STUDIES ON NEUROTROPHIC FACTORS
IN CHICK MUSCLE

by

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ABSTRACT

Experiments have been performed in order to try to elucidate the biological function of intramuscular protein (IMP), a novel substance antigenically related to vasoactive intestinal polypeptide (VIP). IMP was previously shown to be present in chick skeletal muscle during a restricted phase of development and to be secreted by cultured myotubes. This evidence and other findings suggested that IMP might be a trophic factor for motoneurons, and it is this hypothesis which the thesis sets out to test.

Immunocytochemistry showed that IMP is present in ciliary muscle over a time course related to that of naturally occurring cell death in the ciliary ganglion (CG) and raised the possibility that IMP may be a trophic factor for CG neurons in addition to spinal cord motoneurons.

IMP for use in bioassays was partially purified from embryonic muscle extract and myotube conditioned medium (MCM) by column chromatography, using a modified VIP radioimmunoassay to assay for IMP concentration. The molecular weight of IMP in muscle extracted in a neutral buffer is ca.120kD, but when extracted in acid the major form has a molecular weight of ca.35kD. The major form of IMP in MCM also has a molecular weight of ca.35kD.

Fractions containing IMP were tested in _in vitro_

bioassays on CG neurons and motoneurons. Many IMP-containing fractions had survival activity for CG neurons; activities in IMP-containing fractions were also identified which (i) increased ChAT activity of CG neurons, (ii) had a synergistic effect on survival of CG neurons in the presence of ciliary neurotrophic factor, and (iii) had survival activity for spinal cord motoneurons. The role of IMP in these bioactivities was assessed by using fractions that had been depleted of IMP by antibody affinity chromatography, and in no case was activity clearly attributable to IMP. Thus several, possibly novel, trophic activities were identified.

In conclusion, the strong circumstantial evidence for IMP being a neurotrophic factor has not been supported thus far by experiments designed to test this idea _in vitro_,

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CONTENTS

CHAPTER THREE. THE PARTIAL PURIFICATION OF IMP

CHAPTER FOUR. THE ACTIVITY OF IMP AND OTHER FACTORS IN _IN_

VITRO_ NEURONAL BIOASSAYS

LIST OF TABLES AND FIGURES

CHAPTER 4

LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
aFGF	acidic fibroblast growth factor
ALD	anterior latissimus dorsi
Ara-C	cytosine arabinoside
bFGF	basic fibroblast growth factor
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumen
CG	ciliary ganglion
ChAT	choline acetyl transferase
CNTF	ciliary neurotrophic factor
cpm	counts per minute
cMCM	concentrated myotube conditioned medium
DAB	3,3'-diaminobenzidine tetrahydrochloride
DEAE	diethyl aminoethyl
DMEM	Dulbecco's modified Eagle's medium
DRG	dorsal root ganglion
DTT	dithiothreitol
E	embryonic day
EDTA	sodium ethylenediaminetetraacetate
FGF	Fibroblast growth factor
FuDR	5-fluorodeoxyuridine
GIP	gastric inhibitory peptide
hdcMCM	heparin-depleted cMCM
hecMCM	heparin elution of cMCM
ieMCM	ion-exchange purified MCM
ieME	ion-exchange purified muscle extract

Ig	immunoglobulin
IGF-II	insulin-like growth factor II
IMP	Intramuscular protein
kD	kilodaltons
KGF	keratinocyte growth factor
LMC	lateral motor column
LPBS	low phosphate-buffered saline
MCM	myotube conditioned medium
MMC	medial motor column
MW	molecular weight
NGF	nerve growth factor
ONE	rat optic nerve extract
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
pSNE	purified rat sciatic nerve extract
RIA	radioimmunoassay
SBTI	soybean trypsin inhibitor
SEM	standard error of the mean
SNE	rat sciatic nerve extract
St	embryonic stage (Hamburger and Hamilton,1948)
TTX	tetrodotoxin
VIP	Vasoactive intestinal polypeptide
WGA-FITC	fluorescein isothiocyanate coupled to wheat germ agglutinin
α-BTX	alpha bungarotoxin
ß-BTX	beta bungarotoxin

CHAPTER 1

INTRODUCTION

INTRODUCTION

In this thesis I will be considering neurotrophic factors for efferent neurons during development in the chick. The major part of the thesis concerns the putative trophic action of a novel protein antigenically related to vasoactive intestinal polypeptide (VIP) that is present in skeletal muscle during a restricted phase of development. This protein has been named IntraMuscular Protein (IMP). The minor part of the thesis concerns the identification of novel neurotrophic activities that are not due to IMP.

Before describing the background work on IMP, I will first attempt to set the scene by reviewing, firstly, the relevant part of nerve-muscle development, and, secondly, the neurotrophic hypothesis as it relates to nerve-muscle development.

NERVE-MUSCLE DEVELOPMENT

Muscle development

The muscles to be considered here are the skeletal muscles of the trunk and limb. Although we are primarily concerned with chick development, work so far in the rat has revealed only minor differences with the chick and so it is reasonable to include these results where appropriate.

The origin of the muscle cells in both the axial and

limb muscles are cells in the somites, as was demonstrated by chick-quail chimaera experiments (Chevallier et al.,1977; Chevallier,1979; Christ et al.,1977). The axial muscles develop in situ from the myotome whilst the precursor cells of the limb muscles must first migrate to the limb. Under scanning EM the presumptive migrating cells can be seen emerging from the ventrolateral corner of the somites and crossing the short gap laterally to the prospective limb-forming region of the somatopleure (Jacob et al.,1978). This migration occurs very early, before the limb bud has formed; in the chick, the migration of cells to the prospective hindlimb begins at Stage (St) 16 and continues to St 19-20.

For both axial and limb muscles, individual muscles form by a highly stereotyped process of cleavage of primitive muscle masses into sequentially smaller blocks by the interpolation of sheets of connective tissue. In the limbs, there are initially two primitive muscle masses, dorsal and ventral. The cleavage of these masses in the chick hindlimb was described in detail by Romer (1927); cleavage begins at St 27 (Embryonic day (E) 5.5) and is complete by St 30 (E6.5). The mechanism of cleavage is poorly understood.

Muscle development on a cellular scale proceeds by the fusion of myoblasts to form myotubes. Kelly and Zacks (1969) demonstrated that the formation of myotubes occurs in at least two temporally separate waves. Subsequent work has confirmed that there are two waves and named the

products of them primary myotubes and secondary myotubes. Individual primary myotubes form by the rapid fusion of rows of myoblasts spanning the gap between tendons i.e. the whole length of the muscle. After formation, primary myotubes acquire a basal lamina and grow rapidly in width and length. Beneath the basal lamina are mononucleate cells which are mostly comprised of secondary myoblasts. In the chick hindlimb, the first primary myotubes are seen at around St 28 (E6), and formation of primary myotubes continues to St 34 (E8); thus muscle cleavage and primary myotube generation are largely concurrent.

All the events of muscle development described so far are independent of innervation. Early work on transplanted limbs showed that gross morphology was normal in the absence of a nerve supply (Hunt,1932; Hamburger,1939), whilst normal muscle cleavage in the absence of nerves was shown by Shellswell (1977). Harris (1981) demonstrated that primary myotubes develop in normal numbers in the rat following either destruction of motoneurons with ß-bungarotoxin (ß-BTX) or paralysis with tetrodotoxin (TTX); a lack of influence of activity on primary myotube formation has also been shown in the chick, using curare (McLennan,1983a).

The next major event in muscle development is the fusion of secondary myoblasts to form secondary myotubes. In rats, fusion of secondary myoblasts begins near the neuromuscular junction on the adjacent primary myotube (Duxson et al.,1989a); the developing secondary myotubes

then gradually elongate by fusion of secondary myoblasts at random points along their length (Harris et al.,1989a) until they reach the tendons at either end. Secondary myotubes subsequently acquire their own basal lamina and a cross section of a muscle at this stage reveals characteristic "myotube clusters" comprising a central primary myotube surrounded by several secondary myotubes. Secondary myotubes outnumber primary myotubes by about 5 to 1 and thus go on to form the bulk of adult muscle. In the chick hindlimb, the secondary myotubes form between St 38-42 (E12 - E16; McLennan,1983b).

In contrast to primary myotubes, secondary myotube formation is absolutely dependent on a functional innervation (Harris,1981; McLennan,1983a). It appears that this is because innervation provides a mitogenic signal for secondary myoblasts (Ross et al.,1987): after ß-BTX treatment to rats, secondary myotubes failed to develop and few mononucleate cells (presumptive secondary myoblasts) were present; when treatment with ß-BTX was delayed until secondary myotube formation had begun, myotubes continued to form until mononucleate cells were exhausted.

In vitro studies suggest that different populations of committed myoblasts are responsible for production of primary and secondary myotubes, although this has yet to be tested in vivo. Hauschka and colleagues (White et al.,1975; Rutz and Hauschka,1982) cloned myoblasts from chick hindlimbs between St 21-38 (E3-12) and found that (i) clones differed in terms of their colony morphology and

medium requirements, (ii) these differences were stably inherited over many generations and (iii) the spectrum of clones obtained varied with age, so that the types of clone obtained at St 21 were very rare at St 38 and vice versa. Moreover, it appears that the precursors of the late-forming colony types migrate into the limb bud later than precursors of the early-forming colony types (Seed and Hauschka,1984). Miller and Stockdale (1986) extended the phenotypic characterization of clonable myoblasts by showing that myoblast colonies from St 23-27 (E4-E6) limbs included mixtures of cells of three myosin heavy chain phenotypes whereas myoblast colonies from St 36-38 (E10-E12) were all of the same phenotype. A neural dependence on the appearance of clonable myoblasts has also been demonstrated (Bonner,1978): the abundance of late-forming colony types was greatly reduced following either early ablation of the neural tube or curare treatment. Treatment with curare had to be performed some time between St 27-30 (E5.5-6) to have this effect, implying a critical time window of neural dependence (Bonner,1980).

Motoneuron development

In the chick spinal cord motoneurons are found in two columns, the lateral motor column (LMC) supplying the limb muscles and the medial motor column (MMC) supplying the muscles of the trunk. All of the motoneurons projecting to a particular muscle constitute a motoneuron pool; the cell bodies of neurons in a pool are clustered together and

occupy a discrete anatomical location within the motor column. Pools innervating the hindlimb muscles in the chick were mapped by Landmesser (1978a) and Hollyday (1980). The major events of motoneuron development are cell birth, axon outgrowth and pathfinding, target recognition, cell death, synaptogenesis, and elimination of polyneuronal innervation; not all of these need to be discussed in detail.

The birthdates of motoneurons in the lateral motor column of the chick were investigated by Hollyday and Hamburger (1977) using tritiated thymidine autoradiography. Lumbar motoneurons are born between St 17-27 (E3-5.5), with over 95% of them being born in the first one and a half days of this period (St 17-23). Birth of brachial motoneurons starts earlier, at St 15, and thereafter their production follows a similar time course to those in the lumbar region.

The numbers of motoneurons born at different levels of the cord was until recently thought to be uniform. However, Oppenheim et al. (1989a) found that this is not the case: the number of motoneurons present per cross-section at St 23 (E4) in the brachial and lumbar (limb-innervating) regions was 50% higher than in the cervical and sacral regions of the cord, with the motoneuron density in the thoracic cord being intermediate.

After birth, motoneurons rapidly extend axons, the first axons leaving the spinal cord at St 19. The axons traverse the anterior part of the somite (Keynes and

Stern,1984) and in the limb regions then aggregate to form the plexi; for the hindlimb these are the anterior crural and the posterior sciatic plexi. The growth cones remain a relatively long time in the plexi and it is not until St 24 (E4.5) when the major dorsal and ventral nerve trunks emerge to invade the premuscle mesenchyme (Tosney and Landmesser,1985a). The first muscle nerves are recognizable at St 26 (E5) and, by St 28 (E5.5-6), the general pattern of muscle nerves is apparent, before muscle cleavage is complete. There is some evidence that the formation of muscle nerves is dependent on the presence of muscle. Tosney (1987) found that the formation of the dorsal ramus, the nerve which innervates the dorsal axial muscles, is dependent on sufficient target muscle being present. Lewis et al.(1981) found that the nerve trunks in the limb formed normally but muscle nerves were absent when muscle development was prevented by somite ablation, although the presence of motoneurons after the manipulation was not demonstrated.

In the chick hindlimb there is good evidence that the axons form correct projections from the time of initial outgrowth (Landmesser,1978; Lance-Jones and Landmesser,1981; Tosney and Landmesser,1985b). Axon guidance is not merely a passive process; for example, motor axons made to enter novel spinal nerve roots by neural tube reversal still find their correct pathways and muscle targets, provided that the displacement is not too great (Lance-Jones and Landmesser,1980). Thus, motoneurons

are intrinsically specified to project to individual muscles. As the neural tube reversals were carried out very early (St 15-16, E2) it can be concluded that this specification takes place before the motoneurons are born or the limb bud is formed.

The axons in the muscle nerves wait for a period of about 24 hrs. before invading the muscle, at St 30 (E6.5; Tosney and Landmesser,1985b). The development of intramuscular nerve branching within two chick hindlimb muscles, the iliofibularis (a predominantly slow muscle) and posterior iliotibialis (a fast muscle), was recently investigated in detail by Dahm and Landmesser (1989). The spatial and temporal pattern of nerve branching was highly stereotyped for each muscle, and varied with the fibre type being innervated. Treatment with curare caused a defasciculation of intramuscular nerves and greatly enhanced nerve branching.

Between St 29-36 (E6.5-10) between 40-50% of the 20,000 lumbar motoneurons in the lateral motor column die (Hamburger,1975; Chu-Wang and Oppenheim,1978); during this time, many degenerating motoneurons are visible in the LMC. This period of death encompasses the time over which axons ramify and form synapses in muscles but is before the beginning of secondary myotubes formation. Some regional variation in the degree of death within the lumbar region was found by Williams et al.,(1987): death is most extensive at the edges of the lumbar region so that about 65% of motoneurons in lumbar segments 1 and 8 die compared

with about 30% death of motoneurons in lumbar segments 4 and 5. The degree and timing of death within individual motoneuron pools has not been reported thus far, although it is known that the position a pool occupies within the cord does not alter over the period of death (Landmesser,1978). In the brachial region, the period of motoneuron death is much more extended: 60% of the motoneurons die between St 28-46 (E6-21) and only half of this death occurs by St 36 (E10), when death in the lumbar region has already ended (Oppenheim and Majors-Willard,1978).

Although the first weak synaptic contacts are made very early (muscle contraction in the chick hindlimb can be elicited by nerve stimulation at St 27; Landmesser and Morris,1985), the majority of nerve branches reach potential sites of synapses on primary myotubes between St 31-34 (E7-E8) (Dahm and Landmesser,1989). Later (after St 38), nerve branches innervate the newly forming secondary myotubes.

Each endplate site becomes innervated by more than one axon; later, the number of axons innervating each endplate is reduced to one. The formation and elimination of this polyneuronal innervation have not been studied in the chick hindlimb, but data on the anterior latissimus dorsi (ALD), an anterior trunk muscle, were obtained by Bennett and Pettigrew (1974). In the ALD, only single axon profiles are visible at any point on a myotube at St 34 (E9), whilst at St 37 (E11), when acetylcholinesterase

(AChE) deposits (markers of synaptic sites) first appear, between 1-4 axon profiles are visible at each AChE deposit, and the same is true at St 42 (E16). The time course of development of primary and secondary myotubes has not been investigated in the ALD and so it is not known whether primary myotubes become multiply innervated before the secondary myotubes have formed. Physiological experiments indicate that elimination of polyneuronal innervation occurs after E18, and is complete by 4 weeks posthatch. Thus the elimination of polyneuronal innervation is a late event.

TARGET-DEPENDENT NEURONAL DEATH AND TROPHIC FACTORS

I. Introduction

The regulation of naturally occurring neuronal death by production of limited amounts of trophic factor by the target is thought to occur widely within the nervous system (reviewed in Purves and Lichtman,1985). We will firstly consider the evidence for target-dependent cell death in the motor system, then describe some properties of the best characterized trophic factor, nerve growth factor (NGF), before going on to discuss motoneuron trophic factors.

II. Target-dependent neuronal death in the motor system

Death of a substantial proportion of spinal motoneurons is a common feature in vertebrate development: in addition to the chick (described above), it has been

shown to occur in amphibians (Hughes,1967) and rodents (Lance-Jones,1982). The motoneurons that will die differentiate normally up to the time of death and have probably all sent axons to the periphery before they die (Oppenheim et al.,1978; Chu-Wang and Oppenheim,1978). The extent of death is modified by the size of the target. Virtually all motoneurons die when the limb bud is ablated (chick: Hamburger,1958; frog: Prestige,1967; Lamb,1981) or in a limbless chick mutant (Lanser and Fallon,1984); and the onset of death is the same in normal and limb-ablated animals (Oppenheim et al.,1978). Conversely, survival of motoneurons is enhanced by 10-30% when target size is increased by transplantation of a supernumerary limb (Hollyday and Hamburger,1976); thus death of the neurons destined to die is not predetermined.

There is evidence that suggests that the aspect of target size that is important for determining the degree of motoneuron death is the number of primary myotubes. McLennan (1982) showed that the final size of motoneuron pools in the chick hindlimb was correlated with the number of primary myotubes at the start of the death period. Tanaka and Landmesser (1986) measured death of chick and quail motoneurons in normal birds and in birds with cross-species limb bud transplants; an excellent correlation was found between motoneuron survival and number of myotube clusters.

However, some data are inconsistent with the simple view that myotube number determines the degree of cell

death. In <u>Xenopus</u>, motoneuron survival was only slightly reduced when the relative size of the peripheral field was halved by forcing two sciatic nerves to innervate one hindlimb (Lamb,1980). Furthermore, there is evidence that at least some motoneurons in <u>Xenopus</u> die because they fail to make the right connections (Lamb,1979), although this has yet to be quantified; so the situation in <u>Xenopus</u> appears to be somewhat different than in the chick, where we have seen that motoneuron projections are extremely accurate from the earliest times (see above). In birds, survival of motoneurons in the trochlear nucleus was not increased when the peripheral field was enlarged by cross-species transplantation (Sohal <u>et</u> <u>al.</u>,1986). However, the number of myotubes was not quantified in this study and so the result is difficult to interpret.

