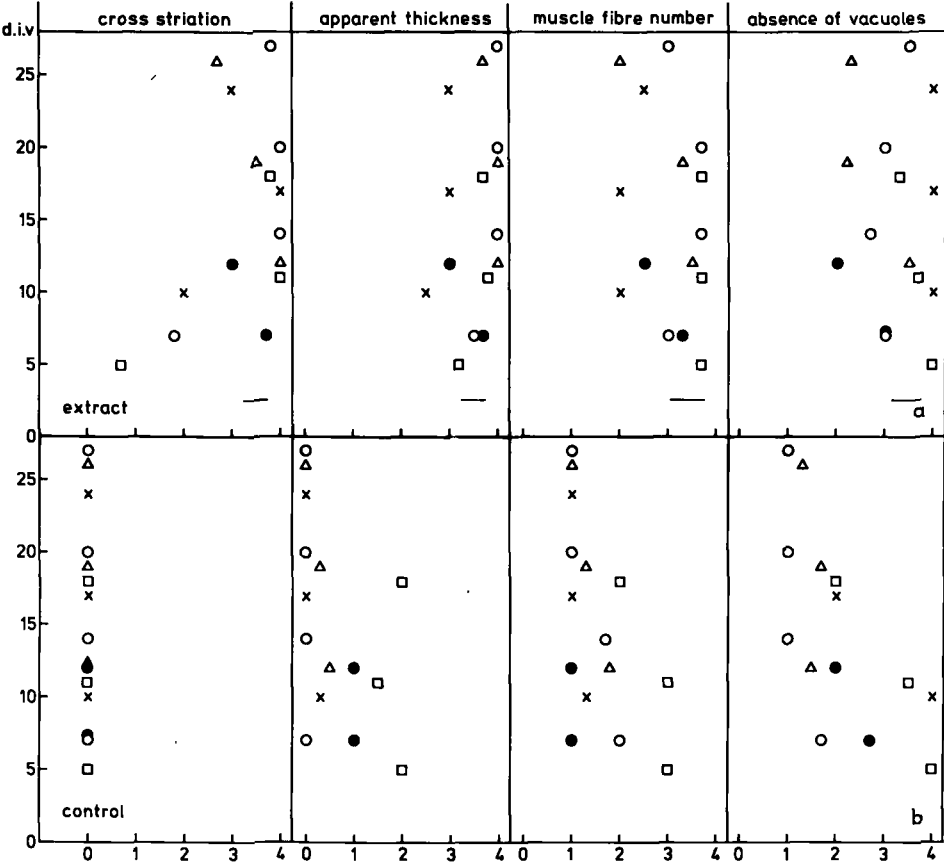


Fig. 23. The effect of chick embryo extract on the morphology of muscle fibres in culture. Abscissa: mean score of the four morphological parameters. Ordinate: days in vitro (d.i.v.). a. morphological properties of muscle fibres in experimental cultures; in medium containing embryo extract. b. morphological properties of muscle fibres in control cultures; in medium without embryo extract from the second day of cultivation onward. ○, ●, □, △ : mean of 3 cultures; x : mean of 2 cultures. bar = 2 S. E. M.

two days in culture. The former muscle fibres became thick and developed pronounced cross striation after 5 days in culture. At 8–10 days in culture most muscle fibres had branches and were several millimetres long. Vacuoles became conspicuous in these muscle fibres after 10–12 days and muscle fibres began to disappear. In control cultures devoid of embryo extract the muscle fibres were thin and usually short and had no cross striation. Vacuoles developed already after 7 days in culture and some muscle fibres had disappeared at that time.

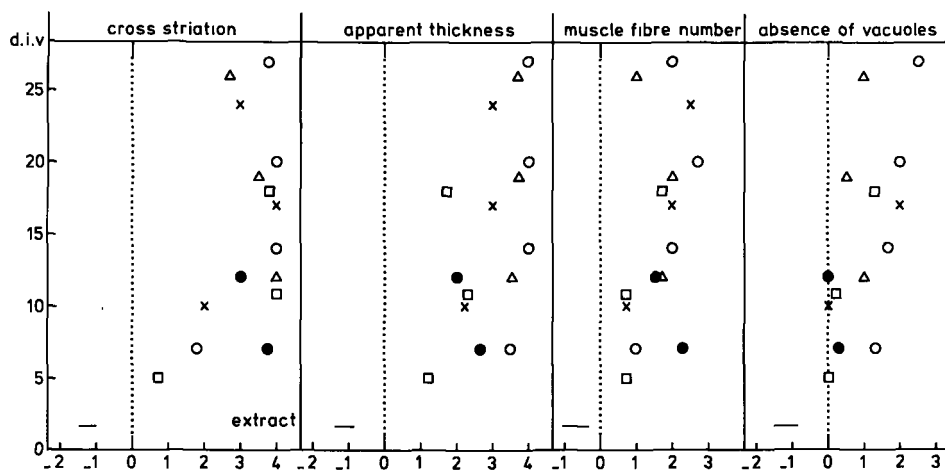


Fig. 24. The influence of chick embryo extract on the morphology of muscle fibres in culture. The values in the graph were calculated from those presented in figure 23. Abscissa: differences between the mean scores of experimental and control cultures estimated on the same day in culture. Further details as in figure 23. All figures up to figure 28 are composed in this way.

From day 5 onwards the morphological properties of the muscle fibres in experimental cultures were scored and compared with those in control cultures. The results were graphically presented in figure 23 (a and b), plotted against time in culture. From the same experimental series figure 24 shows the difference between the scores of the experimental cultures and those of the corresponding controls. A favourable effect of embryo extract on all

parameters was found. These effects were expressed in single figures of merit, calculated by averaging the differences between the experimental and the control scores obtained for a certain parameter. Because in some later experiments the effects of extracts were evaluated only up till 15 days in culture, these figures of merit were calculated only from scores obtained during days 5-15 in culture (table 5, line 1). Comparisons between such figures, as in table 5, must be interpreted with caution because they were not based on comparisons with one and the same series of control cultures.

The culture medium contained about 16 mg/ml protein, 15.5 mg/ml contributed by horse serum and about 0.5 mg/ml protein by embryo extract. It seems therefore unlikely that the effect of the extract was due to the addition of the small amount of protein contained in the embryo extract. In order to investigate the amount of active component in the embryo extract, the effect of media with decreased amounts of embryo extract were studied. In medium with 1.5% embryo extract an effect on cross striation and thickness could still be demonstrated, but for a significant effect on vacuolization and on the number of muscle fibres the undiluted extract was needed. In media with 0.5% or 0.15% embryo extract the development of muscle fibres was similar to that in medium without embryo extract.

Trophic effects of various extracts

It might be that the effect of embryo extract on differentiation and maintenance of muscle fibres was exerted by a growth factor that influences the development of all kinds of chick embryo cells. It might also be, however, that a specific neurotrophic factor is involved which is synthesized in the embryonic nervous system and is transported through the axons to innervated, developing muscle fibres. In that case it might be expected that the brain and the spinal cord contain greater amounts of this factor than the other parts of the body, unless a massive amount of this factor is transported into

the developing muscle fibres. The effect of extracts obtained from the head and the spinal cord, called "brain extract", and extracts from the rest of the body, called "body extract" have therefore been tested and compared with those of embryo extract. The protein content was approximately 10 mg/ml.

As before, the embryo extract considerably improved cross striation and apparent thickness, more muscle fibres survived and vacuolization was delayed and less severe than in cultures devoid of embryo extract. Surprisingly, brain and body extracts had similar effects on all parameters. These results

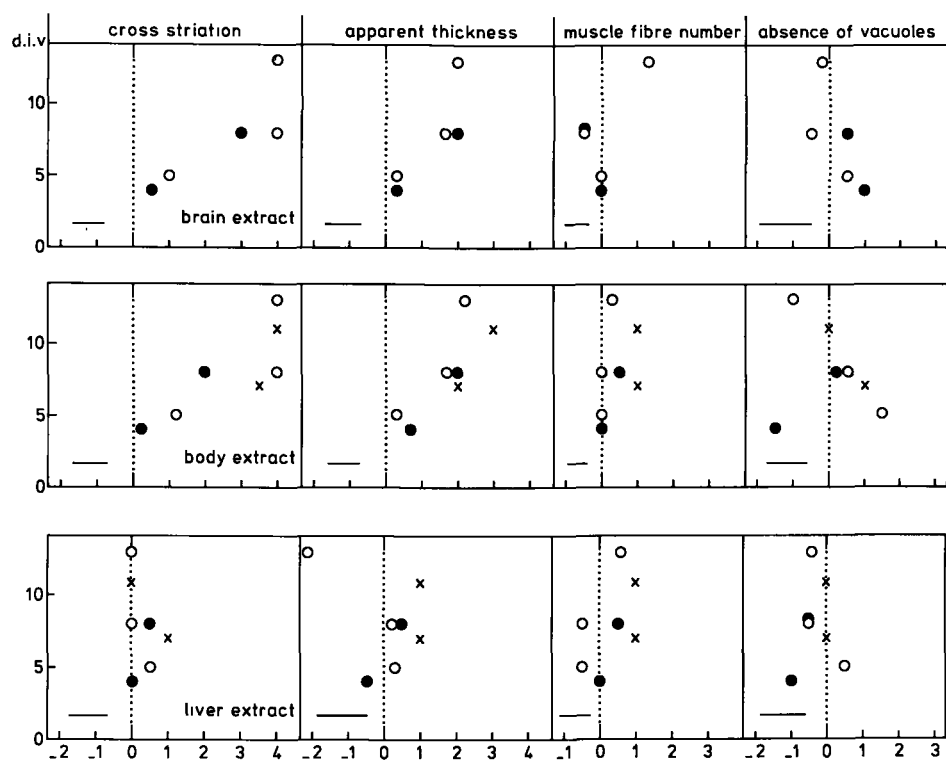


Fig. 25. The effect of brain, body and liver extracts of chick embryos. Differences between experimental and control cultures are plotted as explained in figure 24. All points: mean of 2 cultures. The protein content of the extracts was 3, 8, 4, 3 and 5.3 mg/ml, respectively. bar = 2 S. E. M.

Table 6. Effect of extracts of various embryonic tissues on cultured muscle cells.

Mean of the differences between the scores in cultures cultivated in media with various extracts and those of control cultures. The figures marked ' are significant to at least $P_2 < 0.05$.

extract	protein content mg/ml	cross striation	apparent thickness	muscle fibre number	absence of vacuoles
<u>normal procedure</u>					
brain	3.8	2.5'	1.3'	0.1	0.3
body	4.3	2.8'	1.7'	0.4'	0.1
liver	5.3	0.3	0.5	0.3	0.3
<u>extracts changed after 2 hours</u>					
brain	3.8	2.6'	2.1'	1.8'	0.8'
body	4.3	2.8'	2.0'	1.2'	0.5'
liver	5.3	0.0	0.6'	0.8'	0.4
<u>normal procedure</u>					
adult brain	5.2, 6.8	1.0'	1.3'	0.6'	0.6'
adult muscle	4.9, 5.6	1.0'	1.4'	0.5'	0.6'

suggest that a growth factor present in all embryonic tissues was involved rather than a specific neurotrophic factor. Because liver is free of neuronal

material, extract of embryonic chick liver was tested against brain and body extracts. In these experiments the extracts used had all approximately the same amount of protein, but were less concentrated than those used in the previous experiments. The results are presented in figure 25, and the figures of merit are given in table 6. The cultures with either brain or body extract developed as expected, except that the timing of vacuolization and the number of muscle fibres was almost the same as those in control cultures without embryo extract. Liver extract clearly had no positive effect on

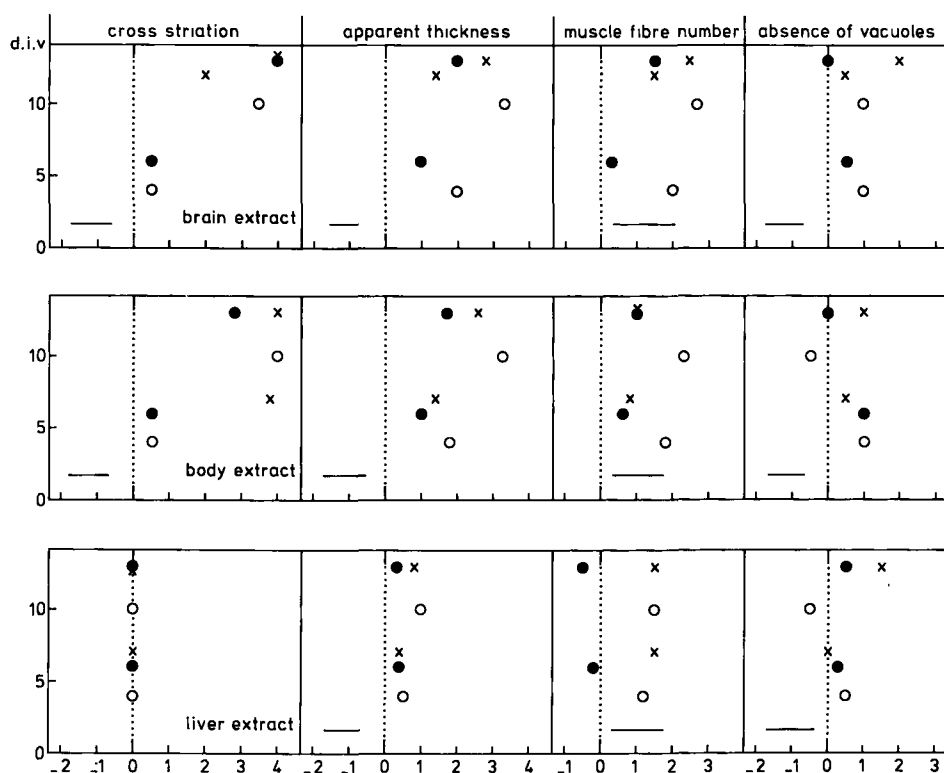


Fig. 26. The effect of brain, body and liver extracts of chick embryos. In these experiments the change from standard to experimental media was made 2 h after plating of the cell suspension instead of after 2 days as usual. All points: mean of 2 cultures. The protein content of the extracts was 3.8, 4.3 and 5.3 mg/ml respectively. Further details see figure 23.

cross striation and thickness, suggesting that the trophic factor is not generally present throughout the embryo.