It should be pointed out that even for chick lumbosacral motoneurons there are other influences on death apart from the target. Okado and Oppenheim (1984) studied the influence of afferent inputs on motoneuron death and found that a combination of early neural crest removal and thoracic spinal transection led to a 37% reduction in numbers of lumbosacral motoneurons at St 36 (E10), i.e. at the end of the normal period of death. In contrast, either experimental manipulation alone led to normal numbers of motoneurons at St 36 (E10) but a 20-25% decrease in numbers by St 42 (E16). Thus both primary afferent neurons and supraspinal neurons can influence motoneuron death, although not in a manner that is easily comprehensible.

Although the influences on motoneuron death may be somewhat different in <u>Xenopus</u>, and factors other than the target can influence death of chick lumbosacral motoneurons, it remains clear that the primary determinant of motoneuron death in the chick lumbosacral cord is the target muscle. The experimental observations support the view that some aspect of the muscle enables motoneurons to survive and that motoneurons compete for a limited supply of this trophic influence. The biological function of motoneuron death is thus thought to be to match the size of the innervating pool to the size of the target. It has been proposed that this trophic influence is a molecule released by the target muscle and taken up by motor axons. An analogy for such a motoneuron trophic factor is provided by the archetypal neurotrophic factor, NGF, but a motoneuron trophic factor has yet to be discovered.

Further insight into the nature of the trophic interaction between muscle and nerve comes from experiments performed with neuromuscular blocking agents. Pittman and Oppenheim (1978,1979), treated chick embryos during the normal period of motoneuron death with a dose of curare that greatly reduced spontaneous muscle contractions and rescued about 50% of the lumbar motoneurons that would normally have died. A similar result was obtained using either α-bungarotoxin (α-BTX) or botulinum toxin, and α-cobratoxin could completely prevent death in a few cases. Thus no motoneurons are predetermined to die, and the results strongly suggested that nerve activity regulates

the ability of the muscle to provide trophic support. Rescue of motoneurons has also been reported in the trochlear nucleus of the duck embryos treated with α-BTX (Creazzo and Sohal,1979), in the spinal cord of rats treated with tetrodotoxin (TTX) (Harris and McCaig,1984) and in the spinal cord of Xenopus embryos treated with α-BTX (Olek,1980); and brachial and lumbar motoneuron survival is 45% greater than normal in the mouse mutant muscular dysgenesis (mdg/mdg) in which there is a deficit in excitation-contraction coupling in skeletal muscle (Oppenheim et al.,1986). Conversely, chronic electrical stimulation of chick hindlimbs caused a 20% increase in lumbar motoneuron death (Oppenheim and Nuñez,1982).

Rescue of motoneurons in the presence of neuromuscular blockade may be due to an increase in the amount of motoneuron trophic factor produced by muscle or, alternatively, it may be the access of motoneurons to the trophic factor that is affected, e.g. by an increase in the number of synaptic sites, with the concentration of the trophic factor itself remaining unchanged. Consistent with this latter explanation, treatment with curare increases the branching of nerves and number of synapses formed within a muscle (Dahm and Landmesser,1989) and there is a correlation between the effects of different neuromuscular blocking agents on motoneuron survival and number of synaptic sites formed (Oppenheim et al.,1989). Also, no measurable increase in either in vitro (Tanaka,1987) or in vivo (Oppenheim,1989b) trophic activity for motoneurons of

muscle extract from chick embryos treated with curare. However, it is not certain that the trophic activity being measured in these experiments is the physiologically relevant one. It will probably not be possible to determine which one of the above explanations is correct until a motoneuron trophic factor is identified.

The search for a motoneuron trophic factor has been heavily influenced by knowledge of the archetypal neurotrophic factor, nerve growth factor (NGF), which we will now consider.

Nerve growth factor

NGF was first purified from mouse submaxillary salivary gland (reviewed in Levi-Montalcini,1987). The active protein (ß-subunit or 2.5S NGF) is a 26.5kD dimer of identical, non-covalently linked monomers which show amino acid sequence similarity to the family of insulin-related proteins.

There is good evidence that NGF is a target-derived survival factor for sympathetic and a subclass of sensory neurons during development (for a review, see Barde,1989). Naturally occurring cell death has been shown to occur in both of these neuronal populations (Levi-Montalcini and Hamburger,1949; Aguayo et al.,1973) and target ablation was found to greatly increase the death of sensory neurons (Levi-Montalcini and Hamburger,op.cit.). NGF has been shown to have effects on survival of these neurons both in vitro (Levi-Montalcini and Angeletti,1963) and in vivo

(Oppenheim et al.,1982; Hamburger et al.,1981). Both NGF and its mRNA are found in low quantities in the targets of sympathetic and sensory neurons, and in the case of sympathetic neurons it has been demonstrated that the levels of protein and mRNA correlate with the density of innervation (Korsching and Thoenen,1983; Shelton and Reichardt,1984; Davies et al.,1987). Most importantly, anti-NGF antibodies have been shown to cause destruction of essentially all sympathetic neurons in neonatal rats (Cohen,1960) and up to 80% of dorsal root ganglion and trigeminal ganglion neurons in guinea pigs in utero (Gorin and Johnson,1979).

More recently, evidence has been obtained that suggests that NGF may also be a trophic factor for a population of CNS neurons, the basal forebrain cholinergic neurons, in the adult rat (reviewed in Thoenen et al.,1987). The death of these neurons that occurs after axotomy can be prevented by intraventricular perfusion of NGF (Hefti,1986; Williams et al.,1986). NGF is synthesised by the target neurons in the hippocampus (Ayer-Lelièvre et al.,1988; Whittemore et al.,1988) and is retrogradely transported by the cholinergic neurons (Schwab et al.,1979). However, a normal rôle of NGF for these neurons in vivo has yet to be demonstrated, and some doubt over such a rôle was recently cast by the demonstration that survival of these neurons in the adult is not affected by ablation of their target (Sofroniew et al.,1990).

The case for NGF in target-dependent neuronal

survival, at least for peripheral neurons, is now well established. Many other effects of NGF on neurons have also been described, mostly in in vitro experiments; these include the regulation of neurotransmitter synthesis by sympathetic neurons (Chun and Patterson,1977a;1977b), a chemotactic action on sensory neurons (Gunderson and Barrett,1979), and the maintenance of terminal arborizations of sympathetic neurons (Campenot,1982). The in vivo relevance of these observations is less clear at present; for example, in at least one system, the mouse trigeminal system, NGF expression occurs too late for NGF to play a rôle in chemotaxis (Davies et al.,1987). It is important to note that the term "trophic" is used to include these effects on the differentiated properties of cells as well as effects on survival.

High and low affinity receptors for NGF have been identified on target neurons (Sutter et al.,1979) and specific retrograde axonal transport of ^{125}I-NGF has been demonstrated (reviewed in Schwab et al.,1981). However, the mechanism of action of NGF remains unclear: although many effects downstream of receptor binding have been described, it is not known which of these, if any, are important for mediating the trophic action of NGF.

Apart from NGF, the only other purified neurotrophic factor shown to have activity in vivo thus far is brain-derived neurotrophic factor (BDNF), which was purified from adult pig brain (Barde et al.,1982). BDNF has a different spectrum of biological activity from NGF: like NGF, it

supports the survival of a large proportion of DRG neurons both in vitro and in vivo (Barde et al.,1982; Hofer and Barde,1988) but unlike NGF it acts on placode-derived sensory neurons, trigeminal mesencephalic neurons and retinal ganglion neurons but does not support the survival of sympathetic neurons (Lindsay et al.,1985; Davies et al.,1986; Johnson et al.,1986).

The BDNF gene has been cloned and sequenced and found to be highly homologous to NGF (Leibrock et al.,1989). A third member of the NGF family, neurotrophin-3, was recently discovered on the basis of DNA sequence homology with NGF and BDNF, using polymerase chain reaction technology (Hohn et al.,1990; Maisonpierre et al.,1990). Neurotrophin-3 supports survival of trigeminal mesencephalic neurons and a subpopulation of neurons in the nodose ganglion that are not responsive to BDNF, and so has a different spectrum of biological activity from both NGF and BDNF.

Motoneuron trophic factors

The basic strategy in the search for motoneuron trophic factors has been to assay skeletal muscle extracts, skeletal muscle conditioned media and known growth factors on motoneurons in vitro. As well as short term survival of motoneurons, Choline Acetyltransferase (ChAT) activity (a measure of the degree of differentiation) and neurite outgrowth have been commonly measured. Since the first reports of an effect of muscle conditioned medium on ChAT

activity (Giller et al.,1977) and an effect of skeletal muscle extract on motoneuron survival (Bennett et al.,1980), many different preparations containing activity for motoneurons have been reported. A list of known molecules and partially purified factors that have been reported to have an action on motoneurons is shown in Table 1.1. We shall now consider the evidence for whether any of these act as a survival factor for motoneurons in vivo during the period of motoneuron death, beginning with the known growth factors.

NGF has not been found to support the survival of motoneurons in vivo: NGF failed to rescue motoneurons in either the chick during the period of naturally occurring motoneuron death NGF (Oppenheim et al.,1982) or in neonatal rats in which death of motoneurons was induced by axotomy (Yan et al.,1988). However, both muscle and the lateral motor column display high-affinity binding sites for NGF between St 23-36 (E4-E10; Raivich et al.,1985) and retrograde transport of NGF by motoneurons has been demonstrated in the chick and rat (Wayne and Heaton,1988; Yan et al.,1988), so an alternative rôle for NGF may exist. NGF was recently reported to have an effect on neurite outgrowth of motoneurons (Wayne and Heaton,1990); however, the effect was small (ca.25% increase in neurite length/neuron) and was obtained using a high concentration of NGF.

Table 1.1. Trophic factors for motoneurons.

Factor/Source	MW(kD)	Biological activity				References
		Sp	Survival	ChAT activity	Neurite outgrowth	
NGF	26.5	Chick	-	-	+	Wayne and Heaton,1990
bFGF	18	Chick	+	?	+	Unsicker et al.,1987
IGF-II	8	Chick	?	-	+	Caroni and Grandes,1990
laminin	400	Rat	-	-	+	Calof and Reichardt,1984
Neonatal rat skeletal muscle	55	Rat	-	+	+	Smith et al.,1986
	1.5	Rat	+	+	-	"
	20	Rat	-	+	?	McManaman et al.,1988
Post-hatch chick leg muscle	50	Chick	-	?	+	Henderson et al.,1984
Embryonic chick leg muscle	400	Chick	-	?	+	"
	<30	Chick	+ (in vivo)	?	?	Oppenheim et al.,1988
neuroleukin	56	Rat	+	-	-	Gurney et al.,1986

Both acidic and basic fibroblast growth factors (aFGF and bFGF) have been reported to have an effect on survival of motoneurons in vitro (Unsicker et al.,1987); however, neither the size nor the statistical significance of the effect are explicitly stated in the paper. bFGF, or a molecule antigenically related to it, is present in skeletal muscle during the period of motoneuron death. Anti-bFGF antibodies were found to stain embryonic chick and rat skeletal muscle cells by immunocytochemistry (Joseph-Silverstein et al.,1989; Kalcheim and Neufeld,1990; Gonzalez et al.,1990); in the chick, staining is inside muscle cells at St 28 (E6) in the limb and at St 31 (E7) in the myotome, but by St 39 (E13) staining is extracellular in the axial muscles. Also, material which competes with iodinated aFGF in a receptor binding assay (as do both aFGF and bFGF) has been obtained from chick limb bud (Seed et al.,1988). bFGF, or another member of the FGF family, are thus still candidates for a motoneuron trophic factor.

Insulin-like growth factor (IGF-II) mRNA has been demonstrated in fetal rat muscle by in situ hybridization (Beck et al.,1987); levels of the mRNA in skeletal muscle increase between E16 to 4 weeks postnatal and then decline to undetectable levels by 8 weeks postnatal (Ishii,1989). Recently, an effect of IGF-II on in vivo nerve sprouting in the adult and in vitro neurite outgrowth of embryonic chick motoneurons was reported (Caroni and Grandes,1990); no effect on motoneuron survival was mentioned.

Laminin is a well-known inducer of neurite outgrowth

of many different types of neuron. It can also potentiate the effects of other trophic factors, e.g. NGF (Edgar et al.,1984) and may well have a similar effect on the action of a motoneuron trophic factor.

Neuroleukin was originally identified as a 56kD protein which was recognised on Western blots by antisera which block motoneuron sprouting (Gurney,1984). Its subsequent cloning (Gurney et al.,1986) and identification as the glycolytic enzyme phosphohexose isomerase make it unlikely to have physiological relevance as a growth factor.

Thus a clear candidate for a motoneuron survival factor, with the possible exception of a member of the FGF family, has not emerged from known growth factors. Of the motoneuron bioactivities obtained from muscle extracts, the only one to be purified to apparent homogeneity to date is a 20 kD protein which increases ChAT activity but not survival of motoneurons in vitro (McManaman et al.,1987). Of the partially purified factors that have an influence on survival, perhaps the best candidate for a physiologically relevant factor to emerge so far is that of Oppenheim et al.(1988), since it is obtained from leg muscle at the right age and is active in vivo.

BACKGROUND WORK ON IMP

Introduction

Helen New (1986) discovered that certain antisera

raised against vasoactive intestinal polypeptide (VIP) stained developing chick skeletal muscle by immunocytochemistry; this staining was specific, as it could be blocked by VIP but not by the VIP-related peptides secretin, glucagon, or gastric inhibitory peptide (GIP). However, not all the anti-VIP antisera tested stained muscle, although they could all stain chick sympathetic ganglia, which contain VIP. An extract of St 35 (E9) chick pectoral muscle made in 2M acetic acid was found to contain material which could displace ^{125}I-VIP in a radioimmunoassay (RIA) for VIP using antiserum L25 supplied by R.Dimaline (Liverpool University) (A.Mudge, pers.comm.). When the extract was run on a column containing Sephadex-G50, the RIA-reactive material eluted as two major peaks of approximately 20kD and 10kD, with a minor peak of ca.6kD sometimes being present. In contrast, synthetic VIP, either run alone or in the presence of the muscle extract, eluted appropriately for its molecular weight of 3.5kD (A.Mudge,pers.comm.). Thus the material in muscle that was antigenically related to VIP appeared to be novel and was named IntraMuscular Protein (IMP).

Anti-VIP and anti-IMP antisera

A list of the antisera used in previous and present studies of IMP is given in Table 1.2. All were raised in rabbits using VIP or VIP fragments coupled to carrier molecules.

The R.I.A.(UK) and Nilaver antisera were the main

antisera used for the original immunocytochemical study (New,1986). The R.I.A.(UK) antiserum stained both sympathetic ganglia and muscle (VIP and IMP) whilst the Nilaver antiserum stained sympathetic ganglia but not muscle (VIP only); the supply of the R.I.A.(UK) antiserum was very limited. Antiserum L25 also stained both muscle and sympathetic ganglia, but the staining could not be blocked by $10^{-5}M$ VIP, leading to doubts over its specificity in immunocytochemistry.

L25 is the serum used in the radioimmunoassay (RIA) and it has already been well characterized by R.Dimaline (Dimaline and Dockray,1978). The Nilaver antiserum can also be used in an RIA and reacts both with VIP and material in muscle extract, but with a shallow displacement curve. This together with its lack of staining of muscle meant that the Nilaver antiserum was not used further for RIA.

Antisera 14 and 2 were raised in rabbits by A.Mudge and N.Mundy using synthetic human VIP fragment (1-14) (synthesized by J.Rothbard, ICRF, London) coupled to thyroglobulin. The antisera were affinity-purified on a column containing VIP_{1-14} by I.Mason and A.Mudge to produce antisera p14 and p2 (p for purified). These antisera are useful for immunocytochemistry but not for RIA: both antisera bind ^{125}-VIP but the labelled VIP is not displaced by either VIP or muscle extract in a useful concentration range. Antiserum p14 labels both sympathetic ganglia and muscle whereas antiserum p2 labels sympathetic ganglia but not muscle.

The antigenic sites on VIP for the different antisera have been determined by using VIP fragments (A.Mudge, pers.comm.; see table 1.2). Staining with the R.I.A.(UK) antiserum is not blocked by VIP_{10-28} so the antibody(s) involved is directed towards the amino terminal of VIP; staining with the Nilaver antibody is blocked by VIP_{10-28} and so the epitope(s) involved is in the carboxy terminal half of VIP. In the RIA using L25, both VIP_{1-14} and VIP_{4-14} are as effective as VIP in displacing ^{125}I-VIP, whilst VIP_{7-14} is more than 10-fold less effective and VIP_{10-28} is ineffective: thus the epitope recognised by L25 includes the region of VIP_{4-10}. Staining of muscle with antiserum p14 at a dilution of 1:300 is completely blocked by $10^{-7}M$ VIP_{1-14} and VIP_{4-14}, partially blocked by $10^{-6}M$ VIP_{7-14} and not blocked by VIP_{10-28}; again the epitope(s) includes the region of VIP_{4-10}. Finally, staining of sympathetic ganglia by antiserum p2 is completely blocked by VIP_{1-14}, partially blocked by VIP_{4-14} and VIP_{7-14}, and unaffected by VIP_{10-28}: so the epitope is in the region of VIP_{1-10}, with part of the epitope at the amino end of this region. Taking these results together, it appears that the region of VIP that is homologous with IMP is in the region VIP_{4-10}, with non-homologous regions lying both towards the amino end and carboxy end of VIP (recognised by p2 and the Nilaver antiserum, respectively). This region of VIP is not homologous with other members of the same peptide family, e.g. secretin, glucagon, GIP.

Table 1.2. Anti-VIP antisera used in the study of
IMP.

Reactivity and specificity of antisera are shown.
Synthetic VIP fragments are all from the human VIP
sequence. For blocking by VIP peptides in
immunocytochemistry, + indicates that staining was blocked
by a 10^{-6}M solution of the peptide. For the RIA, + indicates
that ^{125}I-VIP could be displaced by the peptide in the 10-
100 fmol range, or by muscle extract containing a high
concentration of IMP, (+) indicates that the displacement
curve was shallow in this range, and - indicates that there
was no displacement in this range.

Symp gang= St 41 (E15) sympathetic ganglion; nerve=
St 26 (E5) ventral root; muscle= St 34 (E8) hindlimb
muscle.