The trophic effect was also studied on muscle fibres that had been cultured in medium containing embryo extract for only 2 hours, instead of 2 days. At that time the medium with embryo extract was replaced by medium with extracts to be tested or by medium without extract. As a result of this change in schedule the differences between the experimental and control cultures became more pronounced (fig.26, table 6). The presence of either brain or body extract from the onset of the culture period resulted in a significant positive effect on the number of muscle fibres and in a small positive effect on vacuolization. Even under these circumstances liver extract had no effect on the amount of cross striation. In the experiments described above it was

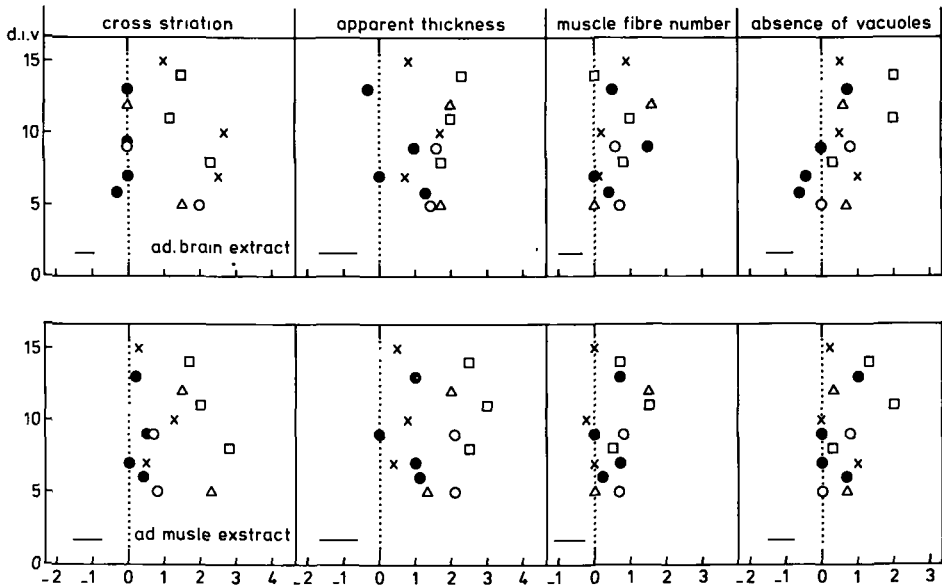


Fig. 27. The effect of the extracts of brain and muscle of adult chicks applied as usual after 2 days in vitro. All points: mean of 3 cultures. The extracts contained 5.2 and 4.9 mg/ml protein respectively. Further details as in figure 23.

demonstrated that a trophic factor is present in extracts of neural and of some non-neural tissues derived from 11 day old chick embryos. The question arose whether this trophic factor is only present in embryonic, i. e. developing tissues or whether it might also be present in tissues of the adult animal. This was studied in cultures of muscle cells grown in the presence of extracts from brain or muscles of adult chicks. These extracts appeared to enhance all four parameters studied (fig. 27, table 6). Thus a factor influencing these parameters is not only present in embryonic tissues but also in the brain and the muscles of adult chicks.

Some properties of the active factor

a. Stability under tissue culture conditions

The possibility existed that the active component in the extract was gradually degraded under the conditions present in tissue culture. Medium containing 1.5% brain extract from chick embryos was used to "feed" 6 day old cultures of differentiating muscle fibres for 2 days and was subsequently harvested. Muscle fibres were grown as usually for two days in the presence of embryo extract and henceforth cultured in regularly harvested pre-used medium. In two series of experiments in triplo it appeared that pre-used medium was still active (table 7, line 1). It can be concluded that the trophic factor is neither degraded nor exhausted during 48 hours in the presence of growing muscle fibres.

b. Stability at elevated temperatures

The stability of the trophic factor at elevated temperature was studied under two conditions. Two portions of embryo extract containing 6.5 mg/ml protein were heated to 56° and 70° C respectively for 30 min and then centrifuged to remove the aggregated denaturated material. The protein

Table 7. Effect of treatment of embryo extract on its effect on cultured muscle cells.

The mean of the differences between the scores in experimental and control cultures. Experimental cultures were grown with extracts treated as described in the text.

Marked ' figures were significant to at least $P_2 < 0.05$.

treatment	protein content before/after treatment mg/ml	cross striation	apparent thickness	muscle fibre number	absence of vacuoles
pre-used	3.8/	2.0'	1.6'	0.4	-0.1
56°C	6.5/5.6	2.6'	1.9'	0.9'	0.8'
70°C	6.5/3.3	2.0'	1.5'	0.4	0.3
dialyzed	6.5/6.5	2.3'	1.5'	0.3'	-0.6

contents of the supernatants obtained were 5.6 and 3.3 mg/ml, respectively. As shown by the figures of merit in table 7, line 2 the extract heated to 56°C still had a positive effect on all 4 morphological parameters. Even the extract heated to 70°C still had a positive effect on 2 of the 4 morphological parameters.

c. Dialysis

A rough estimate of the molecular weight of the active factor has been obtained from dialysis experiments. A portion of embryo extract containing 6.5 mg/ml protein was dialysed in Visking dialysing membrane against Tyrode's solution. The dialysed extract still had a positive effect on cross striation, on thickness of the muscle fibres and on the number of the muscle fibres, however, the timing of the vacuolization was not affected. Generally, proteins with a molecular weight of over 20,000 Daltons do not pass this type of dialysis membrane. The trophic factor is therefore supposed to be a heat stable macromolecule.

Electrophysiological experiments

a. Resting potential

After denervation of an adult skeletal muscle fibre its membrane resting potential decreases (Redfern and Thesleff, 1971). A humoral factor may be involved in the maintenance of the normal resting potential. It was therefore investigated whether the factor that improved the muscle fibre morphology might also exert an influence on the resting potential of the muscle fibre. The mean resting potentials of muscle fibres grown in medium without embryo extract were the same as those of fibres grown in brain extract, body extract or whole embryo extract, notwithstanding their very poor morphological appearance (table 8). The resting potentials of muscle fibres grown in medium containing embryo extract were surprisingly high already after 3 days in culture and remained at the same level until at least 25 days in culture. Moreover, the resting membrane potentials of the muscle fibres grown in media with the various extracts did not change during the period of observation.