Antiserum		Immunocytochemistry						Radioimmunoassay		
		Stains			Staining blocked			^{125}I-VIP displaced		
Name	Source	Symp gang	Muscle	Nerve	VIP	VIP 1-14	VIP 10-28	VIP	VIP 1-14	VIP 10-28 Muscle extract
	R.I.A.(UK)	+	+	+	+	+	-	?	?	(+)
	R.Nilaver	+	-	-	+	?	+	(+)	?	+
L25	R.Dimaline	+	+	+	-	+	-	+	+	+
p14	A.Mudge	+	+	+	+	+	-	-	+	-
p2	A.Mudge	+	-	-	+	+	-	-	-	-

It should be emphasised that the definition of what constitutes IMP is operational. For immunocytochemistry, IMP is defined as a combination of positive staining with either the R.I.A.(UK) or p14 antisera and lack of staining with either the Nilaver or p2 antisera. In the RIA with antiserum L25, a combination of the source of material, the slope of the standard curve in the RIA (-2.7 for VIP and -2.2 for IMP in muscle extract) and data from size exclusion chromatography are used to indicate that IMP rather than VIP is being measured.

Immunocytochemistry of IMP

IMP staining in the chick was present in both axial and limb muscle during a restricted phase of development. The results of New (1986) using the R.I.A.(UK) antisera are described first. In the myotome, staining was first seen in cells at St 14 (50-53 hrs.) in the brachial region, and intense staining was present throughout the myotome between ca.St 16-34 (E2½-8); staining intensity declined thereafter, so that it was faint by St 37 (E11) and rarely visible at St 43 (E17). In the hindlimb, the first staining was seen later, at St 26 (E5), in a few cells in the muscle mass; by St 27 (E5.5), staining was intense and clearly in myotubes. At St 34 (E8) unlabelled cells (presumptive secondary myoblasts) could be seen lined up in contact with labelled myotubes. As in the myotome, staining intensity declined in the limb after St 34 (E8) and was rarely

visible by St 43 (E17). Thus IMP staining in both the dorsal muscle mass and limb was present in the primary myotubes and probably absent from the secondary myotube population. IMP staining was also seen in myoblast-like cells and myotubes in cultures prepared from St 37 (E11) chick pectoral muscle. In addition, the R.I.A.(UK) antiserum stained the ventral root up to St 23 (E3.5) and the dorsal ramus at St 26 (E5); this staining was also IMP-specific since the nerves were not stained with the Nilaver antiserum. An IMP-specific pattern of staining was also seen in the radial glia in the spinal cord and in Müller cells in the retina.

The results of staining with antiserum p14 (E.Clarke and A.Mudge,pers.comm) are in broad agreement with those obtained with the R.I.A.(UK) antiserum. The disappearance of staining in hindlimb muscles with p14 has been studied in some detail. At St 34 (E8) all thigh muscles are intensely stained and at St 38 (E12) no staining is present; at intermediate ages some muscles show staining in all myotubes and other muscles are unstained, and the temporal pattern of this loss of staining from particular muscles is consistent from embryo to embryo. The nerves to the hindlimb stain more intensely and for a longer time with p14 than with the R.I.A.(UK) antiserum: with p14 the ventral root is still stained at St 26 (E5) and muscle nerves are stained at St 34 (E8). However, this may simply reflect the ability to use higher concentrations of p14 than the R.I.A.(UK) antiserum, which was in very short

supply. Antiserum p2 did not stain the nerves. The time course of p14 staining has been examined in the pectoral muscle. Staining of myotubes is far more prolonged than in the hindlimb, with staining present at St 42 (E16) and disappearing by St 47 (E21). At St 42 (E16), the staining is intense in the primary myotubes, which by this age are at the centre of myotube clusters, and apparently absent from the secondary myotubes. Finally, IMP-specific staining is also seen in the neural tube with p14 and p2, but unlike staining with the R.I.A.(UK) antiserum, staining is not confined to radial glia but is present throughout a transverse section of neural tube except for the roof and floorplates.

The antibody staining described thus far is for results in the chick. Staining for IMP has also been found in developing rat and mouse muscles. In the rat, antiserum p14 stains myotome at E14, limb myotubes at E17, and at postnatal days 0-7 (P0-P7) a few hindlimb muscles contain a blotchy pattern of extracellular staining, a pattern which has never been seen in the chick. In normal mouse at a late fetal age, staining with p14 in hindlimb muscle is weak; in the mouse mutant muscular dysgenesis (mdg/mdg) staining at the same age is much more intense.

IMP secretion

Conditioned medium from myotubes cultured from St 37 (E11) chick pectoral muscle for 4 to 7 days was found to contain material reactive in the VIP RIA. This IMP is

probably secreted since (i) the amount of IMP collected in the medium each hour for four consecutive hours is similar to the amount of IMP extractable from the myotubes at the beginning of the collection period and (ii) the ionophore monensin, a non-specific inhibitor of exocytosis, inhibits the accumulation of IMP in the medium (P.Harkness and A.Mudge,pers.comm.).

Electron microscopic staining using antibody p14 and peroxidase on sections of myotubes from muscle revealed a generalized distribution of peroxidase product in the cytoplasm, unassociated with myofibres (E.Clarke and A.Mudge,pers.comm.); this staining pattern is consistent with a molecule secreted by the constitutive exocytotic pathway.

Effect of curare treatment on IMP

When eggs were treated with curare between St 28-34 (E6-9), according to the protocol of Pittman and Oppenheim (1978), the concentration of acetic acid-extractable IMP per mg protein in pectoral muscle was increased twofold (New,1986).

Biological function of IMP

These observations led to the hypothesis that IMP may be a trophic factor for motoneurons during development. The evidence that this might be so can be summarised as follows:

(i) IMP is present in muscle at the right time and in the

right place for it to be a trophic factor for motoneurons, in both the fore and hindlimbs. Motoneuron death in the lumbar region occurs between St 28-36 (E6-10), whilst IMP expression in the hindlimb occurs from St 26-38 (E5-12). In the brachial region, both motoneuron death and IMP expression occur over a longer time course, with death of brachial motoneurons being from St 28-46 (E6-E20), and IMP expression in the pectoral muscle being visible up to St 42 (E16). In both fore and hindlimbs, IMP is found in the primary myotubes, whose number is known to be correlated with motoneuron death (see above).

(ii) IMP is secreted by cultured myotubes.

(iii) IMP staining is seen in muscle nerves. This may represent retrograde transport of IMP from muscle to cell body, known to occur for NGF.

(iv) IMP concentration is increased under conditions of impaired neuromuscular function that are known to increase motoneuron survival. We have seen that the concentration of extractable IMP is increased in curare-treated chick embryos and that the staining intensity of IMP is increased in mice with muscular dysgenesis. The upregulation of trophic factor concentration by neuromuscular dysfunction is one possible explanation for the effect seen on motoneuron survival (see above).

The aim of this thesis is to test the hypothesis that IMP is a motoneuron trophic factor.

CHAPTER 2

THE DISTRIBUTION OF IMP

IN THE TARGETS OF

CILIARY GANGLION NEURONS

INTRODUCTION

In this chapter some experiments to determine the distribution of IMP in the targets of ciliary ganglion (CG) neurons will be described. CG neurons are attractive for investigating the potential trophic actions of IMP: there is evidence that CG neurons may share trophic factors with motoneurons and they offer certain advantages over motoneurons for in vitro studies (see below). The rationale behind the experiments described in this chapter was to see whether the spatial and temporal distribution of IMP in the targets of CG neurons was consistent with a possible role for IMP as a trophic factor for CG neurons.

I will begin by considering the development of the ciliary ganglion and the justification for believing that CG neurons may be a useful system for studying the putative trophic actions of IMP. The development of the targets of CG neurons, whose IMP staining pattern is examined in this chapter, will then be described.

The ciliary ganglion (CG) is a parasympathetic ganglion which innervates the intrinsic muscles of the eye. In the chick, the neural crest precursors of the CG have migrated to their final position behind the orbit by St 9 (E2), and birth of CG neurons is complete by St 25 (E4.5), when some axons are already in close proximity with their target myoblasts (Landmesser and Pilar, 1978). 50% of CG neurons die between St 34-40 (E8-E14), the number of

neurons in each ganglion falling from ca.6500 to ca.3200 (Landmesser and Pilar, 1974b).

There is good evidence that target size can regulate death of CG neurons. Virtually all CG neurons die following early removal of the optic vesicle (Landmesser and Pilar, 1974a) whilst neurons that were destined to die can be rescued by grafting an extra eye (Narayanan and Narayanan, 1978) or by cutting one of the ciliary nerves, thereby increasing relative target size (Pilar et al., 1980). Thus it is likely that death of CG neurons, like death of spinal motoneurons, is regulated by a target-derived trophic factor.

CG neurons are cholinergic, like spinal motoneurons, and in the chick half of them share a further property with motoneurons in that they project to striated muscle. Furthermore, cultured CG neurons survive in the presence of skeletal myotubes, and synapse onto them (Hooisma et al., 1975; Nishi and Berg, 1977); and skeletal muscle extract can promote cholinergic differentiation of CG neurons (McLennan and Hendry, 1978). Thus CG neurons and motoneurons may share trophic factors in common.

Finally, as mentioned above, CG neurons offer certain advantages over motoneurons for in vitro studies. Firstly, they are easier to dissociate and maintain in culture than motoneurons. Secondly, unlike motoneurons, CG neurons can be dissected away from other neurons and so special techniques are not required to identify them in culture.

The anatomy and development of the targets of CG

neurons will now be considered. There are two populations of neurons in the CG, the ciliary and choroid neurons (Marwitt et al., 1971), which have different projections within the eye (see fig.2.1). The ciliary subpopulation project to the striated muscles of the iris and ciliary muscle, whilst the choroid subpopulation project to smooth muscle cells in the wall of blood vessels in the choroid layer.

The ciliary muscle is derived from the neural crest (Johnston et al.,1979), but its development in the chick has not been well studied. Ciliary myoblasts are first visible at St 32 (E7) as small clumps of cells lying between the cornea and retina (Romanoff,1960) The clumps rapidly attain confluence to form a continuous muscle mass; ciliary myotubes are first seen at St 36 (E10).

The mature avian iris comprises 3 muscular components- myoepithelial cells at the pupillary margin, and the striated constrictor and dilator muscles. All are ectodermal in origin, but, whereas the myoepithelial cells are derived from the pigment epithelium, the constrictor and dilator muscles are derived from neural crest (Nakano and Nakamura,1985). The myoepithelial cells are the first to develop - at St 35 (E9), myoepithelial cells predominate (Pilar et al.,1987). By St 37, many myotubes are present and by St 39 (E13) most myofibres are well-differentiated (Narayanan and Narayanan,1981).

CHOROID SMOOTH MUSCLE

CILIARY
GANGLION

IRIS

CILIARY
NEURON

CHOROID
NEURON
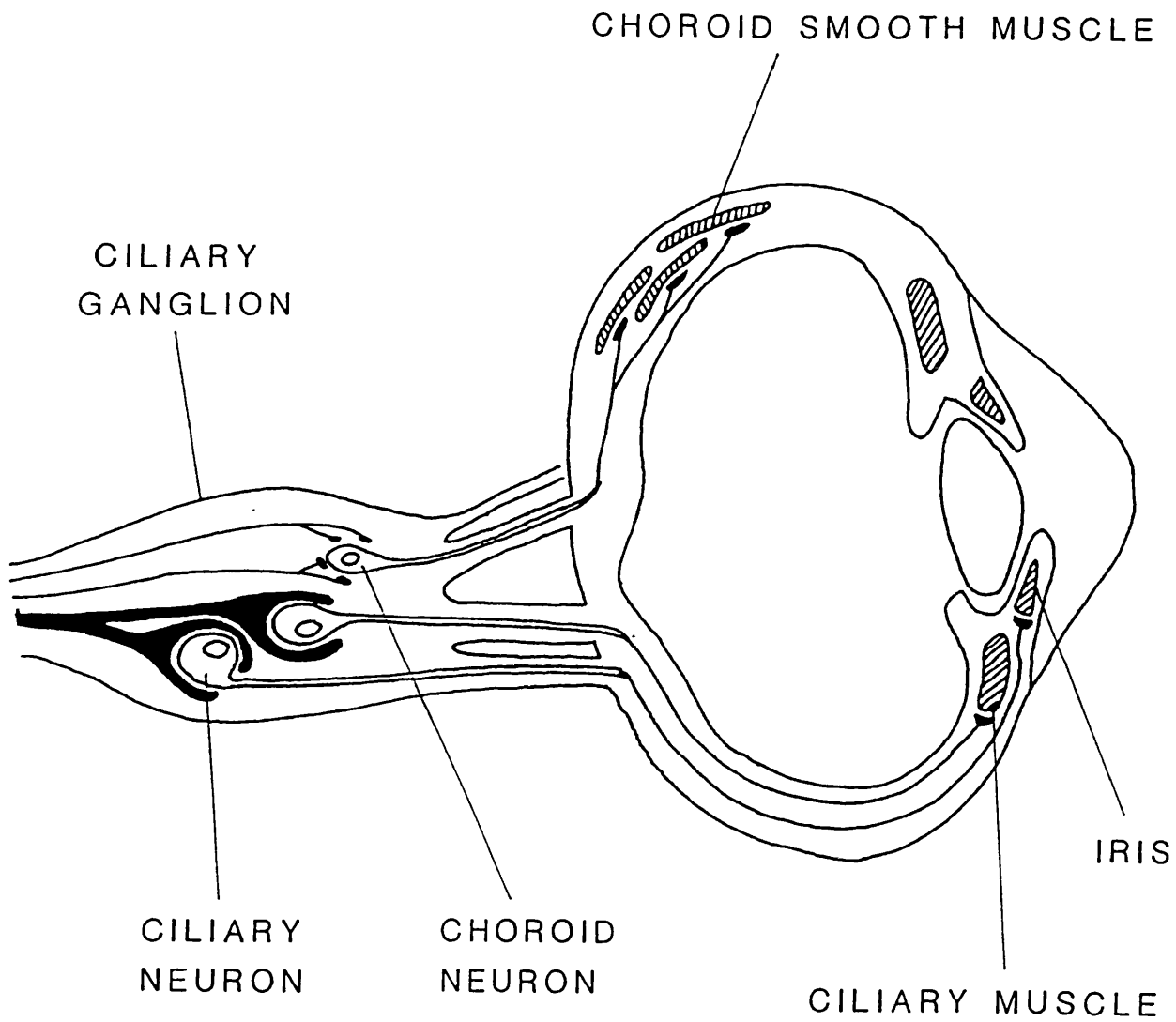
CILIARY MUSCLE

Fig. 2.1. Ciliary ganglion neurons and their targets in the

avian eye

(after Landmesser and Pilar, 1974a)

Neither the embryological origin nor the morphological development of the choroid smooth muscle cells have been investigated. However, contraction of the choroid smooth muscle can be elicited from St 36 (E10; Meriney and Pilar,1987).

I therefore set out to determine whether any of the targets of CG neurons express IMP during development and, if so, whether the temporal pattern of expression was consistent with a role for IMP as a trophic factor for CG neurons.

MATERIALS AND METHODS

Rhode Island chicks were incubated in a forced draft incubator. Embryos were staged according to Hamburger and Hamilton (1951). Pieces of tissue were fixed overnight in 4% (w/v) paraformaldehyde in PBS (Phosphate buffered saline : 150mM NaCl, 50mM sodium phosphate, pH 7.0) and placed in 30% (w/v) sucrose in PBS for 4 to 6 hours. They were embedded in OCT (Miles Scientific) and frozen by plunging into liquid nitrogen. $10\mu m$ sections were cut on a cryostat at -20°C and melted onto slides coated with 1% (w/v) gelatin / 10mM chromic potassium sulphate in water. The slides were air dried for a few hours and stored for short periods at -20°C before staining.

The immunoperoxidase method was used for staining. Sections were incubated in 0.3% (v/v) hydrogen peroxide in PBS for 30 mins., washed in PBS and incubated overnight at

4°C with first antibody in staining buffer (4% (v/v) calf serum (Gibco), 0.1% (v/v) Triton X-100 (Sigma), 0.1% (w/v) sodium azide in PBS). For peptide blocking, the antibody was preincubated for 1 hour with 10^{-6}M VIP (human synthetic; Bachem) before addition to sections. The remainder of the procedure was performed at room temperature. Sections were rinsed in staining buffer, incubated in 1:40 Sheep anti-rabbit Ig (Miles Scientific) for 1 hour, rinsed in staining buffer, incubated in 1:100 peroxidase-antiperoxidase (Dakopatts) for 1 hour, rinsed in Tris buffer (50mM Tris-HCl, pH 7.5), and the peroxidase reaction performed with 50mM 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences) in Tris buffer containing 0.009% (v/v) hydrogen peroxide. The production of the brown reaction product was monitored under the microscope and the reaction terminated with excess Tris buffer. Sections were mounted in glycerol and viewed under Nomarski optics with a Zeiss microscope.

The antisera used in this study were p14, which recognises IMP in skeletal muscle as well as VIP in dorsal root ganglia, and p2 , which recognises VIP but not IMP (see chapter 1). p14 was used at 1:300 and p2 was used at 1:150.

RESULTS

All staining described below was completely blocked by preincubation with 10^{-6}M VIP (see fig.2.2).

The earliest labelling seen with p14 in the targets of CG neurons was very faint staining in the region of the presumptive ciliary muscle at St 35 (E9). By St 36 (E10), staining could be seen in small clumps of cells midway between the anterior border of the sclera and the retina (fig.2.2); these cells are presumably ciliary myoblasts. At St 37 (E11), ciliary myotubes stain with p14 and stronger staining in these myotubes is seen at St 39 (E13) (fig.2.2). All myotubes are stained, but there is some variation in the intensity of staining in different myotubes in the same section ; no consistent pattern to this variation was detected. By St 41 (E15), staining in ciliary myofibrils is weak, and no staining was detected at St 44 (E18). Antibody p2 did not label ciliary muscle at any stage and so by definition the staining seen with p14 corresponds to IMP.