Table 8. Effect of extracts of embryonic tissues on the resting membrane potential.

The resting membrane potentials of muscle fibres grown in the presence or the absence of extracts of various embryonic tissues.

Extract	days in culture	range V_m mV	number of fibres	mean $\pm$ S. D. mV
embryo	3-25	61-94	104	75.1 $\pm$ 10.2
without	10-15	60-96	57	79.2 $\pm$ 8.9
brain	7-24	64-90	80	81.3 $\pm$ 6.6
body	7-15	53-96	79	81.4 $\pm$ 8.3

b. Acetylcholine sensitivity

The development of the sensitivity to ACh of the muscle membrane outside the end plate region is an important parameter in the study of the events following denervation and re-innervation of skeletal muscle in vivo. After denervation the entire muscle membrane becomes highly sensitive to ACh (Axelsson and Thesleff, 1959; Miledi, 1960). If a trophic factor is involved in the suppression of ACh-sensitivity in the innervated muscle it might be assumed that this factor will be present in the spinal cord. It might than be expected that brain extract would suppress this sensitivity, for it contained spinal cord material and that body extract would not do so. The sensitivity

to ACh was measured in 87 muscle fibres at 261 sites in 14 different cultures grown either with brain extract or body extract. The range of the sensitivities found was equal in both types of muscle fibres, i. e. 14-2980 mV/nC with a mean ($\pm$ S. D.) of 200 ± 200 mV/nC and 300 ± 500 (n=143 and 118), respectively. The scatter of these values is so large, that it can only be concluded that differences in sensitivity on day 8-13 in culture were not obvious.

c. Membrane resistance

The membrane resistance and consequently the input resistance of muscle fibres are reported to be influenced by innervation *in vivo* (Albuquerque and Thesleff, 1968) as well as *in vitro* (Engelhardt et al., 1976). In the

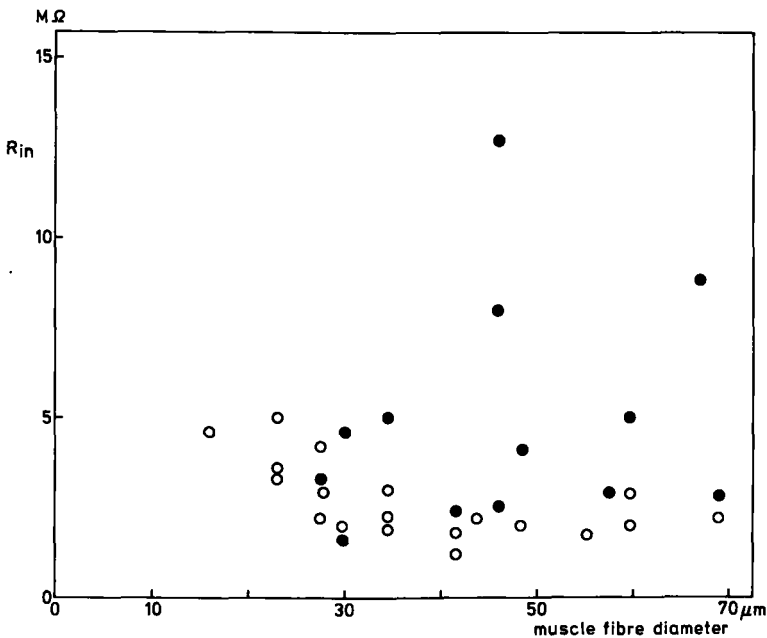


Fig. 28. The input resistance (R_{in}) of muscle fibres grown in medium either with (○) or without (●) embryo extract applied after two days in culture. In total 13 cultures were used. Abscissa: diameter of the muscle fibres. Ordinate: input resistance.

present experiments the input resistance has been studied in cultured muscle fibres grown in medium either containing embryo extract or without extract. A total of 32 muscle fibres was studied in 6 experimental and 6 control cultures. Figure 28 presents the input resistances (R_{in}) plotted against the muscle fibre diameter for experimental and control muscle fibres. A statistically significant difference appeared to exist between the input resistance of fibres grown in the presence of embryo extract: $2.8 \pm 1.0 \text{ M}\Omega$ (mean $\pm$ S. D.), and that in fibres grown without extract: $4.8 \pm 3.1 \text{ M}\Omega$ ($P_2 < 0.05$; Wilcoxon rank-correlation test). The diameter of fibres grown in the presence of embryo extract: $40 \pm 16 \mu\text{m}$ (mean $\pm$ S. D.) was not different from that of muscle fibres grown without extract: $55 \pm 29 \mu\text{m}$ ($n=19$ and 13 , respectively).

Discussion

Addition of chick embryo extract to the tissue culture medium led to an increase in the amount of cross striation and in the thickness of cultured chick muscle fibres. Moreover, the survival of muscle fibres was prolonged in the presence of the extract, the onset of vacuolization was delayed and more muscle fibres were present. Extracts prepared from the head and the spinal cord and from the rest of the body had comparable effects. Liver extract, however, had no positive effect on any of the morphological properties. Extracts obtained from muscle or brain of adult chick also had a positive influence on the properties mentioned above. The fact that body extract of chick embryos and extract of adult muscle have a trophic effect raises doubt as to the nervous system as the sole source of trophic factor. The result could also mean that such a factor is synthesized in the nervous system and is transported to and accumulated in skeletal muscle tissue. Transfer of material from the motor nerve terminal to the muscle has been

demonstrated (Appeltauer and Korr, 1975). Alternatively, the same or a similarly acting factor might be synthesized by peripheral tissues.

The active factor is not degraded in culture. It is probably a large molecule with a molecular weight over 20,000, which is not degraded in culture and is remarkably resistant to heating.

The results presented should be compared with those reported by Oh (1975) on the morphological development and the survival of cultured muscle fibres. In his experiments extracts of the brain and the spinal cord of chick embryos enhanced the development and the differentiation of chick muscle fibres. The mitotic activity of myoblasts was increased, and fusion of myoblasts and maturation occurred earlier in treated cultures. Cross striation developed only in the presence of neuronal extract and survival of the muscle fibres was considerably prolonged. Similar effects were found in cultures treated with extracts of the brain and the spinal cord or extract of the sciatic nerve of the adult chick (Oh, 1976). The experimental data presented here are different in a few essential aspects; whereas Oh has found that the neurotrophic activity is exerted exclusively by extracts of neuronal tissue, the data presented here show that the factor affecting the morphological properties studied is also present in extracts from which the central nervous system has been excluded. The negative results of the experiments with liver extract either mean that the present factor is not ubiquitous in the chick embryo or that liver extract contains a substance which antagonizes the active factor. Experiments are in progress to clarify this point.