In the iris, labelling with p14 was seen at St 37 (E11) and St 39 (E13) (fig.2.3), but not at St 41 (E15). Staining was in scattered single cells in the main body of the iris and in a larger area with poorly defined boundaries at a greater distance from the pupil. The former cells are probably myotubes but the nature of the latter staining is not clear. Again, p2 did not label the iris and so the staining described is IMP staining.

Cells associated with blood vessels in the choroid layer -presumably smooth muscle cells - could be clearly identified, but no staining was seen in these cells with either p14 or p2 at any age examined (St 37-41; E11-15).

Fig.2.2. Staining for IMP in the developing ciliary muscle.

All sections were stained using antiserum p14 at 1:300. All photos are oriented with anterior facing down.

(a)   Ciliary myotubes at St 39 (E13). Scale bar = 25μm.

(b)   As (a), but antiserum preincubated with 10^{-6}M VIP.

(c)   (overpage) St 36 (E10) eye showing presumptive ciliary myoblasts (in centre of field) weakly stained and the retina more intensely stained. Scale bar = 25μm.

(a)
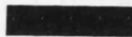
(b)

(c)

Fig.2.3. IMP staining in the developing iris.

Sections were stained using antiserum p14 at 1:300. Sections are oriented with anterior facing down.

(a) St 37 (E11) iris, low power field. Scattered cells in the main body of the iris show faint staining and stronger staining is seen in a more peripheral region. Scale bar = 100μm.

(b) Higher power view of (a). Scale bar = 25μm.

(a)

(b)

In addition to the staining by p14 of the CG neuron targets described above, p14 also stained the retina from St 35 (E9) to St 44 (E18) (fig.2.2). Staining was considerably more intense than ciliary muscle staining and was present in all retinal layers. p2 did not stain the retina at any stages examined.

DISCUSSION

The major finding of this study is that IMP is present in ciliary muscle during a restricted phase of development i.e. between St 35-41 (E9-E15). IMP is also expressed in the iris for a restricted period (St 37-39; E11-E13) but is not found in choroid smooth muscle. Thus IMP is found in the targets of the ciliary subpopulation of CG neurons but not the target cells of the choroid subpopulation.

The timing of IMP expression in the ciliary muscle is nearly coincident with the time of cell death of CG neurons (St 34-40; E8-E14) and so this is consistent with the possibility that IMP may be a trophic factor for CG neurons, or at least the ciliary subpopulation. The fact that cell death in the CG begins before IMP staining might be explained by earlier lower levels of IMP being undetectable by the present staining method. Alternatively, it could be that the death in the ciliary subpopulation of CG neurons begins later, as Landmesser and Pilar (1974b) did not look at death in the two subpopulations quantitatively. In any case, the majority of cell death in

the CG occurs between St 36-39 (E10-E13) and the trophic influence can act to save neurons within a day (Pilar et al.,1980) so IMP could play a major role as a trophic factor.

The putative trophic role for IMP for ciliary neurons innervating the iris is more tenuous as a large proportion of the iris muscle in any one section does not show IMP staining. However, some staining is seen at the right time in what are almost certainly myotubes and it is possible that IMP staining occurs in all iris myotubes but is transient; or there may be a sensitivity problem with the staining method.

The lack of IMP staining associated with choroid smooth muscle cells excludes IMP as a natural trophic factor for choroid CG neurons. There is recent evidence which indicates that target production of trophic support is regulated differently in ciliary and choroid CG neurons: Pilar's group have shown that blockade of target muscles results in increased survival of ciliary but not choroid neurons (Meriney et al.,1987). This suggests that there may be other basic differences between the trophic regulation of choroid and ciliary neurons e.g. the trophic factor itself. It is thus not unreasonable that IMP may be a trophic factor for the ciliary population alone.

Staining by the R.I.A.(UK) antiserum in the retina was described by H.V.New (1986). In that study the staining was confined to radial cells that were probably Muller cells. In the present study, staining with p14 was much more

widely distributed, with cell bodies in all layers of the retina being stained. This result therefore parallels the situation in the spinal cord, where the R.I.A.(UK) antiserum stains only radial glia whereas staining with p14 is much more extensive (see chapter 1).

To conclude, the experiments in this chapter raise the possibility that IMP may be a trophic factor for CG neurons. This led to the bioassay experiments for CG neurons described in chapter 4 using the partially purified IMP fractions discussed in the next chapter.

CHAPTER 3

THE PARTIAL PURIFICATION OF IMP

INTRODUCTION

This chapter describes the fractionation of IMP for use in the bioassays which are presented in chapter 4. The two sources of IMP utilized were embryonic chick skeletal muscle and conditioned medium from cultured chick myotubes. Concentrations of IMP were measured using a VIP radioimmunoassay.

As the putative biological activity of IMP might be unstable, the aim was for fractionation steps to be rapid and mild. Thus (i) simple column fractionation methods such as size exclusion and ion exchange chromatography were favoured; (ii) procedures were carried out at low temperature in the presence of protease inhibitors where possible; (iii) extremes of pH were avoided; and (iv) dialysis over long periods was avoided where possible.

The fractionation experiments were performed in parallel with the bioassays described in chapter 4 and, although the two sets of experiments will be discussed separately, it should be pointed out that the results of the bioassays influenced the fractionation strategy and so some of the rationale for the purification procedures will not become clear until the next chapter.

It should be stressed that the aim was not to purify IMP to homogeneity but to perform fractionation procedures that would concentrate IMP and separate it from other bioactivities present, thus enabling the putative biological activity of IMP to be determined with antibody-affinity depletion experiments.

MATERIALS AND METHODS

Radioimmunoassay

Concentrations of IMP were measured on the basis of the ability of IMP to compete for the binding of ^{125}I-VIP to antiserum L25 in the VIP radioimmunoassay (see chapter 1). The buffer used for the radioimmunoassay (RIA) was as follows: 10mM EDTA ,0.1% (w/v) sodium azide, 0.05% (w/v) Polybrene (Sigma) in PBS with either 0.3% (w/v) BSA (Sigma; RIA grade) or 0.1% BSA and 0.1% (w/v) gelatin in water from an Elgastat UHQ (Elga).The RIA was performed in borosilicate glass tubes using antibody L25 at 1:20,000 to 1:50,000 and ca.5,000 cpm/tube (ca.1 fmol/tube) of ^{125}I-VIP (Amersham; specific activity 2,000 Ci/mmol). The antibody and ^{125}I-VIP (the trace) were added in 0.2ml RIA buffer to 0.3ml of sample (either in RIA buffer or a different buffer) to make a final volume of 0.5ml/tube. The tubes were incubated at 8°C for 24-72 hours. Trace that was bound to antibody and free trace were separated on the basis that free trace is adsorbed by activated charcoal whereas bound trace is not. The charcoal mixture used was as follows: 3.2% (w/v) activated charcoal (Sigma), 0.32% (w/v) Dextran T70 (Pharmacia), 0.1% (w/v) sodium azide in PBS. At the end of the incubation period, 0.5ml of charcoal mixture were added to each tube, the tubes were centrifuged at 2,000g for 20 mins. and the supernatant poured off for counting. The counts obtained therefore represent the trace bound to

antibody (the bound fraction). Samples were counted in a Nuclear Enterprises NE1600 gamma counter.

In each RIA, a standard curve for VIP was obtained in the range of 2.5-40 fmol VIP/tube and, usually, sufficient serial dilutions of IMP samples were assayed to enable IMP sample curves to be plotted. Standard and sample curves were calculated using the logit-log linearisation method (Rodbard et al.,1969). If D is the damaged trace (i.e. the counts in the bound fraction in the absence of antibody), B_0 is the maximum binding (i.e. the counts in the bound fraction in the presence of antibody but without VIP or sample), and B the binding with sample (i.e. the counts in the bound fraction in the presence of antibody and either VIP or IMP) then the fractional binding is $(B - D)/(B_0 - D)$.

The slope obtained for VIP in the RIA was -2.7, which is larger than the theoretically expected value of -2.3 (Rodbard,1969), indicating that VIP has a higher affinity for L25 than iodinated VIP (see Appendix). This is presumably because the radiolabelled VIP is iodinated on tyrosine-7, which falls within the inferred antigenic site (see chapter 1).

fractional binding of 50%) to be determined.

Tissue extracts

Tissue for IMP extraction was frozen on dry ice immediately after dissection and stored in liquid

Fig.3.1. Example of curves obtained for IMP and VIP in the RIA.

The source of IMP used here was neutral trunk extract. Log dose is plotted against logit fractional binding for pairs of sample points and regression lines fitted [logit(x) is defined as $\log\{x/(1-x)\}$]. The slope and 50% displacement value (where logit fractional binding = 0) are then measured. In this case, for VIP, 50% displacement value = 12.2 fmol, slope = -2.8; for trunk extract, 50% displacement value = $36.8\mu l$, slope = -2.0. Note that the relative positions of the two curves along the x-axis is not meaningful.
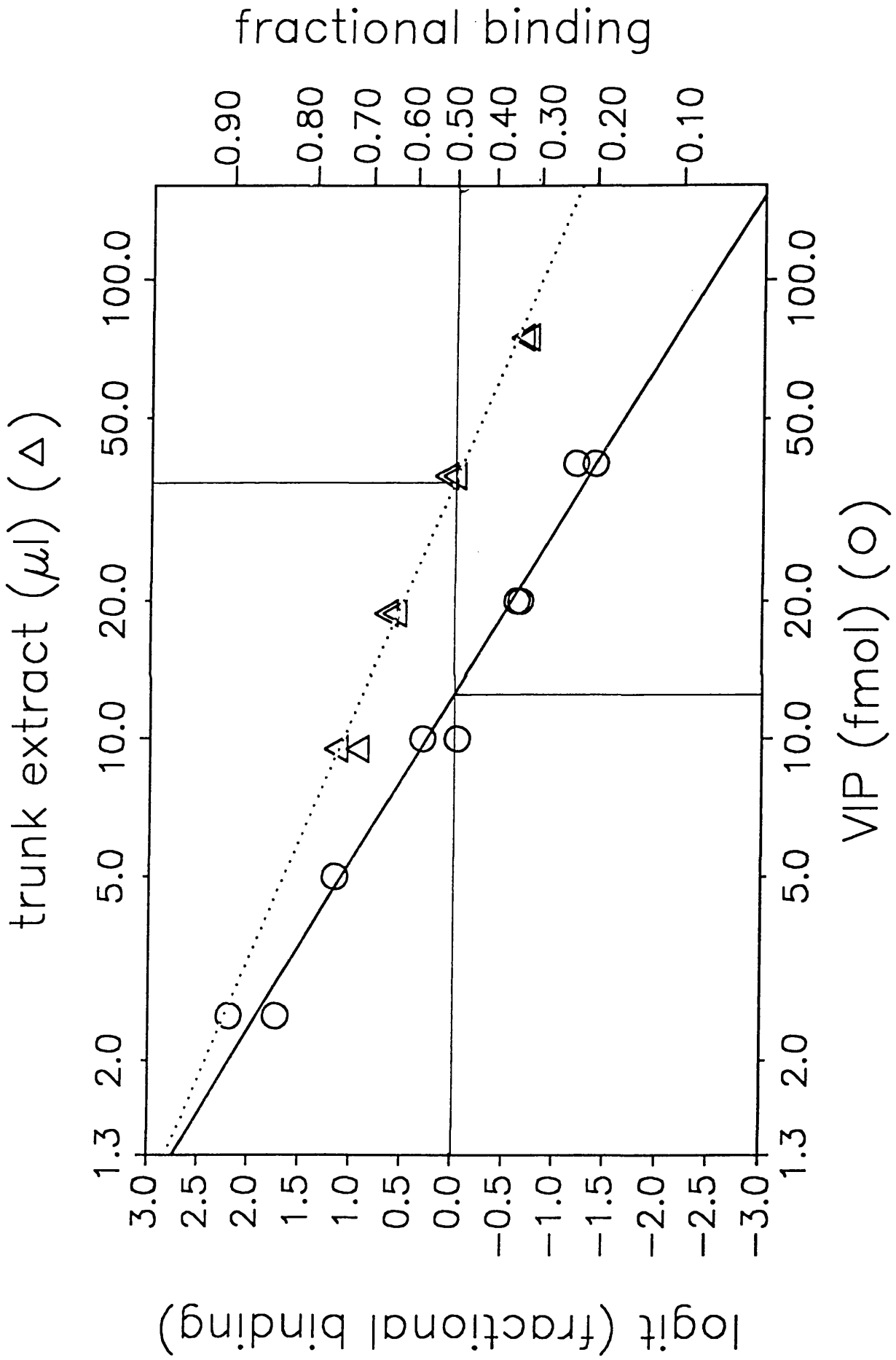
nitrogen for up to a few months. A 1:4 (v/v) mixture of tissue and buffer was homogenized on ice using an electric homogenizer (Ultra-Turrax with UT-dispersing tool 10G; Janke and Kunkel) in the presence of 2mM Phenylmethylsulphonylfluoride (PMSF; Sigma), added in two aliquots from a solution in ethanol. Unless otherwise stated the extract was made in 50mM Tris pH 7.0. Small volumes of extract for pilot studies were then spun at 13,000g for 2 mins. on a Microcentaur (MSE) centrifuge; larger volumes for purification runs were spun at 500,000g for 30 mins at 4°C on an ultracentrifuge (Prepspin 50; Fisons). Extracts made in 2M acetic acid were boiled for 3 mins. before spinning. Extracts were always made immediately prior to use.

Conditioned medium

Conditioned medium containing IMP was produced from chick myotube cultures. The assistance of P.Harkness and E.Clarke is gratefully acknowledged. Pectoral muscles from St 37 - St 39 (E11-E13) embryos were dissected and cleaned of connective tissue, incubated in Ca-Mg-free buffer for 30 mins. at 37°C and triturated with a fire-polished pasteur pipette. The dissociated material was filtered through nylon gauze, spun for 5 mins. at 1000g , and the cells plated at 8-10x10^6 cells/flask in 10ml into 200ml flasks coated with 1% (w/v) gelatin. Cells were plated in the following medium: Dulbecco's modified Eagle's medium (DMEM; Gibco) with sodium bicarbonate at a concentration of 2.2g/l

containing 10% (v/v) heat-inactivated horse serum (Gibco),
2mM glutamine (Sigma), 50 U/ml penicillin (Gibco) and
50mg/ml streptomycin (Gibco). After 24-36 hrs., cytosine
arabinoside (Sigma) was added to 5×10^{-6}M. After a further 24
hrs. the medium was changed to a chemically defined medium
without horse serum or chick embryo extract (see Table 3.1
below) and collection of conditioned medium began. 3-4
collections were made from each flask every 24 hrs. for up
to 7 days, the replacement medium always being the
chemically defined medium. Myotube conditioned medium (MCM)
was frozen and stored at -20°C.

Table 3.1 Composition of the chemically defined medium used
for collecting myotube conditioned medium (MCM)

DMEM

Bovine insulin	$5\mu g/ml$
Bovine serum albumin	$20\mu g/ml$
Conalbumin	$30\mu g/ml$
Progesterone	6pg/ml
Putrescine	$16\mu g/ml$
Selenium	5ng/ml
Glutamine	2mM
Penicillin	50 U/ml
Streptomycin	50mg/ml

All obtained from Sigma except for DMEM (Gibco)

Purification procedures

1.Materials and equipment

All chemicals were Analar grade ; water was from an Elgastat UHQ or an Elgastat Spectrum (Elga). All procedures except for early antibody affinity column runs were performed at 4°C. Most samples were stored at -20°C, for up to a few weeks ; antibody affinity depleted samples were stored at -70°C. Sephacryl S-200, Sephadex-G25, DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Heparin-Sepharose CL-6B and CNBr-activated Sepharose 4B were obtained from Pharmacia ; DEAE-Trisacryl was obtained from IBF biotechnics. Pharmacia columns were used together with a 2111 Multirac fraction collector, 2238 Uvicord SII UV recorder, and 2210 2-channel chart recorder, all from LKB. All fractions were collected into borosilicate glass tubes (Corning). Bovine thyroglobulin, Cytochrome C (Type VI) and BSA (RIA grade) were from Sigma. Protein concentrations were measured with the Biorad Protein Assay (Biorad). The lyophilizer was an Edwards Modulyo.

2.Size-exclusion and ion-exchange chromatography

Size-exclusion and ion-exchange chromatography procedures were standard (Pharmacia,1979;1989). The following size exclusion columns were used: 1800ml Sephacryl S-200 (sample volume 50ml), 400ml Sephacryl S-200 (sample volume 10-12ml and 1000ml Sephadex G-25 (sample

volume up to 1000ml). Sephacryl S-200 separates molecules in the molecular weight (MW) range 5kD-250kD; Sephadex G-25 separates in the MW range 1-5kD. Unknown molecular weights were interpolated from the position of molecular weight standards by plotting log MW vs.(Ve-Vo)/(Vt-Vo), where Ve is the peak elution volume, Vo the void volume and Vt the total column volume; MW markers used were thyroglobulin (MW 669kD), BSA (MW 66kD) and cytochrome c (MW 12.4kD). For ion exchange chromatography, salt gradients were made using a Pharmacia gradient-former and salt concentrations measured using a conductivity meter.

3.Rebinding and eluting DEAE-purified IMP

Pooled IMP fractions were diluted 1:4 in Tris buffer (to dilute the salt) and run on a small DEAE-Sepharose column (5-10ml of gel in a syringe blocked with glass wool), followed by 5 column volumes of 2mM Na phosphate, pH 7.4 and then up to 5 batch elutions with one column volume of 2mM Na phosphate, 250mM NaCl, pH 7.4.

4.Methods for dissociating molecular complexes

In all cases, treated samples were run on a 400ml Sephacryl S-200 column (sample volume 10ml); the buffer used varied as indicated below.

Samples for acidification were either (i) lyophilized, resuspended in 2M HAc and boiled for 3 mins. (the conditions under which an acetic acid extract of tissue is made), or (ii) acidified with HCl to pH 2.5 (the pH of 2M

HAc). The samples were then run on a Sephacryl column in 1M HAc and the fractions lyophilized before measurement in the RIA.

For salt treatment, tissue was extracted in 50mM Tris pH 7.0 containing 2M NaCl and run on the column in the same buffer. For high pH treatment, the sample was adjusted to 10.0 with NaOH and run on the column in 0.05M Glycine-NaOH buffer pH 9.6. For guanidine treatment, 6M Guanidine hydrochloride (Sigma) and 1mM EDTA were added to an extract in Tris buffer and run on the column in Tris buffer. For reduction, 20mM Dithiothreitol (DTT; Sigma) and 1mM EDTA were added to an extract in Tris buffer, the pH brought to 8.3 with HCl and the sample incubated in nitrogen for 2hrs. at 37°C. Reformation of disulphide bonds was inhibited by addition of 20mM fresh iodoacetamide (Sigma) for 4 hrs. on ice and the sample was run on the column in Tris buffer. In all cases controls were performed to check that IMP could still be measured in the RIA after the treatment in question.

5.Concentration of myotube conditioned medium (MCM)

Concentration of unfractionated MCM was performed using either 20kD Polyethylene glycol (PEG; BDH) or a Minitan concentrator (Millipore). For concentration using PEG, MCM was put into 8-10kD MW cut off dialysis tubing (Spectrapor), dialysed against water in the presence of protease inhibitors (1mM EDTA (BDH), 1μM leupeptin (Boehringer), 1μM pepstatin (Boehringer) and 2mM PMSF), the

volume reduced with PEG, and the concentrated MCM dialysed against LPBS. Concentration on the Minitan system was performed on undialyzed MCM in the presence of $1\mu M$ pepstatin and 2mM PMSF using 10kD MW cut off filters, with a change of buffer being done at the same time if necessary.

Concentration of pooled ion exchange fractions of IMP in MCM was performed using centrifuge concentration devices with 10kD cut off filters (Centriprep concentrator 10; Amicon). The buffer was changed at the same time to LPBS.

6.Heparin affinity chromatography

Small columns containing 1-10ml of swollen Heparin-Sepharose CL-6B gel were made with syringes or disposable columns (Biorad). The gel was washed with LPBS, and the sample run over the gel repeatedly for 2-3 hrs. (2-5 times). The column was washed with one column volume of LPBS and then eluted with high salt buffers in batches, typically 3 elutions with one column volume of 0.5M NaCl 2mM Na phosphate pH 7.4 and 3 elutions with one column volume of 3M NaCl 2mM phosphate pH 7.4.

7.Antibody affinity chromatography

Coupling of antibody to cyanogen bromide-Sepharose beads was based on the method of Hudson and Hay (1980). Dry gel was activated by suspension in 1mM HCl and washed in a sintered glass filter with 1mM HCl for 15 mins. The gel was washed in coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl pH 8.5),

added to antibody in coupling buffer in the ratio 1:1 (v/v) to a total volume of 1-2ml, and incubated on a rotary shaker for 2 hrs. at 37°C. After washing in coupling buffer, the remaining active groups were blocked by a 2hr. incubation in 0.1M Tris pH 8.0. Non-covalently bound molecules were then removed by alternate washes in 0.1M acetate buffer 0.5M NaCl pH 4.0 and 0.1M Tris 0.5M NaCl pH 8.0. Control gel was always made simultaneously by the same protocol but without addition of antibody. The concentration of antibody used varied from 1-4mg/ml of active gel. Because of limited antibody availability, in some instances coupling was performed on less than 1mg of antibody, and, to keep the volume of gel manageable (i.e. large enough), in these cases up to 75% of the total gel used in the coupling reaction was replaced by inactive gel (i.e. gel that had already been through the control coupling procedure). Coupling efficiency was greater than 90 % in all cases.

Antibody affinity depletion experiments were usually performed with the gel in a column throughout the procedure. Duplicate samples were run in parallel on disposable columns (Biorad) containing 1ml of gel coupled to antibody or 1ml of control gel. The protocol used was as follows: the columns were washed with 10ml of LPBS, 1ml of horse serum (Gibco) was added to inhibit non-specific binding followed by a further 10ml LPBS; the samples containing IMP were cycled over the columns between 8-10 times (sample volume varied between 1.5-10ml); the columns

were washed with 10ml of LPBS and eluted with 2.5ml 0.2M Glycine-HCl pH 2.5; finally, 10ml of LPBS were added followed by 10ml LPBS containing 0.1% (w/v) Na azide. The horse serum used for blocking was always the same as that used in the subsequent bioassay. Eluted material was immediately neutralised using concentrated Tris. For a few experiments, the gel was incubated with the IMP sample on a rotary shaker overnight, with washing, addition of horse serum and elutions all performed with the gel packed in a column.

RESULTS

1. CALCULATION OF IMP CONCENTRATIONS

To calculate relative amounts of IMP in a given RIA, when a particular form of IMP was being fractionated, the fractionated samples containing IMP were read against the standard curve obtained with the IMP sample. In many instances, a curve was plotted with the fractionated sample itself, allowing a calculation of IMP yield from the 50% displacement value, and a comparison of the slope with the sample slope. Calculation of percentage depletion of IMP in antibody depletion experiments, for example, was done in this way. These calculations are independent of the sensitivity of the RIA and the relative affinities of antiserum L25 for IMP and VIP.

To calculate relative IMP concentrations between

different RIAs, the sensitivity of different RIAs must be compared. This was done by comparing the sensitivity for VIP, measured as the 50% displacement value, between different RIAs and scaling the IMP concentrations accordingly. For convenience, these amounts of IMP are expressed in arbitrary units such that one unit of IMP is defined as the amount of IMP that gives 50% displacement in an RIA with a 50% displacement of 1fmol VIP. Or, in general, if the 50% displacement occurs in an RIA at x fmol VIP, then x units of IMP will also give 50% displacement. Note that this method assumes that the sensitivity of the RIA for VIP and IMP will vary in parallel, which seems reasonable.

As an example, the results of the RIA shown in Fig.3.1 give 50% displacement values at 12.2 fmol VIP and 36.8 μl trunk extract. Therefore 36.8μl of trunk extract contains 12.2 units of IMP and so the concentration of IMP is 332 units/ml.

To estimate absolute concentrations of IMP in a particular sample, one needs to know something about the relative affinities of antibody L25 for IMP and VIP. The slope of the standard curve varies with affinity - the greater the slope, the higher the affinity, and, for the given RIA conditions, this can be quantitated (E.Mroz, pers.comm.). For the present RIA, the slope obtained for

Table 3.2. Slopes of IMP in the RIA obtained using

different sources and fractions of IMP

Source of IMP	Slope ± SEM (n)
Neutral muscle extract:	
muscle extract	2.2 ± 0.1 (5)
after gel filtration	2.2 ± 0.1 (2)
after anion exchange	2.3 (1)
after anion exchange and re-anion exchange	2.0 ± 0.1 (2)
Myotube conditioned medium (MCM):	
MCM	2.2 ± 0.1 (6)
concentrated MCM	2.3 ± 0.1 (3)
heparin-depleted MCM	2.1 ± 0.1 (7)
elution from heparin column	2.2 ± 0.1 (2)

All examples are included in which the standard
error of the slope obtained in the RIA was
acceptable.

Applying the method to Fig.3.1, the concentration of IMP is at least 3×10^{-9}M. All concentrations of IMP quoted in this and subsequent chapters represent this estimated lower limit of IMP concentration and it should be borne in mind that the actual molarity of IMP may be much higher. It should perhaps be stressed that it is not of great importance to know the exact concentration of IMP but what is important is to know that the concentration is sufficient to be able to see bioactivity if it were present.

The final point about calculation of concentrations concerns samples below the sensitivity level of the RIA. The sensitivity level is taken to be samples out of the 2 logits range i.e. with fractional bindings of greater than 88%. For such samples the amount of IMP is simply expressed as less than the amount of IMP that would give 88% displacement.

2. IMP IN MUSCLE EXTRACT

Choice of muscle extract

The muscle extract in early studies of IMP was made using pectoral muscles of St 35 (E9) embryos in a buffer of 2M acetic acid (see chapter 1). 2M Acetic acid had been chosen for its efficacy in extracting peptides. However, the low pH might well affect bioactivity of IMP and so all purification for bioactivity was performed on IMP extracted in neutral buffers. As original purification attempts

Table 3.3. IMP content in neutral extracts of embryos at different stages.

Stage	no.	Wet weight of tissue (mg)	IMP content/ 4 embryos (units)	IMP content/ wet weight (units/mg)
27	1	550	420	0.76
	2	584	415	0.71
31	1	710	657	0.93
	2	705	611	0.87
33	1	960	652	0.70
	2	900	629	0.70
35	1	110	70	0.63
	2	115	50	0.48
36	1	220	272	1.24
	2	210	205	0.98

Two extracts were made at each stage each using material from four embryos. The tissue used was as follows: Stage 29-33 - trunk minus spinal cord; Stages 35 and 36 - pectoral and leg muscles.

required lyophilization, extracts were initially made in 0.1M ammonium acetate, pH 7.0.

Extracts of embryos of different stages were made in this buffer in order to determine the best source of IMP for large scale purification (see Table 3.3). For older stages (St 35 and 36), the pectoral and hindlimb muscles were used; at younger stages (Stage 33 and younger) the pectoral and hindlimb muscles were difficult to dissect and, instead, the trunk minus spinal cord was used (recall from chapter 1 that the spinal cord contains IMP staining). Stage 36 (E10) embryos had the highest concentration of extractable IMP, followed by St 31 (E7) embryos. Dissection of the separate muscle masses was slow compared with trunk dissection, and so it was decided to use St 31 embryos for all further purification of IMP in muscle extract. The choice of younger embryos had the additional advantage of allowing more rapid turnover in the egg incubator.

Purification by size exclusion chromatography

When 50ml of St 31 trunk extract made in ammonium acetate buffer was run on a 1800ml column of Sephacryl S-200 , the IMP ran as a single peak just after the main peak in absorbance at 280nm (Fig.3.2). From the profile of the molecular weight markers thyroglobulin and BSA, the apparent molecular weight of IMP is ca.120kD. This is in striking contrast to the MW of IMP in an acidic extract run on a Sephadex-G50 column in acetic acid, where the estimated MWs were 20kD and 10kD (see chapter 1). This

Fig.3.2. Neutral muscle extract run on a Sephacryl S-200 size exclusion column.

Typical result from a run of 50ml trunk extract made in 0.1M ammonium acetate run on the 1800ml Sephacryl S-200 column in the same buffer. IMP concentration is expressed as the IMP concentration in a fraction relative to the IMP concentration in the extract, expressed as a percentage. Fraction size 9.9ml, no. of fractions 180, every 3rd fraction assayed for IMP.
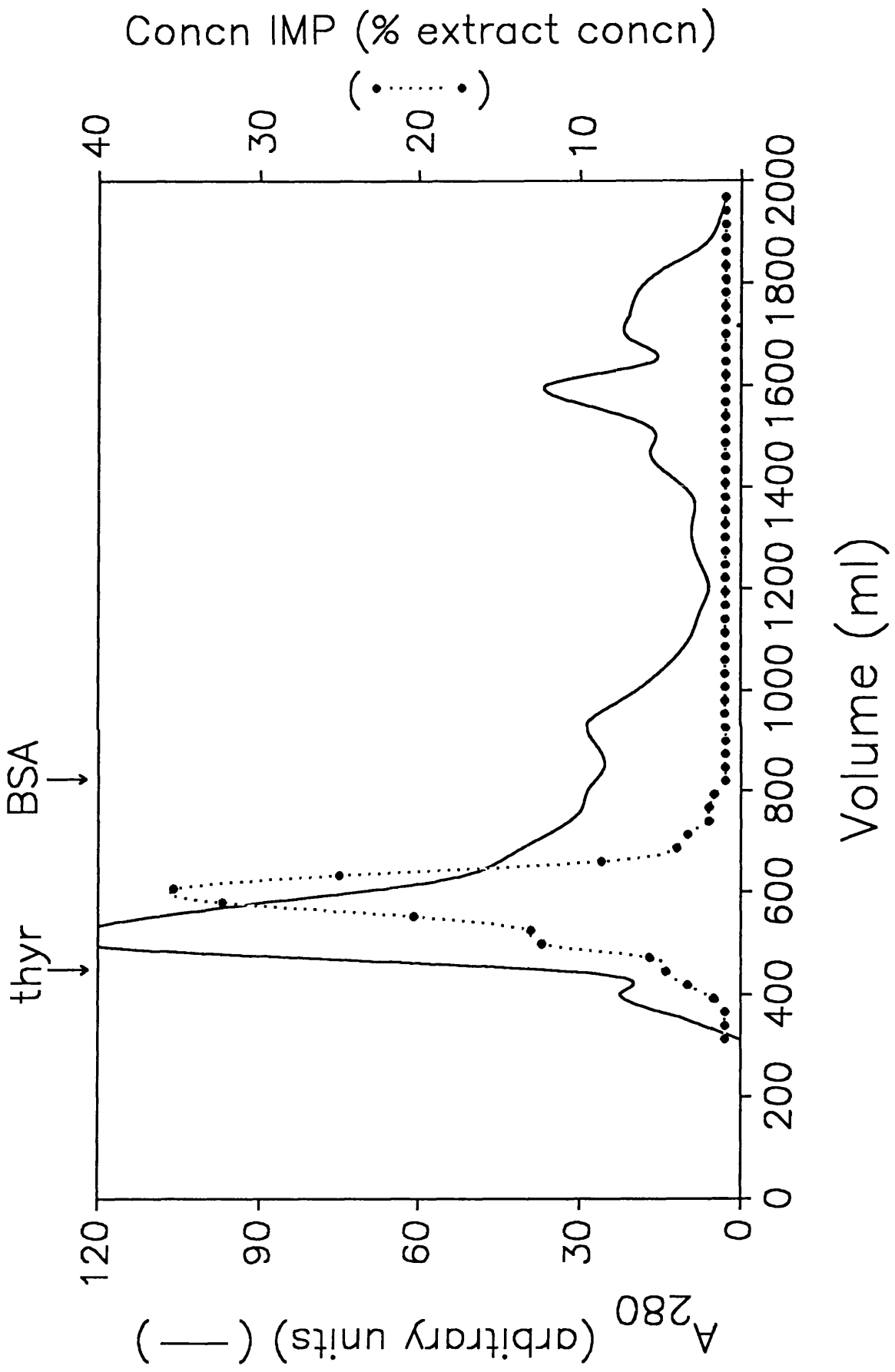
question of the relationship between IMP in neutral and acidic extracts is addressed in a later section.

The recovery of IMP over the Sephacryl S-200 column was between 50-90% in different runs. Pilot bioassays suggested that IMP would need to be concentrated relative to its concentration in muscle extract in order to be able to study bioactivity. Attempts were therefore made to concentrate IMP in Sephacryl fractions by (i) lyophilizing the fractions and resuspending in a smaller volume or (ii) reducing the volume using 20kD PEG. However, in both cases, the recovery of IMP was poor (15% in (i) and 6% in (ii)), due presumably to poor resuspension in (i) and the presence of a precipitate in (ii). Thus an alternative strategy was required.

Purification by anion exchange chromatography

Pilot experiments performed with Trisacryl anion exchange beads and CM-Sepharose CL-6B cation exchange beads suggested that IMP in muscle extract could bind to and elute from anion exchange but not cation exchange beads in the mid pH range (data not shown). This led to the development of the following protocol for the purification of IMP. Muscle extract made in Tris buffer (50mM Tris pH 7.0) was run on a 60ml DEAE-Sepharose CL-6B column, followed by one column volume of Tris buffer, a 0-250mM gradient of NaCl in Tris buffer over 10 column volumes, and 1M NaCl in Tris buffer for one column volume. This gave a single peak of IMP in the salt range 60-100mM with

Fig.3.3. Neutral muscle extract run on a DEAE-Sepharose column.

Typical result from a run of 200ml muscle extract made in 0.1M Tris pH 7.0 on a 60ml DEAE-Sepharose column in the same buffer, with elution by a 0-250mM salt gradient over 10 column volumes and a step to 1M salt. IMP concentration expressed in units/ml. 190 fractions of 10ml each collected; IMP assayed in every 5th absorption fraction and every 2nd elution fraction. In this example, the peak of IMP elution is at 100mM NaCl.
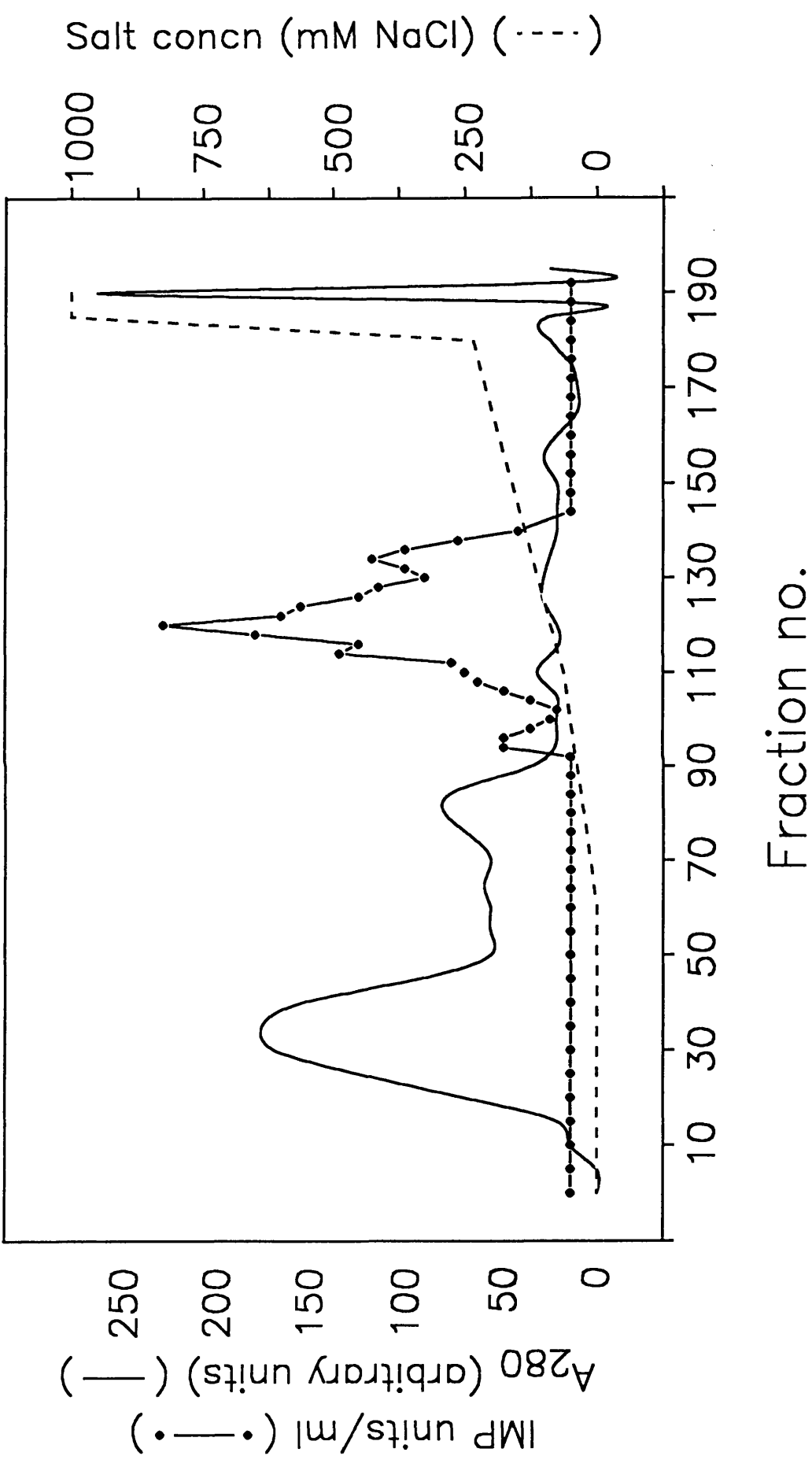
apparently good purification (see Fig.3.3). The recovery of IMP was over 50% (% recovery 62.3 ± 7.7; mean ± SEM, n=8; see Table 3.4). Initial runs were performed with 50ml of muscle extract made with 50 trunks; later this was scaled up to 200ml extract made with 200 trunks.