The factor described by Oh (1975) partially lost its neurotrophic action after being heated during 30 min at 60°C. In the present experiments heating to 56°C for 30 min produced no measurable reduction of the neurotrophic action and even heating to 70°C only abolished the effect on the number of muscle fibres and on the vacuolization. The latter results may mean that 2 factors were present in the extract which have a different heat resistance.

It could also mean that some morphological properties were more sensitive than others to a reduced concentration of one neurotrophic factor. The results obtained with dialyzed and with diluted extracts, showing that the 4 morphological properties are unequally affected by the changes brought about in the extracts, also leave room for the same 2 interpretations. In this context the observations of De la Haba and Amundsen (1972) are interesting, these authors found that chick embryo extract contains at least 2 factors: one which enhances the fusion of myoblasts and another that promotes the development of muscle fibres in culture. The first factor does not pass a membrane with cut-off at molecular weight 50,000, whereas the second factor passes that membrane. Both factors were needed for the survival of the muscle fibres in culture.

The fact that the presently studied embryo extract made that a larger number of muscle fibres was observed in the cultures could be the result of either i an increased formation of muscle fibres or ii a delay of degeneration. The experimental conditions chosen do not favour the first possibility. In all except one series of experiments the embryo extract was omitted or replaced by another extract after 48 hours, at the time that most of the mitotic activity and much fusion had already taken place (Slater, 1976). Moreover, in the subsequent 48 hour period mitotic activity was suppressed by ara-C. The observed effect of embryo extract on the number of muscle fibres can therefore be regarded as a result of a delay in degeneration.

Another observation points in the same direction. When liver extract was tested in the usual way, i.e. added to the medium after 2 days, it had no favourable effect on the morphology of the muscle fibres. If, however, this extract was already added 2 h after plating, that is at a time when mitotic activity of the myoblasts is high, it increased the number of fibres formed. Thus the extract probably had a positive effect on mitosis but such an effect had no consequences for the number of muscle fibres when exerted after

2 days in culture.

Although there are no indications that muscle fibres grown in medium with extract more actively contract than those grown in medium without extract, it must be kept in mind that the morphological effects of extracts could be exerted by the induction of more spontaneous activity. Activity induced in denervated muscles *in vivo* can prevent or reverse changes of functional and morphological properties caused by denervation (Lömo, 1976).

The influence of extracts on some electrophysiological properties of the muscle fibres was investigated. The presence of the extract had no influence on the resting membrane potential. Lömo (1974) and Westgaard (1975) demonstrated that the membrane resting potential of muscle fibres *in vivo* is influenced considerably by direct electrical stimulation. Kuromi and Hasegawa (1975) found that spinal cord extract affected the rate of rise and fall of the action potential of denervated muscle fibres in organ culture, but that the extract had no effect on the membrane resting potential. In the present experiments these potentials were already high (80 mV) as soon as fusion of myoblasts had started and was maintained at that level even in cultures grown without extract, notwithstanding the poor morphology of the muscle fibres in those cultures. The high resting membrane potential presently found should be compared with the values of around 58 mV found in developing chick thigh muscles *in vivo* (Karzel, 1968) and with 50–60 mV found in embryonic pectoral muscle fibres in culture (Fischbach, 1970, 1972) and in embryonic thigh muscle fibres (Harris et al., 1973). Omission of the factor that influences the morphology and the survival of cultured muscle fibres obviously does not lead to a lower membrane resting potential.

The mean input resistances of muscle fibres grown in medium with or without embryo extract were 2.8 and 4.8 M Ω , respectively. These values are significantly different but are both well within the range found by Harris et al. (1973) and Engelhardt et al. (1976). Engelhardt and co-workers

demonstrated that chick muscle fibres cultured together with spinal cord explants had a lower input resistance than those cultured without neurons. Apparently the effect of the presence of explants on this property can be mimicked by embryo extract. Obata (1977) on the other hand found no difference in input resistance between innervated and non-innervated muscle fibres. It is not likely that the difference in input resistance found in the present experiments can be explained solely by the difference in thickness of muscle fibres grown in medium with or without extract. Harris et al. (1973) have proposed the formula

$$R_m = \frac{4R_{in}^2 \times A \times B}{R_i}$$

in order to calculate the specific membrane resistance of cultured chick muscle fibres; where

R_m = the specific membrane resistance per unit area, R_{in} = the input resistance, A = the cross-sectional area of the cell, B = the circumference of the cell and R_i = the specific resistance of the myoplasm.

The mean values found for the input resistance and the diameter were used to calculate the difference in thickness required to explain the difference in mean input resistance; it was assumed that R_m and R_i were equal in all cells. It appeared that muscle fibres grown in medium with embryo extract had to be 3 times thicker than those cultured in medium without extract. As can be seen in fig. 28 it might be that the latter group of fibres consisted of two subgroups. Although the total number of experiments did not allow such a classification to be made, it was tentatively tried to calculate the difference in thickness between the fibres with the largest input resistance and those cultured in medium with extract. In that case a 7-fold difference in thickness would be needed to explain the differences. Such large differences do not seem to exist between the fibres that were either grown in

medium with or without embryo extract. Consequently, the assumption that the specific resistances R_m and R_i were equal, was probably wrong.

The present results demonstrate that factor(s) are present in extracts of embryonic and adult chicks that influence the morphology and survival of chick muscle fibres in culture. The factor is a large, rather heat stable molecule that does not influence the resting membrane potential but it decreases the input resistance of the muscle fibres.

S u m m a r y

1. The effects of extracts obtained from tissues of embryonic or adult chicks on the morphology and the survival of chick muscle fibres in culture were studied.
2. Extract of brain + spinal cord of the embryo and extract of the remainder of the embryo were equally effective. Extract of the liver of the embryos had no effect.
3. Extract of muscles and brains of adult chicks had both a favourable effect.
4. The effects of heat treatment and of dialysis of embryo extract suggest that the active factor is a rather heat stable macromolecule.
5. The resting membrane potential and the sensitivity to ACh of the muscle fibres were not affected by extract.
6. The input resistance of muscle fibres grown in medium with embryo extract was lower than of that of fibres grown in medium without extract.

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S U M M A R Y

The structure and the physiological condition of a skeletal muscle are under control of its innervating neurons. This trophic maintenance function of the neuron has been demonstrated *in vivo*. In mixed cultures of explants of spinal cord tissue and skeletal muscle the formation of electrophysiologically active neuromuscular junctions and the trophic support of muscle cells by neurons have been demonstrated. The investigations described in this thesis have been designed to obtain more information about this trophic relationship. The attention has been focused on the following questions: i. is it possible to quantify trophic influences of neuronal tissues on the morphology of muscle fibres in culture, ii. are these influences exerted by the electrical or contractile activity induced at the neuromuscular junction or is the trophic support mediated by a humoral factor and iii. if a humoral factor is involved is it possible to mimic the trophic support by extracts of embryonic material as a first step in the isolation and identification of the active substance involved? In these studies cultures of nerve and muscle cells were used.