The trace of absorbance at 280nm from a run of muscle extract on the DEAE column showed a large peak in material that appeared after one column volume, i.e. in material that had not interacted with DEAE. Measurement of absorbance at 260nm demonstrated that this was due to the presence of nucleic acid in these fractions; there was little nucleic acid present in IMP-containing fractions. This meant that the purification of IMP attained was much less than that anticipated from the UV trace. Attempts to make the absorbance trace at 280nm more meaningful by precipitating the nucleic acid in extract with protamine sulphate resulted in a low yield of IMP.

As Tris is toxic to cells, the buffer in pooled IMP fractions had to be changed before use in a bioassay. This was achieved by rebinding the IMP to DEAE-Sepharose and eluting it in batches in a phosphate buffered saline with low phosphate which can be added to cells (LPBS). The details of the method are described in the Materials and Methods section. The majority of the IMP was eluted in either the 2nd or 3rd batch; the method has the advantages that it is fast and results in a concentration of IMP, although the yield was somewhat variable. It also resulted in a slight purification of IMP in terms of IMP

Table 3.4. Compilation of IMP purification results from experiments in which neutral muscle extract was run on a DEAE column, and rerun on a DEAE column.

Values for units of IMP and amounts of protein represent the following: for the tissue extract, the total amount present; for the first ion exchange run, the total amount in IMP-containing fractions; and for the rerun on DEAE, the total amount in that particular fraction. Degree of purification is calculated on a units of IMP/mg protein basis. Percentage recoveries of IMP represent the recovery for that step.

Run no.	Tissue Extract		Ion Exchange				Rerun on Ion exchange					
	Units IMP x10^3	Prot (mg)	Units IMP x10^3	Prot (mg)	% recovery	fold purification	elution no.	Units IMP x10^3	Prot (mg)	fold purification	% recovery	Concn IMP x10^{-9}M
1	73	1180	72	336	99	3.5	2	9.1	ND	–	26	11.3
							3	10.0	37	4.4		12.5
2	26	1000	15	147	57	3.9	2	1.3	ND	–	42	1.3
							3	3.1	16	7.5		3.1
							4	1.8	ND	–		1.8
3	43	850	37	300	86	2.4	2	1.2	ND	–	14	0.8
							3	3.8	36	2.1		2.5
4	43	ND	21	ND	49	–	2	2.7	ND	–	65	3.2
							3	8.0	ND	–		9.4
							4	2.4	ND	–		1.8

concentration/mg protein. A summary of ion exchange recoveries is shown in Table 3.4.

Further purification of DEAE-purified IMP was attempted by running pooled IMP fractions in Tris on a 400ml Sephacryl S-200 column. The IMP ran in a similar position to that on the larger Sephacryl column and most of the absorbance at 280nm was in the same fractions, so no significant further purification was achieved (data not shown).

Antibody affinity depletion of DEAE-purified IMP

Agarose beads coupled to antibody p14 were used to try to deplete IMP in order to test for IMP-specific bioactivity (described in the next chapter). In a few attempts to deplete IMP containing fractions by either overnight rotation with antibody-coupled beads or by repetitive passage through a column containing the beads, the maximum depletion by antibody-coupled beads versus control beads was 30%. As the same beads could deplete IMP from MCM samples efficiently (see later), this low value was not due to the antibody-coupled beads.
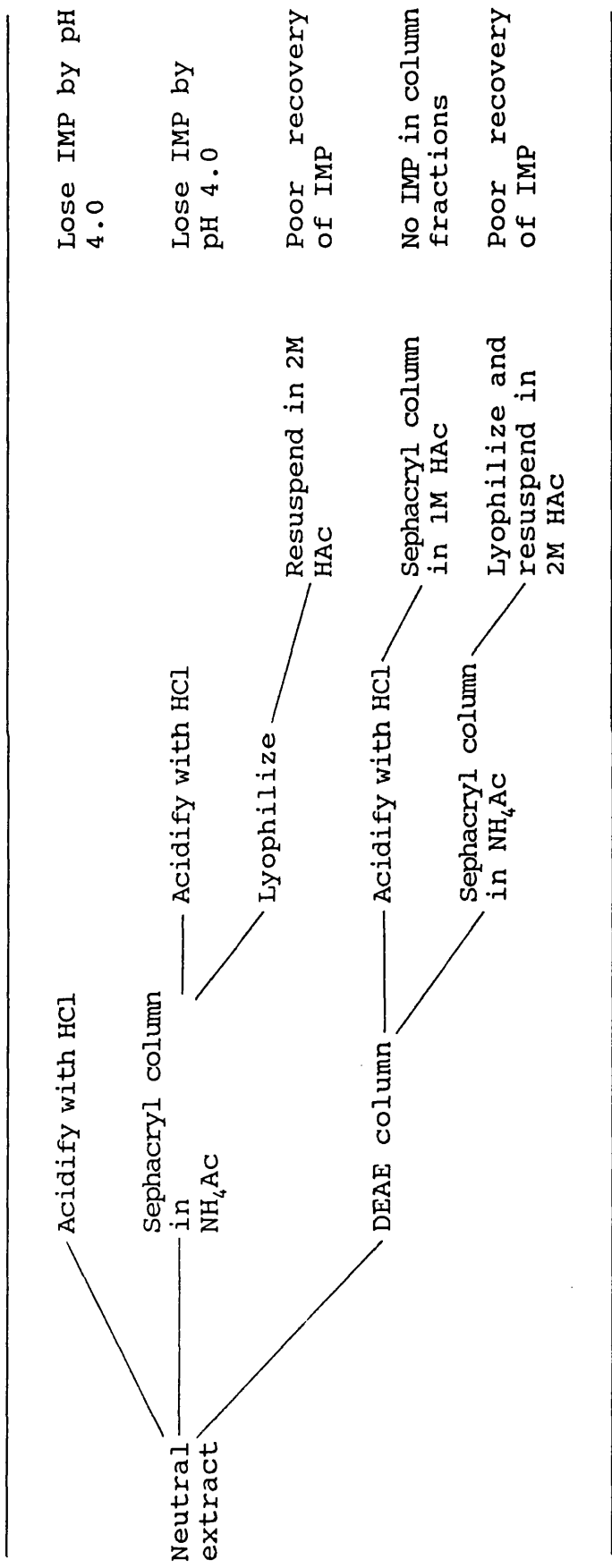
Relationship between IMP in neutral and acidic extracts

The possibility of converting the high MW IMP in neutral muscle extract to the lower MW forms of IMP in muscle extracted in acetic acid was investigated. The first strategy that was adopted in order to try to convert high MW IMP to low MW IMP was to acidify neutral extract and

fractions of neutral extract containing IMP and run the resulting material on a Sephacryl S-200 column in 1M acetic acid to determine the size of IMP. The different protocols are summarised in Table 3.5. As a standard, muscle extract made in 2M acetic acid was run on the Sephacryl S-200 column in 1M HAc: the IMP eluted as two peaks, a major peak corresponding to a MW of 35kD and a minor peak corresponding to a MW of about 13kD (see Fig.3.4). However, it was found that acidification to pH 4.0 using HCl of neutral extract, Sephacryl-purified or DEAE-purified IMP resulted in a precipitate and loss of assayable IMP. As the original acid extract was made using acetic acid, it was possible that it was necessary to use acetic acid itself rather than HCl. Lyophilized fractions were resuspended in 2M acetic acid, but recovery of IMP was poor, and insufficient for measurement in Sephacryl column fractions.

As attempts to alter the MW of IMP by acidification were inconclusive, other methods were employed which can dissociate both non-covalent and covalent (disulphide-bonded) molecular complexes. Neutral extract or DEAE-purified neutral extract were the starting material for these experiments, and in order of increasing strength the methods used were (i) high salt (extract made in Tris buffer with 2M NaCl) (ii) high pH (extract brought to pH 10 with NaOH) (iii) a chaotropic agent (6M Guanidine HCl) (iv) a reducing agent (Dithiothreitol) (see Materials and Methods for details). Meaningful results were only obtained in the first 2 cases as IMP could not be measured in the

Table 3.5. Summary of protocols used in the acidification of neutral muscle extract

Neutral extract

— Acidify with HCl → Lose IMP by pH 4.0

— Sephacryl column in NH₄Ac — Acidify with HCl — Lyophilize
  — Lose IMP by pH 4.0
  — Resuspend in 2M HAc → Poor recovery of IMP

— DEAE column
  — Acidify with HCl — Sephacryl column in 1M HAc → No IMP in column fractions
  — Sephacryl column in NH₄Ac — Lyophilize and resuspend in 2M HAc → Poor recovery of IMP

RIA after treatment with Guanidine or DTT (even after extensive dialysis). In both high salt and high pH, the IMP remained in a high MW form (data not shown).

The failure to convert IMP in neutral extract into a lower MW form raises the question of whether it is in fact a different substance from the IMP measured in an acidic extract. Pellets from a neutral extract in Tris buffer were re-extracted either in Tris buffer or in 2M acetic acid: if a substantial amount of IMP was re-extracted in acetic acid then this would be very suggestive that 2 different species of IMP were involved. However, re-extraction with acetic acid yielded a quantity of IMP equivalent to 8% of the IMP present in the initial extract, whilst Tris re-extraction yielded 4% of the initial IMP.

3. IMP IN MYOTUBE CONDITIONED MEDIUM (MCM)

Size exclusion chromatography

When MCM was run on a Sephacryl S-200 column in a neutral buffer, the major IMP peak ran between BSA and cytochrome c, at a position corresponding to a MW of ca.35kD. This value was the same as that obtained for the main peak of IMP in an acetic acid extract of muscle and so the IMP profiles of MCM and acidic muscle extract were compared in parallel runs on the Sephacryl S-200 column run in 1M acetic acid (Fig.3.4). The major peak of IMP was coincident in the two runs, again at ca.35kD and so IMP may be present in the same molecular form in these sources.

Fig.3.4. Sephacryl S-200 size exclusion chromatography of IMP in MCM and acidic muscle extract.

10ml of MCM and 10ml of trunk extract made in 2M acetic acid were run sequentially on the 400ml Sephacryl S-200 column in 1M acetic acid.

IMP concentration is expressed as units/fraction sample. Fraction size 8ml.
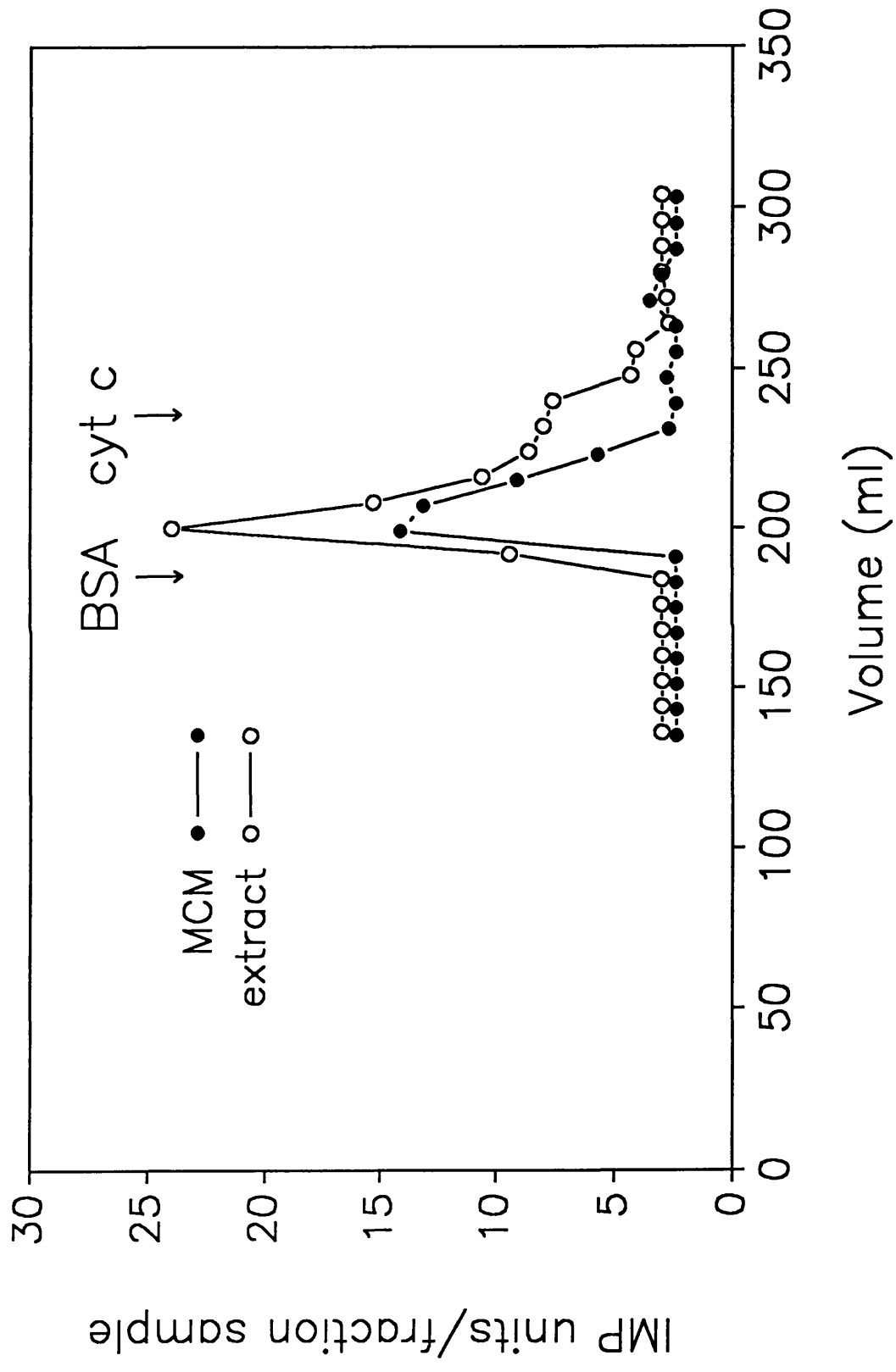
A second, smaller form of IMP was also identified in MCM. This form was usually much less abundant than the 35kD form. Most of the data comes from runs of MCM on a Sephadex-G25 column (performed in order to change the buffer, see below). On this column, the 35kD form of IMP eluted in the void volume of the column, whilst the smaller form was chromatographed. The maximum amount of the smaller form seen was 20% of the total IMP (mean 7.0% ± 3.1% SEM; n=7), whilst none of the smaller form was detected in nearly 50% of cases . In the one run on a Sephacryl S-200 column in which the smaller form of IMP could be clearly seen it eluted well after the cytochrome c peak into a MW range in which the resolving power of Sephacryl S-200 is poor. In fact, the IMP coeluted both with amino acids and ^{125}I-VIP (MW 3.5kD); it is therefore not possible to put an accurate MW estimate on the smaller form, except that it is likely to be below 5kD. Measurement of the slope of the smaller form in the RIA was not possible because of the low amounts present.

Concentration of MCM

Bioassay results demonstrated that concentration of IMP in MCM was necessary. The first approach was to run MCM on a Sephadex-G25 column in 0.1M ammonium acetate, collect and lyophilize the void volume, and resuspend the lyophilized material in a small volume. However, this procedure destroyed bioactivity and was not pursued.

The second method avoided lyophilization by using PEG.

MCM was dialysed against water (to remove salt so as to avoid excessive salt concentrations occurring during concentration), the volume reduced 10- to 30-fold with 20kD PEG and the resulting sample dialysed against LPBS (see Materials and Methods for details). The third method used a Minitan concentrator which is specifically designed for concentrating supernatants, and was much faster than the 2nd method. Again the volume was reduced 10- to 30-fold.

The results were as follows: with PEG, 7- to 28-fold concentration of IMP was achieved, with a recovery of 52.3 ± 13.7% (mean ± SEM, n=4); with the Minitan concentrator, 5- to 21-fold concentration was obtained, with a recovery of 52.2 ± 7.1% (n=5). Thus substantial concentration of IMP with a good recovery was achieved with both PEG and the Minitan concentrator.

It should be noted that as 8-10kD MW cut off dialysis tubing was used for the PEG concentration , and as the Minitan concentrator was used with a 10kD MW cut off filter, only the 35kD form of IMP was concentrated, and the smaller form was lost. As the smaller form accounted for at most 20% of the total IMP and was often not present it seemed unlikely to be a significant contributor to possible bioactivity.

Heparin affinity chromatography

Heparin-Sepharose affinity chromatography was performed in order to see whether it could separate IMP from any heparin-binding growth factors that might be

present, especially members of the FGF family, most of which bind to heparin with high affinity. Concentrated MCM (cMCM) was the starting material for these experiments. In early experiments, depletion alone was measured whilst in later experiments elution of any bound IMP was also performed, using high salt buffers.