Initially, it has been investigated whether it was feasible to study trophic relationships between muscle fibres and individual motoneurons in cultures of muscle cells and cells obtained by the dissociation of spinal cord material. It was tried to recognize different types of neurons according to their morphology in culture and to find morphological parameters to identify a certain type of neuron as a motoneuron. Radical changes in cell shape, cell diameter and number of neurites of the neurons during the culture period did not allow classification of the neurons. The morphological classification of neurons, obtained by dissociation of the anterior

horn of the spinal cord and maintained for several weeks in culture, also appeared impossible.

Another approach was, therefore, chosen and chick muscle cells were cultured together with explants of mouse spinal cord. Neurons in the spinal cord explants formed functional junctions with the muscle fibres after two days of co-cultivation. Spontaneous miniature end plate potentials (mepp's) were generated at the junctions by random release of acetylcholine and in many junctions end plate potentials (epp's) arose as a result of spontaneous neuronal activity. Both types of activity could be blocked with d-tubocurarine. Tetrodotoxin inhibited the conduction of action potentials in the neurite, resulting in a block in the generation of epp's. Extracellular stimulation of neurites evoked epp's. The spontaneous end plate depolarizations occurred at random or in rather definite repetitive patterns. The similarity of these patterns suggested that they were generated by neurons in the explant belonging to a neuronal network that generated repetitive bursts of activity.

A 20-fold increase of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio in the perfusion fluid resulted either in an increase of the amplitude or in the frequency of the epp's, but had no effect on the mepp's. A 40-fold decrease in the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration ratio reduced the amplitude of the epp's and the frequency of the mepp's. In neuromuscular junctions on muscle fibres that had been together with explants for more than 18 days functional AChE could be demonstrated, showing a certain maturation of those junctions in culture.

The presence of spinal cord explants favoured the morphological development and the survival of muscle fibres cultured in medium without embryo extract. The resting membrane potential of muscle fibres was not influenced by innervation by neurons in spinal cord explants. The amount of cross striation, however, was well correlated with the frequency of the epp's

recorded in such fibres. This seemed to indicate that activity of the muscle fibre played an important role. The spinal cord explants, however, appeared equally effective in trophic support when the generation of epp's was abolished by the continuous presence of d-tubocurarine in the culture medium. Moreover, ciliary ganglia and sympathetic ganglia of chick embryos that contain neurons that innervate muscle fibres in culture provided less effective support than cortex slices of mouse embryos that do not contain such neurons. Since the trophic support was correlated with the functioning of the junction between spinal cord neurons and muscle fibres but appeared independent of the mechanisms which produce the end plate potential, it is likely that the trophic action was mediated by a humoral factor released by the nerve terminal. On the basis of the present results it cannot be excluded that the trophic factor might be ACh, provided this substance would not need the postsynaptic ACh receptor for its trophic action.

The idea that a humoral factor is involved was strengthened by the results of experiments which demonstrated that the trophic support by explanted neuronal material can be mimicked by extracts obtained from various chick tissues. Extracts of chick embryos and adult chicks exerted a striking positive effect on the morphology of cultured muscle fibres. The factor or factors which produce this effect did not seem to be present in the liver of the chick embryo. The factor consisted of a rather heat resistant macromolecule which was definitely not acetylcholine. Embryo extract did not influence the membrane resting potential of the muscle fibres or their sensitivity to ACh. The input resistance of muscle fibres grown in medium without extract was higher than that of fibres grown in medium with extract.

The effects of explants and extracts were evaluated by estimating the condition of the muscle fibres according to four morphological parameters. These four parameters were chosen because they seemed reliably to

represent the morphological development and the survival of muscle fibres in culture. If it is stated arbitrarily that for a certain parameter a biologically significant effect is present if the mean difference between control and experimental cultures (the figure of merit) is at least 1.5 times as large as the S. E. M. for that parameter, the results show that in general cross striation and thickness were affected in parallel and that the same was true for number of fibres and of vacuolization. In almost all cases an effect or the absence of an effect was found for cross striation together with thickness or for number of fibres together with number of vacuolated muscle fibres.

As mentioned above neither explants nor extracts had an influence on the resting membrane potential. This resulted in the rather unexpected observation that well developed muscle fibres in this respect were no better than very thin, highly vacuolated fibres. In vivo the fall of the resting membrane potential after denervation appears even earlier than the changes of the morphology. Whether different neurotrophic influences are involved or whether the resting membrane potential of developing fibres is less sensitive remains to be seen.

The experiments reported in this thesis demonstrate that neurotrophic influences can be studied quantitatively in tissue culture and suggest that these influences are exerted by humoral factors.

KORTE BESCHRIJVING VAN HET ONDERZOEK

Inleiding

In het ruggemerg van gewervelde dieren bevinden zich motorische zenuwcellen. Deze cellen zijn door hun lange uitlopers (axonen), gewoonlijk gebundeld in zenuwen, verbonden met de spiervezels waaruit de skeletspieren zijn opgebouwd. Elk van deze uitlopers maakt gewoonlijk contact met meerdere spiervezels. De contactplaats van zenuwuitloper en spiervezel, de motorische eindplaat, heeft een gespecialiseerde structuur. Zenuwimpulsen, een soort elektrische signalen, kunnen worden opgewekt in de motorische zenuwcel, ze worden dan voortgeleid langs de uitloper naar deze eindplaat en maken daar de overdrachtstof (transmitter) acetylcholine vrij, die zeer snel een kortdurende binding aangaat met gespecialiseerde receptoren in de membraan van de spiervezel. Als gevolg van deze tijdelijke binding ontstaan er tijdelijk kleine openingen in de membraan en kunnen elektrisch geladen deeltjes, o.a. Na^+ en K^+ ionen, er door heen. De binnenkant van de spiervezel wordt hierdoor tijdelijk minder negatief geladen. Deze ladingsverandering veroorzaakt op zijn beurt elektrische impulsen in andere delen van de membraan, die de spiervezel laten samentrekken.

Het is gebleken, dat de motorische zenuwcel behalve dit zeer kort

durende effect (onderdelen van een seconde) ook een veel langer durende invloed heeft op de skeletspieren (weken). Als de verbindingen tussen de motorische zenuwcellen en de skeletspieren, bijv. ten gevolge van een ongeluk, langdurig verbroken wordt, ontstaat niet alleen een verlamming, maar treden er ook een aantal langzame veranderingen op in de spiervezels. Het meest opvallend hierbij is het verlies aan volume als gevolg van het dunner worden van de spiervezels en het uiteindelijk afsterven van spiervezels.

De verschijnselen, die na een dergelijke denervatie in de spiervezels optreden, hebben geleid tot de conclusie dat de spieren een "trofische" d.w.z. voedende invloed van de zenuwcellen ondergaan. Als trofische invloeden kunnen worden beschouwd alle invloeden afkomstig van de motorische zenuwcel, die langdurig de structuur en de werking van de spier beïnvloeden. De manier waarop de neurotrofische beïnvloeding tot stand komt is ondanks uitvoerig onderzoek nog onbekend. Sommige onderzoekers veronderstellen dat een spiervezel, die vaak wordt aangezet tot samentrekken, juist door deze activiteit in een goede conditie blijft. Anderen menen daarentegen dat die spiervezel in stand wordt gehouden door één of meer speciale stoffen, die door de zenuwcel in de motorische eindplaat aan de spier worden overgedragen. Er zijn aanwijzingen dat de transmitterstof zelf deze rol vervult. Er zijn echter ook argumenten dat een andere, nog hypothetische "neurotrofische factor" het effect bewerkstelligt. Na denervatie treden, volgens de ene opvatting, de lange termijn veranderingen in de spier op, doordat deze niet meer samentrekt, volgens de andere hypothese zijn deze veranderingen het gevolg van het wegvallen van de bevoorrading met transmitterstof of neurotrofische factor. Het kan ook heel goed zo zijn dat beide mechanismen een rol spelen.

Tot voor kort is vrijwel al het onderzoek naar neurotrofische beïnvloeding gedaan met proefdieren. Enkele jaren geleden echter bleek het mogelijk

zenuwcellen uit embryos geruime tijd buiten het lichaam in leven te houden in zgn. weefselkweek. De zenuwcellen hechtten zich op de bodem van een kweekschaaftje met voedingsmedium en vormden uitlopers, die contact maakten met andere zenuwcellen. De afzonderlijke zenuwcellen in dergelijke culturen zijn te zien met een fase-contrast microscoop. Zo is het mogelijk veranderingen van de zenuwcellen te volgen omdat een cultuur op achtereenvolgende dagen steeds opnieuw bekeken kan worden zonder dat de zenuwcellen daarbij worden beschadigd. Door zeer dun uitgetrokken glazen elektroden aan te brengen in de zenuwcellen en hiermee de elektrische activiteit van de cellen af te leiden, bleek dat er functionerende verbindingen tussen de zenuwcellen waren ontstaan. Elektrische impulsen toegediend aan de ene zenuwcel werden voortgeleid door de pas aangelegde uitlopers en in de contactplaats aan andere zenuwcellen overgedragen. Het was ook mogelijk in culturen van spiercellen en zenuwcellen uit het ruggemerg, waarin zich immers de motorische zenuwcellen bevinden, werkende contacten tussen zenuwcellen en spiercellen aan te tonen.

Al spoedig werd duidelijk dat er niet alleen contacten werden gemaakt, maar dat er ook een zekere mate van trofische beïnvloeding van de spiercellen te zien was, die zich uitte in een betere ontwikkeling en een langere overleving van de spiercellen. Daardoor leek de mogelijkheid aanwezig neurotrofische invloeden in weefselkweek te onderzoeken. Twee vragen lagen er voor de hand, nl. i. is het mogelijk de gevolgen van neurotrofische invloeden te kwantificeren en ii. zijn deze invloeden een gevolg van activiteit veroorzaakt door de zenuwcellen of zijn ze een gevolg van afgescheiden stoffen?

Samenvatting van de resultaten van het onderzoek

Bij het begin van dit onderzoek was er over de mogelijkheden van weefselkweek van zenuwcellen nog veel onbekend en het lag voor de hand te proberen het onderzoek te doen in culturen die alleen motorische zenuwcellen en spiercellen bevatten. Daarom is eerst nagegaan of er in culturen van ruggemercellen van het embryo van de muis, waarin naast de motorische zenuwcellen ook andere soorten zenuwcellen voorkomen, verschillende klassen zenuwcellen te onderscheiden zouden zijn op grond van hun vorm, hun aantal uitlopers of de grootte van hun celoppervlak. Door van een groot aantal zenuwcellen elke twee of drie dagen een foto te maken konden de vormveranderingen van een cel in kweek gevolgd worden. Het bleek dat de eigenschappen van de zenuwcellen gedurende de 1-2 maanden kweekperiode zo veranderlijk waren dat een zinvolle indeling in klassen niet mogelijk was. Slechts één ding werd duidelijk, nl. dat er twee groepen zenuwcellen te onderscheiden waren; zenuwcellen behorend tot de eerste groep hadden gemiddeld een kleiner oppervlak en stierven voor de 30^e dag in cultuur. Zenuwcellen uit de tweede groep waren groter en waren tenminste tot de 48^e dag in goede conditie. Tijdens de vroege ontwikkeling van het levende dier vormen zenuwcellen uitlopers, die trachten contact te maken met andere cellen. Lukt het echter niet de juiste cel te bereiken, dan gaat zo'n zenuwcel vaak te gronde. Het zou dus kunnen zijn dat ook in kweek het afsterven van een deel van de cellen iets te maken heeft met het ontbreken van de juiste cellen om contact mee te maken (hoofdstuk 2).

Nadat duidelijk was geworden, dat motorische zenuwcellen niet te herkennen waren op grond van hun vorm of celoppervlak werd gekozen voor een andere proefopzet. In dunne plakjes, gesneden van het ruggemerg,

blijft in weefselkweek de positie van de verschillende zenuwcellen ten opzichte van elkaar maanden lang hetzelfde. Op die manier is het in de toekomst misschien mogelijk aanwijzingen te krijgen welke zenuwcellen functionele contacten maken met spiercellen, als deze plakjes (explantaten) gekweekt worden tezamen met spiercellen. Het was eerst nodig vast te stellen dat zulke contacten inderdaad werden gemaakt en te onderzoeken in hoeverre die contacten leken op die in het levende dier.