The results are summarised in Table 3.6. The proportion of IMP that bound to heparin was usually between 10-30%, although in extreme cases as little as 0% or as much as 78% of the IMP bound; these differences were not correlated with the method of concentration of cMCM. When the column was eluted, all of the IMP that eluted was in the 0.5M NaCl fractions and no IMP was detectable in 3M NaCl fractions. All members of the FGF family for which the data are available require at least 1M NaCl to be eluted from heparin and so heparin-affinity chromatography produces two IMP-containing fractions that should be free of FGFs i.e. concentrated MCM that has been depleted over the heparin column and the 0.5M NaCl eluate from that column. The bioactivity of IMP in these two fractions may be different.

Ion exchange chromatography of MCM

Ion exchange chromatography of MCM was investigated as another strategy to try to concentrate IMP and separate it from fibroblast growth factor family members. Pilot experiments were performed to see in what pH range IMP in MCM would bind to CM-Sepharose and DEAE-Sepharose: although

Table 3.6. Summary of results of heparin-affinity

chromatography of IMP in concentrated MCM (cMCM)

Concentration method	% IMP depletion by heparin	% of total sample IMP in elutions		
		no.	0.5M	3M
PEG	30			
PEG	31			
PEG	14			
PEG	71	1	67	0
		2	0	0
		3	0	
Minitan	30	1	21	0
		2	6	0
		3	0	
Minitan	10	1	6	0
		2	1	0
		3	1	

The % depletion of IMP in cMCM passed over a heparin column is shown, as well as the amount of IMP eluted with 0.5M and 3M salt elutions, the amount of IMP in each elution being expressed as a % of the amount of IMP in the starting material.

there was some variability in the results, in general IMP bound strongly to CM-Sepharose at pH 5.5 and below and to DEAE-Sepharose at pH 8.0 and above (data not shown). It was therefore decided to continue with DEAE chromatography as this would allow the pH to remain nearly physiological.

The buffer used was 50mM Tris pH 8.0 and the MCM was changed to this buffer by passage over the 1000ml Sephadex-G25 column; thus the low molecular weight form of IMP in MCM was lost. The following were run through a column containing 30ml DEAE-Sepharose CL-6B: the sample, one column volume of Tris buffer, a 0-500mM gradient of NaCl in Tris buffer over 10 column volumes, and 1M NaCl in Tris buffer for one column volume. The results are shown in Table 3.7. The IMP generally eluted somewhere between 100-200mM NaCl, but the point within this range where it eluted was variable, and in one case about 40% of the IMP did not bind to the gel and most of the IMP that did bind eluted at around 70mM NaCl. The recovery of IMP was generally excellent.

Attempts were made to concentrate and change the buffer of the IMP that was eluted from the DEAE column using the same method as was used for IMP in muscle extract, i.e. to rebind the sample to DEAE and elute in a different buffer. However, this gave a very poor recovery of IMP, and, instead, a centrifuge concentration device with a 10kD MW cut off filter was used (see materials and methods for details). The volume was concentrated 5-10x and the buffer changed to LPBS with good recovery of IMP.

Table 3.7. Compilation of IMP purification results from runs of MCM on a DEAE column, and subsequent concentration.

The recovery of IMP and salt concentration at which IMP eluted are shown for four runs of MCM on a 30ml DEAE-Sepharose column. Also shown are the recovery of IMP and concentration of IMP following concentration of the DEAE IMP pool using a centrifuge concentration device.

Run no.	mM NaCl at which IMP eluted	IMP recovery over DEAE column	IMP recovery after concn.	[IMP]x10^{-9}M after concn.
1	100-150	>90%	–	–
2	150-200	>90%	78%	6.1
3	125-220	51%	59%	7.7
4	0	40%	–	–
	70	35%	–	–
	100-150	15%	67%	1.4

Antibody affinity depletion of IMP in MCM

These experiments were performed in order to be able to test IMP-containing fractions for IMP bioactivity (see next chapter). The affinity purified antiserum p14 was used to reduce the possibility of non-specific interactions.

A method in which CNBr-Sepharose beads coupled to antibody were in a column throughout the procedure was found to be most efficient. The results of the earlier experiments are shown in Table.3.8. The p14-coupled column could deplete IMP in a variety of supernatant fractions efficiently, with >90% depletion of IMP in the starting material being usual, and a resulting IMP concentration in p14-depleted fractions of usually <15% that of control-depleted fractions. Horse serum was found to reduce non-specific binding to acceptable levels, whilst BSA was less effective. The material eluted from the column with Glycine-HCl buffer had a high concentration of IMP.

However, in later experiments the efficiency with which the p14-coupled CNBr-Sepharose column could deplete IMP dropped dramatically; this was true even with new CNBr-Sepharose beads freshly coupled to p14 antibody. Instead, it seems that it was the MCM that had changed: an old aliquot of MCM could be depleted of IMP by both old and new antibody columns, whereas newer MCM could not be depleted of IMP by either column. The timing of this change in the MCM did not correspond with any identifiable changes in the way the MCM was prepared.

Table 3.8   Antibody affinity depletion of IMP in MCM and MCM fractions using p14

Source of IMP	[IMP]x10^{-9}M in starting material	% depletion by control beads	% depletion by p14 beads	[IMP] in p14 vs. control depletion	[IMP]x10^{-9}M in control depletion
MCM	1.3	16%	>80%	<24%	0.87
Heparin-depleted cMCM	11.0	37%	95%	8%	5.8
Heparin-depleted cMCM	11.9	40%	92%	14%	7.1
Heparin elution of cMCM	20.0	46%	94%	11%	6.4
DEAE-purified MCM	7.7	60%	95%	13%	2.5

DISCUSSION

The major results described in this chapter are (i) the preparation of several different fractions of partially purified IMP for use in bioassays and (ii) data concerning the biochemical nature of IMP. These will be discussed separately.

Partial purification of IMP

Several fractions containing partially purified IMP were obtained from the two available sources of IMP, i.e. muscle extract and MCM (see Table 3.9). These fractions were all prepared using mild methods and the estimated concentration of IMP that they contain is 10^{-9}M or greater (based on a 10-fold difference in affinity between IMP and VIP in the RIA); thus they should contain a reasonable concentration of IMP to test for putative bioactivity. Furthermore, specific depletion of a large percentage of IMP, necessary to determine the rôle of IMP in bioactivity, was achieved in most cases, using an antibody column.

IMP in neutral muscle extract was purified ca.5-fold on a DEAE-Sepharose column followed by rebinding to and eluting from DEAE-Sepharose. This IMP-containing fraction will be termed ieME (for ion exchange-purified Muscle Extract). The maximum depletion of IMP in ieME obtained using antibody p14 was only 30%.

Several fractions containing IMP were prepared from

Table 3.9 Summary of fractions of IMP for use in bioassays

Source	fraction	abbreviation	MW forms of IMP	[IMP] (M)	Maximum depletion of IMP by p14
Neutral muscle extract	DEAE-purified and rerun on DEAE	ieME	120kD	ca.10^{-9}	30%
MCM	-	-	35kD <5kD	ca.10^{-9}	80%
MCM	DEAE-purified + concentrated	ieMCM	35kD	ca.10^{-8}	87%
MCM	concentrated	cMCM	35kD	ca.10^{-8}	-
MCM	heparin-depleted cMCM	hdcMCM	35kD	ca.10^{-8}	92%
MCM	heparin-elution of cMCM	hecMCM	35kD	ca.10^{-8}	89%

MCM. The aim here was to separate IMP from bFGF and other members of the FGF family that might be present. Heparin-affinity chromatography of cMCM led to two IMP-containing fractions: the material that did not bind to heparin, and the material eluted with 0.5M NaCl. These will be termed hdcMCM and hecMCM (for heparin-depleted cMCM and heparin-elution of cMCM). All members of the FGF family bind heparin with high affinity except for keratinocyte growth factor (KGF), which elutes from a heparin column with 0.6M NaCl (Rubin et al.,1989). Therefore, hdcMCM should be free of FGFs and hecMCM is likely to be free of most FGFs, and should certainly be free of bFGF. IMP-containing fractions were also obtained from DEAE-Sepharose chromatography of MCM: this will be referred to as ieMCM (for ion exchange-purifed MCM). Efficient depletion of IMP from all MCM fractions was obtained using antibody p14; IMP could also be eluted from the antibody column.

Biochemical nature of IMP

1. The relationship of IMP from different sources

Most of the data obtained concerning the relationship of IMP from different sources are from runs on size exclusion columns; these data on the different MWs of IMP in different preparations are summarised in Table 3.10. The slopes obtained in the RIA for IMP in neutral muscle extract, acidic muscle extract and MCM were all the same (ca.-2.1): this strongly suggests that the same antigenic site is involved.

IMP in neutral muscle extract was found to have a MW of ca.120kD. In contrast, IMP in an acetic acid extract of muscle run on a Sephacryl column ran as a major peak at ca.35kD and a minor peak at ca.13kD (this compares with figures of approximately 20kD and 10kD previously obtained on a Sephadex G-50 column: the differences can be attributed to the poor resolution of the G-50 column above 15kD and the poor resolution of Sephacryl S-200 below 20kD). All attempts to try to convert the 120kD form to the smaller form by acidification were inconclusive because of poor recovery; high salt and alkalinization failed to change the size of the 120kD form; and use of reducing and chaotropic agents resulted in an inability to measure IMP in the RIA. However, it still seems likely that the two forms are related (i.e. that the smaller molecule is contained within the larger one), since after extraction of IMP in neutral buffer, only a small amount of acetic acid-extractable IMP is present in the pellet. Assuming that the two forms are related, there are two possible explanations: (i) IMP is normally present in muscle in a high MW form which can be converted to lower MW forms with acid. The failure to recover low MW IMP on acidification of neutral muscle extract must then be due to the different environment IMP is in in comparison to muscle; this could, for example, mean that the IMP is precipitated before the acid can act. (ii) IMP is normally present in muscle in a low MW form which binds abnormally to other material in a neutral muscle extract. The high MW form so produced is

resistant to acid treatment. There are at present no data to distinguish between these possibilities.

IMP is present in MCM in 2 forms, a major form with MW ca.35kD and minor form with MW <5 kD. The 35kD form coelutes with the major form of IMP in an acetic acid extract of muscle, and so these may represent the same molecule. The <5kD form is not consistently present in MCM and seems most likely to represent a product of proteolytic breakdown of the 35kD form.

Table 3.10. Summary of the different molecular weight forms of IMP from different sources, as determined by size exclusion chromatography.

Source	MW
Neutral muscle extract	120 kD
2M acetic acid muscle extract	35 kD, 13kD
Myotube conditioned medium	35 kD, <5kD

2.Heterogeneity of IMP in MCM

Apart from the different MW forms present in MCM, there is considerable evidence that the 35kD form is itself heterogeneous: (i) A variable proportion of this form binds to heparin, (ii) the salt concentration at which it eluted

from the DEAE column was variable, and (iii) its ability to bind to antibody p14 in depletion experiments suddenly declined from excellent to poor. The reason for any of these variations is currently unclear; it is important to note that they may be reflected in differences in bioactivity.

CHAPTER 4

THE ACTIVITY OF IMP AND OTHER

FACTORS IN <u>IN</u> <u>VITRO</u>

NEURONAL BIOASSAYS

INTRODUCTION

The experiments described in this chapter address the question of the bioactivity of IMP and other putative trophic factors in simple neuronal systems in vitro. The two systems used were cultures of dissociated ciliary ganglion neurons and cultures of dissociated ventral spinal cord neurons in which the motoneurons were labelled by backfilling. Neuronal survival was the main biological effect that was assayed; in addition, Choline Acetyl Transferase (ChAT) activity, a measure of the degree of cholinergic differentiation, was assayed in some cases. Such assays have been widely used in the past to measure neurotrophic activity (e.g. Nishi and Berg,1977; McLennan and Hendry,1978; Giller et al.,1977; Bennett et al.,1980).

MATERIALS AND METHODS

Ciliary Ganglion cell culture

15-30 St 34 (E8) chick ciliary ganglia were dissected and placed in dissociation buffer (145mM NaCl, 5mM KCl, 5mM Na phophate, 33mM glucose, pH 7.4). The ganglia were cleaned of connective tissue and ciliary nerves, and incubated in 1ml of 0.15% (w/v) collagenase (Sigma Type IA) in dissociation buffer for 45 mins. at 37°C. They were then washed twice in serum-containing medium before being dissociated by triturating 15-20 times with a fire-polished

pasteur pipette (tip bore ca. 0.5mm). The cells were then centrifuged at 500g for 5 mins. in 10ml medium containing 10% horse serum. The pellet was resuspended in basal medium and the cells plated in 100μl into wells of 24-well plates (Falcon) containing 400μl medium/well (final volume 500μl/well); this gave an even plating density.

The wells were coated with 100Kd poly-L- or poly-D-Lysine (Sigma) and laminin (Sigma); for assays lasting beyond 30 hrs. poly-D-lysine was always used. Wells were incubated in 10μg/ml poly-lysine overnight, dried for several hours in a tissue culture hood and kept for up to 7 days; they were then incubated with 2μg/well laminin in DMEM overnight at 37°C on the day prior to use.

Cells for survival assays were plated at two-thirds of a ganglion equivalent/well; for some ChAT assays, cells were plated at one ganglion equivalent/well. The plates were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2. After 18 hrs., mitotic inhibitors were added to inhibit growth of non-neuronal cells. In early experiments, 2×10^{-6}M cytosine arabinoside (Ara-C; Sigma) was used. After a report appeared that Ara-C was toxic for CG neurons (Wallace and Johnson,1989), the Ara-C was replaced by 10^{-5}M 5-fluorodeoxyuridine (FuDR; Sigma) and 10^{-5}M uridine (Sigma). For long term cultures, the medium was replenished after 5 days by removal of 250μl medium from each well and addition of 250μl fresh medium containing the same components in the same concentrations that the well began with.

The basal medium consisted of DMEM with 10% (v/v) horse serum (heat-inactivated; Gibco), 0.3% (w/v) conalbumin (Sigma), 2mM Glutamine, 50 U/ml penicillin (Gibco) and 50mg/ml streptomycin (Gibco). For some ChAT assays, the components of a chemically defined medium were also included in the basal medium (for the composition of the chemically defined medium see Chapter 3 Materials and Methods). Basal medium made up $400\mu l$ out of the total of $500\mu l$/well, the remaining $100\mu l$ containing the experimental additives. The buffer for this $100\mu l$ was nearly always phosphate buffered saline with low phosphate (LPBS; 150 mM NaCl, 2mM Na phosphate, pH 7.4). In a few cases the $100\mu l$ consisted of DMEM with chemically defined medium components. All conditions were repeated in triplicate in each assay.

IMP-containing fractions were prepared in the manner described in the previous chapter- see Table 3.9 for a summary of the IMP-containing fractions used in bioassays. The NaCl concentration was adjusted to ca.150mM and the pH checked where necessary, and all fractions were sterilized by treatment with UV light for 5 mins. Two sources of ciliary neurotrophic factor (CNTF) were used: rat optic nerve extract (ONE) and rat sciatic nerve extract (SNE). Rat ONE was a gift of L.Lillien. Partially purified CNTF from rat sciatic nerve was prepared by P.Harkness using a DEAE column according to the method of Manthorpe et al.(1986). It will be referred to as pSNE (for purified SNE). Between $0.25-2.0\mu l$/well of ONE or pSNE were used.

Partially purified basic FGF (bFGF) from bovine brain was obtained from RND; bovine insulin was from Sigma and synthetic human VIP from Bachem.

Ciliary neuron survival assay

Neuronal survival was measured by counting phase-bright cells with a smooth outline 4 to 6 hours after plating and again 30 hours after plating; in some cases counts at later times were also performed. Using a Leitz inverted phase-contrast microscope, 20 high power fields on a 5x4 grid were counted for each well, representing 7% of the total surface area. During counting the plates were sealed with tape to maintain the CO_2 concentration, and kept warm with a fan heater. Survival is expressed as a percentage relative to the neurons that attached, i.e. the 4-6hr. count. In some assays where only the presence or absence of survival needed to be known, survival was assessed by visual inspection.

Choline Acetyltransferase (ChAT) assay

The ChAT assay was set up by P.Harkness using a modification of the method of Berg and Fischbach (1977), which itself was based on the method of Fonnum (1974). The basis for the method is the conversion of ^{3}H-acetyl CoA to ^{3}H-acetyl choline by ChAT. The ^{3}H-acetyl choline is then transferred to the non-aqueous phase of the scintillation fluid by sodium tetraphenylborate, leaving unreacted ^{3}H-acetyl CoA, and any ^{3}H-carnitine CoA that may have formed,

in the aqueous phase of the scintillation fluid and so not contributing to the count.

The buffer used for both homogenization and the assay reaction was 200mM NaCl, 50mM Na phosphate, 0.5% (v/v) Triton X-100 (Sigma), 5mg/ml BSA (RIA grade; Sigma), pH 7.5. For extraction from cultures, the culture supernatant was aspirated and the extraction performed with 2x45μl buffer, using a micropipette tip as a scraper; samples were stored for up to 2 weeks at -20°C. The reaction mixture consisted of 25μl of sample, 2.5mM choline chloride (Sigma), 240μM acetyl CoA (Sigma), ca.5x10^5 cpm/ca.5μM ^{3}H-acetyl CoA (specific activity 4.3Ci/mmol; Amersham), and either 70mU/ml acetylcholinesterase (AChE; Sigma) or 5mM BW 284 C51 (an AChE inhibitor), in a total volume of 30μl. ChAT from Sigma was used as a standard. Procedures up to the start of the reaction were performed on ice. The reaction was started by addition of the choline chloride, acetyl CoA and ^{3}H-acetyl CoA to the other components, and the tubes were incubated at 37°C for 30 mins. The reaction was terminated by addition of 0.5ml of 10mM Na phosphate pH 7.4 at 4°C. The samples were transferred to scintillation vials with 2x 0.375 ml washes of the phosphate buffer and then to each vial was added 0.5ml acetonitrile containing 5mg/ml Na tetraphenylborate (Sigma), and 2.5ml of toluene-based scintillant (Optiscint hi-safe; LKB). The vials were gently inverted once, to aid mixing, and were counted after 10 mins. in a beta counter (LKB 1214 Rackbeta). The counts per minute (cpm) varied linearly with ChAT concentration

over the range measured. ChAT activity per neuron was calculated from the ChAT activity and neuronal count for individual wells.