Spiercellen, die door enzymatische behandeling met trypsine waren vrijgemaakt uit pootspieren van embryos van de kip, werden in cultuur gebracht. Deze spiercellen vermenigvuldigden zich en versmolten vervolgens tot langwerpige spiercellen (spiervezels), die 10-100 celkernen bevatten. Na 4 dagen begonnen de vezels dwarsstreping te vertonen en als het juiste voedingsmedium werd gebruikt was het mogelijk dergelijke culturen gedurende een aantal weken in goede conditie te houden. Bij 2 dagen oude culturen van spiercellen werden explantaten gevoegd van het ruggemerg van het embryo van de muis. Deze explantaten hechtten zich te midden van de spiercellen. De zenuwcellen in de explantaten vormden uitlopers, waarvan vele contact maakten met de spiervezels. Spoedig hierna werden er snelle samentrekkingen van de spiervezels gezien, die in culturen gekweekt zonder explantaten nooit voorkwamen. Dit wees er al op, dat de zenuwcellen in staat waren de spiervezels tot samentrekken te prikkelen. Na enkele dagen bleek in de spiervezel een elektrische activiteit aanwezig te zijn, die mogelijk werd veroorzaakt door zenuwcellen. Door tetrodotoxine toe te voegen aan de cultuur kon dit verder worden onderzocht. Deze stof blokkeert de voortgeleiding van zenuwprikkels, die lopen van de zenuwcel naar het zenuwuiteinde. Door het toevoegen van deze stof verdween een deel van de elektrische activiteit van de spiervezel, maar andere kleinere veranderingen van de elektrische activiteit werden niet beïnvloed. Deze kleine veranderingen

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zijn waarschijnlijk een gevolg van het spontaan uitlekken van de transmitterstof uit het zenuwuiteinde, zoals dat ook in het levende dier gebeurt.

Curare blokkeert de speciale eenheden in de spiervezelmembraan waaraan de transmitterstof bindt. Als curare gevoegd wordt bij de cultuur dan moet dus alle activiteit verdwijnen die veroorzaakt wordt door transmitterstof, dit bleek inderdaad het geval. Uit deze proeven en uit een aantal andere over het effect van veranderingen van de concentraties van Ca^{2+} en Mg^{2+} ionen en de invloed van physostigmine, een stof die de afbraak van de transmitterstof vertraagt bij goed ontwikkelde eindplaten, bleek dat er functionerende verbindingen werden gemaakt tussen zenuwcellen in het explantaat en spiervezels, die in veel opzichten overeenkomst vertoonden met normale eindplaten (hoofdstuk 3).

Nadat aldus gebleken was dat dit model systeem bruikbaar was voor de bestudering van het innervatie proces kon worden begonnen met het onderzoek van de twee gestelde vragen namelijk of het mogelijk is het effect van neurotrofische invloeden te kwantificeren en vast te stellen waarvan deze invloeden het gevolg zijn. Het kwantificeren van de vorm en structuur (morfologie) en de overleving van de spiervezels in de cultuur bleek mogelijk. Voor dit kwantificeren werden vier eigenschappen van de spiervezels bestudeerd: i. de dwarsstreping, ii. de dikte, iii. het aantal spiervezels met vacuoles en iv. het totale aantal spiervezels per oppervlakte eenheid. Omdat in elke cultuur een groot aantal spiervezels aanwezig was, was het niet mogelijk deze stuk voor stuk te bekijken. Daarom werd een schatting gemaakt van de hele cultuur. De toestand van elk van deze eigenschappen werd gewaardeerd met een cijfer van 0-4. In een groot aantal culturen is gekeken wat deze schatting betekende en hoe betrouwbaar deze was. Hiertoe werd in deze culturen een aantal individuele spiervezels een waardering gegeven volgens een schaal van a-e (zie fig. 1 en 2, hoofdstuk 4). Op deze wijze werd voor elk van de bovengenoemde

waarderingsgetallen van 0-4, geldend voor hele culturen nagegaan hoeveel individuele spiervezels met de waarden a tot e in representatieve culturen aanwezig waren.

De methode van schatten van de morfologie in hele culturen werd gebruikt in het verdere onderzoek. In de eerste plaats werd de invloed onderzocht van explantaten van het ruggemerg van kippe- en muizeëmbryos. Beide soorten van explantaten hadden grote invloed op de morfologie en de overleving van de spiervezels in de buurt van het explantaat. Electrofysiologisch werd in deze spiervezels de elektrische activiteit (post-synaptische potentialen) gemeten die het gevolg waren van het contact met zenuwcellen. Deze activiteit bleek goed gecorreleerd met de mate van dwarsstreping. Dit betekent niet noodzakelijkerwijs, dat deze activiteit op zich belangrijke bijdragen levert aan de kwaliteit van de morfologie van de spiercel. Het trofisch effect van innervatie zou ook kunnen komen door een grotere overdracht van een humorale trofische factor tussen zenuwcellen en spiervezel, waartussen veel prikkels worden overgedragen. Daarom werd de volgende proef gedaan. Curare, de stof die de overdracht van prikkels van de zenuwcel naar de spiervezel blokkeert, werd in het groeimedium gedaan vanaf het moment dat het explantaat werd toegevoegd aan de cultuur. Daardoor werd voorkomen dat zenuwcellen elektrische prikkels aan de spiervezels overdroegen. Het bleek nu dat het explantaat ook onder deze omstandigheden nog steeds een gunstige invloed had op de morfologie en de overleving van de spiervezels. De gunstige invloed op de morfologie bleek dus niet gecorreleerd met spieractiviteit. Er moeten dus andere factoren in het spel zijn en het is zeer waarschijnlijk dat het hier gaat om een humorale trofische stof.

Dat er andere factoren aanwezig zijn in embryos en volwassen dieren bleek ook uit experimenten, waarbij spiercellen werden gekweekt in media met extracten van verschillende weefsels van embryos van de kip

en van spieren en hersenen van volwassen kippen. Vergeleken met spiervezels gekweekt in media zonder extract ontwikkelden de met extract gekweekte vezels zich veel beter en bleven langer in leven. De hier beschreven experimenten wijzen erop dat er bepaalde stoffen in embryos en volwassen dieren aanwezig zijn, die een trofische invloed uitoefenen op spiervezels in weefselkweek (hoofdstuk 5).

CURRICULUM VITAE

Jacob Hooisma werd in 1945 geboren in Dantumawoude. Vanaf 1958 bezocht hij het Stedelijk Gymnasium te Leeuwarden, deed in 1964 eindexamen gymnasium- β , begon de studie in de biologie te Groningen en behaalde het kandidaatsexamen in 1968. Het doctoraal examen, met als hoofdvak biofysica en de bijvakken neurofysiologie en capita uit de wiskunde, werd afgelegd in 1972.

De studie voor het hoofdvak biofysica onder leiding van Dr. H. H. van Barneveld in de stimulerende omgeving van de afdeling biofysica geleid door Prof. Dr. J. W. Kuiper sterkten hem in zijn belangstelling voor het wetenschappelijk onderzoek. De mogelijkheid tot het doen van dit onderzoek werd hem geboden in het Medisch Biologisch Laboratorium TNO door steun van de stichtingen FUNGO en PROMESO. In dit laboratorium werd het in dit proefschrift beschreven onderzoek gedaan onder leiding van Dr. W. F. Stevens en Dr. E. Meeter. Sinds 1975 is hij in tijdelijke dienst van het Rotterdamsch Radio-Therapeutisch Instituut werkzaam in het Medisch Biologisch Laboratorium en sinds kort betrokken bij neurotoxicologisch onderzoek.