Retrograde labelling of motoneurons

Retrograde labelling of lumbar motoneurons was performed using a 0.9% solution (w/v) of fluorescein isothiocyanate coupled to wheat germ agglutinin (WGA-FITC; Sigma) in LPBS. A small window was made in a St 26 (E5) egg, the embryo exposed, and ca.2μl/leg of WGA-FITC injected into ca.10 sites in the muscle masses of one or both hindlimbs. The egg was sealed with parafilm and returned to the incubator for 18 hrs. Between 20-30 embryos were backfilled at one time.

The lumbar regions of a few backfilled embryos were dissected at St 28 (E6) and processed for cryostat sectioning (see chapter 2 for method).

Ventral Spinal Cord Culture

Ventral lumbar spinal cords of St 28-29 (E6-6.5) embryos that had previously been backfilled were dissected in dissociation buffer (as for ciliary ganglia). The cords were chopped with a fine scalpel and incubated in 0.15% (w/v) trypsin (Type III;Sigma) in 2ml dissociation buffer for 30 mins. at 37°C. The trypsin was then inhibited by addition of 0.5ml of a solution containing 0.5mg/ml soybean trypsin inhibitor (SBTI; Sigma), 0.5mg/ml DNAse (Sigma) and 15mM $MgSO_4$ in dissociation buffer. After 2 washes in serum-

containing medium, and further addition of SBTI/DNAase solution, the pieces of cord were dissociated by triturating 10x with a fire-polished pasteur pipette (tip diameter ca.1mm). The cells were centrifuged at 500g for 5 mins. in 10ml serum-containing medium, and the pellet resuspended in basal medium (or DMEM if going on a metrizamide gradient, see below). Viable cells were counted in a haemocytometer, viability being based on the ability to exclude 0.05% (w/v) Trypan blue; both total cells and fluorescent cells were counted, using a Zeiss microscope with phase and fluorescent optics.

50-60,000 cells in 120μl basal medium were plated onto 13mm glass coverslips in 24-well plates and the cells allowed to attach for 4-6hrs. before the addition of the rest of the medium, to a final volume of 500μl. Conditions were repeated in triplicate for each time point examined. The coverslips had been coated previously with poly-D-lysine and laminin by the same method used for coating wells for ciliary neuron culture (see above). The basal medium was the same as that used for ciliary neurons, and the composition of the 500μl in each well was again 400μl basal medium and 100μl experimental additives, in which LPBS was used as a balancing buffer. After 24hrs. of culture, mitotic inhibitors were added: either 2×10^{-6}M Ara-C or 10^{-5}M FuDR and 10^{-5}M uridine. Materials were obtained form the same source as for ciliary cultures.

Coverslips were fixed in 4% (w/v) paraformaldehyde in PBS and mounted on slides with Citifluor (Citifluor Ltd.)

before fluorescent cells were counted. 10% of the total surface area of the coverslip was counted in three parallel strips across the widest part. Three coverslips were fixed 3-4 hrs. after plating to obtain a measurement of the number of cells that had attached. The remaining coverslips were fixed at various times thereafter, the most common time being after 3 days. Fluorescent cells were always counted within a day of fixing.

Fractionation of Ventral Spinal Cord Cells

Ventral spinal cord cells were enriched for motoneurons on the basis of their buoyant density in a metrizamide step gradient, following the method of Dohrmann et al.(1986), which itself was based on the method of Schnaar and Schaffner (1980). To a 15ml Falcon tube was added 1.5ml 15% (w/v) metrizamide (Sigma) in DMEM, followed by careful addition of 5ml 7.5% metrizamide in DMEM and finally 5ml resuspended dissociated ventral spinal cord cells in DMEM, to form three layers. The tube was centrifuged at 500g for 15 mins. after which time two bands of cells were visible at the 0-7.5% and 7.5-15% metrizamide interfaces. Following Dohrmann's terminology, the cells in the 0-7.5% band are fraction I and those in the 7.5-15% band are fraction II. Cells in each fraction were collected in 1-2ml using a pasteur pipette and total, large and fluorescent cells counted, large cells being identified on a subjective basis.

For culturing, the metrizamide was removed by dilution

in 16ml basal medium followed by centrifugation at 500g for 5 mins. and resuspension of cells in basal medium for plating. Cells were plated at 5,000-30,000 cells/coverslip and all other culture and counting methods were as described for total ventral spinal cord cells, except that mitotic inhibitors were not added.

RESULTS

I. CILIARY NEURONS

The ciliary neuron survival assay

Essentially all cells had attached by 4 hours after plating and non-neuronal cells already had a flat morphology and so were easily distinguishable from neurons; also, most neurons had short neurites by this stage. Post-attachment counts indicated a typical yield of 5500-6500 neurons/ganglion.

After 30 hours in culture, all surviving neurons had one or more neurites. In basal medium with LPBS ca.10% of neurons survived whilst in the presence of a large dose of CNTF in either ONE or pSNE between 85-100% survival was seen (see Fig.4.1). This difference was very reproducible from assay to assay and enabled ONE or pSNE to be included in assays as a positive control. The dose of ONE used was based on the supramaximal dose of the same ONE in the rat optic nerve type II astrocyte induction assay, measured by L.Lillien; the supramaximal dose of pSNE was measured by
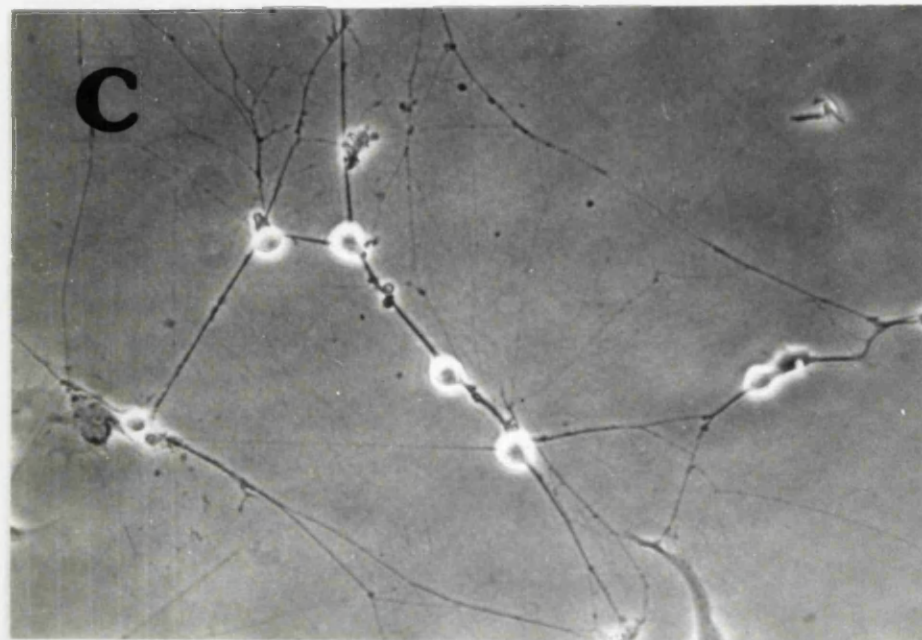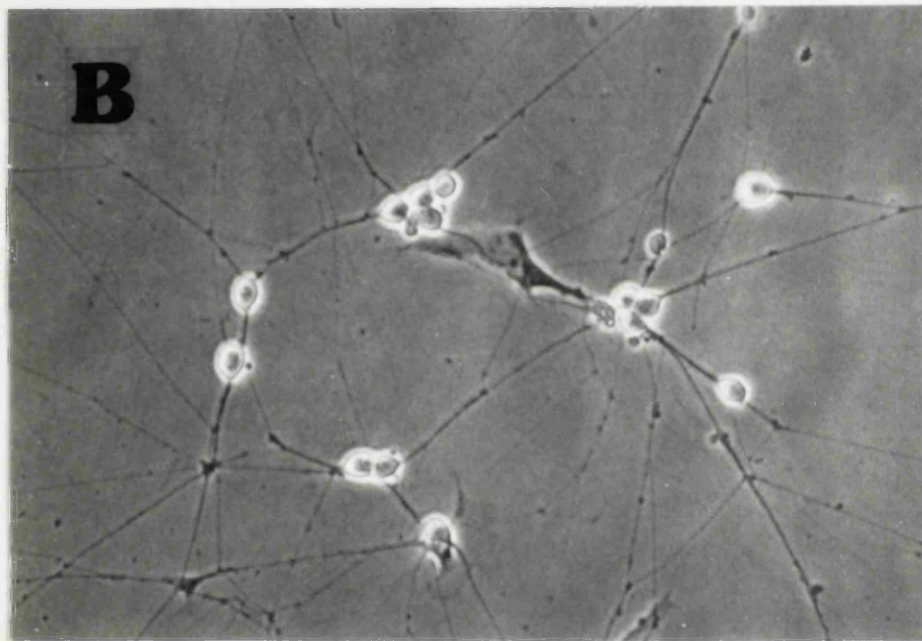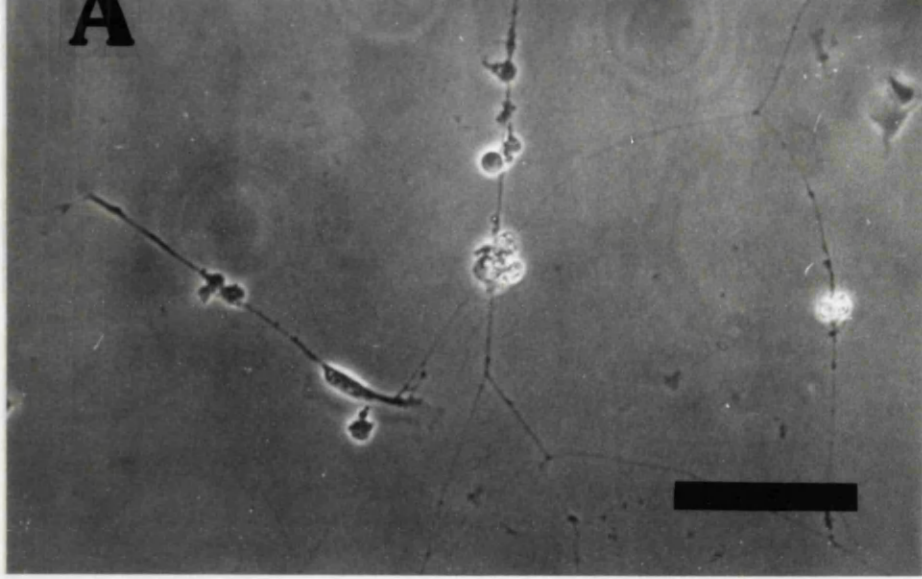
Fig.4.1. The 24-hr. ciliary ganglion neuron survival assay.

Typical fields of CG neurons after 24 hr. in culture, viewed under phase contrast optics. Experimental additives as follows:

(A) Control conditions (100μl of LPBS/well). Scale bar = 100μm.

(B) Optic nerve extract (ONE) at 1.5μl/well.

(C) Heparin-depleted cMCM (hdcMCM) at 100μl/well.

serial dilution in the ciliary assay.

There were also differences in the morphology of surviving neurons in the presence and absence of CNTF: neurons with ONE or pSNE had larger, more bulbous cell bodies and longer and more numerous neurites than those without.

Another molecule which is known to have trophic activity for CG neurons, basic fibroblast growth factor (bFGF), was tested and found to have activity in this assay. With 5ng/ml bFGF, a large dose, the survival at 30hrs. varied between 24% to 47% in different assays (mean=39.2%; s.e.m.=5.8%; n=5).

Synthetic human VIP tested at 10^{-6}M and 10^{-8}M had no effect on neuronal survival (data not shown).
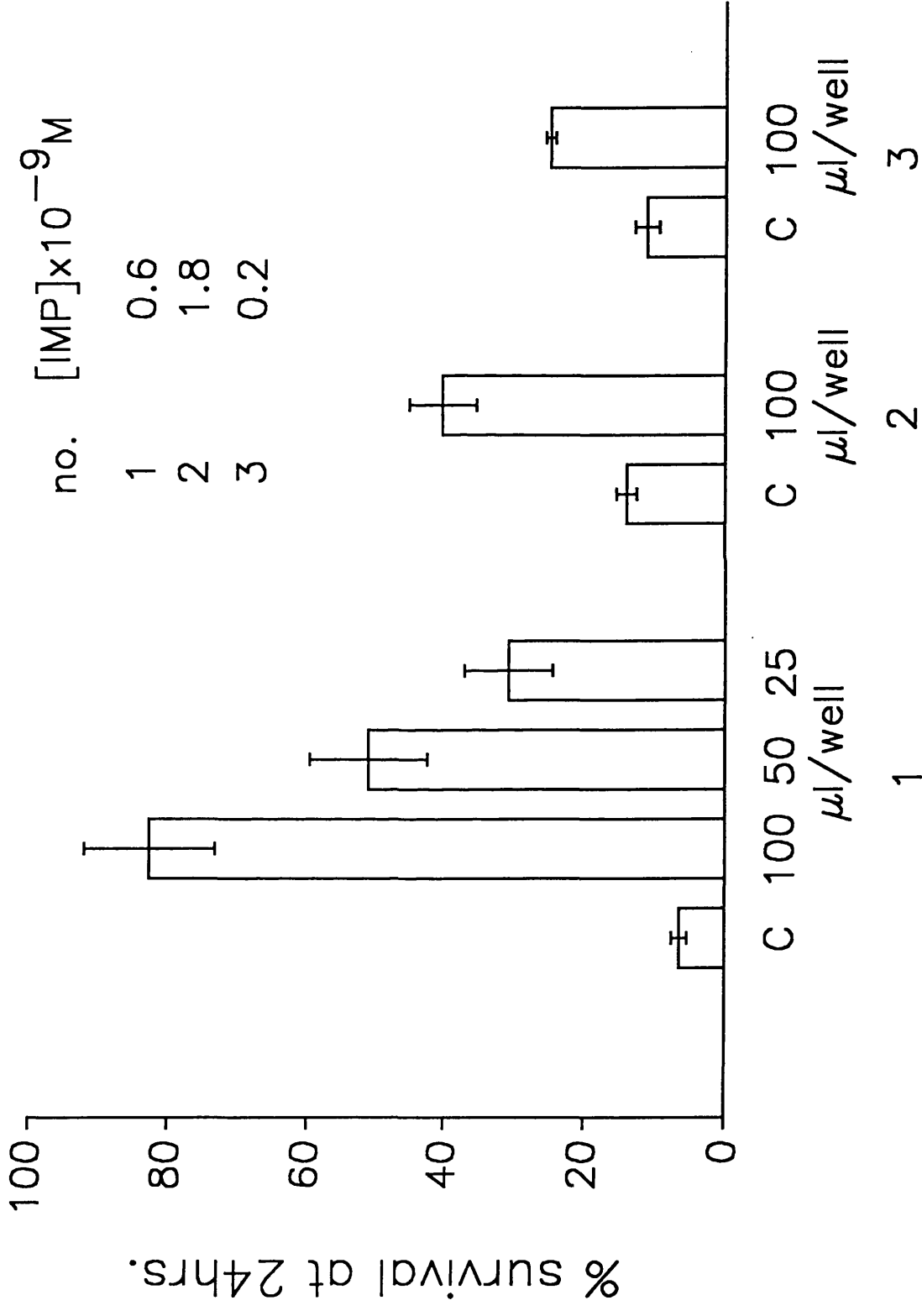

Activity in muscle extract

IMP-containing fractions of neutral muscle extract purified by anion exchange chromatography (ieME) were tested for activity in the survival assay. All preparations tested had activity in the assay (see Fig.4.2); the maximum survival seen varied with different preparations, with no correlation between survival activity and estimated IMP concentration.

The failure to immunoprecipitate a significant proportion of IMP using antibody p14 (see chapter 3) meant that the possible rôle of IMP in the survival activity could not be assessed by this means. However, IMP that had been eluted from the antibody p14 column was tested and

Fig.4.2 Activity of purified neutral muscle extract in the 24hr. ciliary survival assay

Results are shown for 3 different IMP-containing preparations of neutral muscle extract purified by anion exchange and rerun on anion exchange (ieME). The concentration of IMP in the 3 preparations is shown when added at $100\mu l$/well. "C" refers to control conditions ($100\mu l$ LPBS). "μl/well" refers to the volume of purified extract added.

The error bars in this and all subsequent figures represent the standard error of the mean of triplicate data points. Unless otherwise stated, when an error bar is not visible this is because the error is too small.

% survival at 24 hrs.

$[IMP] \times 10^{-9} M$

no.	
1	0.6
2	1.8
3	0.2

C 100 μl/well 1 50 25 μl/well

C 100 μl/well 2

C 100 μl/well 3

found to have no effect in the assay (not shown).

Acetic acid extract of muscle was assayed for survival activity after lyophilization and resuspension in LPBS. Marginally more neurons were seen compared to control conditions (data not shown).

Activity in myotube conditioned medium (MCM)

Myotube supernatants had a consistently large effect in the survival assay, with both percentage survival and morphology similar to that seen with ONE when MCM was added at the highest concentration. For four different preparations of MCM, the % survival with $100\mu l$ MCM/well was 85.2 +/- 5.3 (mean +/- s.e.m.); the minimum concentration of MCM that gave a measurable difference in survival over controls was $10\mu l$/well (see Fig.4.3).

The rôle of IMP in this survival activity in MCM was Note that the IMP that remains in the antibody-depleted sample cannot be sufficient to account for the bioactivity, since the bioactivity when the antibody-depleted sample is added at $100\mu l$/well is far greater than that seen in the control-depleted sample with an equivalent concentration of IMP i.e. at a dose of less than $24\mu l$/well.

not to have activity in the assay (data not shown).

Thus there had to be at least one substance other than IMP present in MCM that had survival activity. Given the circumstantial evidence favouring IMP as a neurotrophic agent, it was important to pursue the possible bioactivity of IMP further. In order to do this, (i) this substance had

Fig.4.3 Activity of MCM in the 24hr. ciliary survival assay

Typical dilution curve obtained with MCM (open circles). Survival with ONE in the same assay is also shown (closed circle).

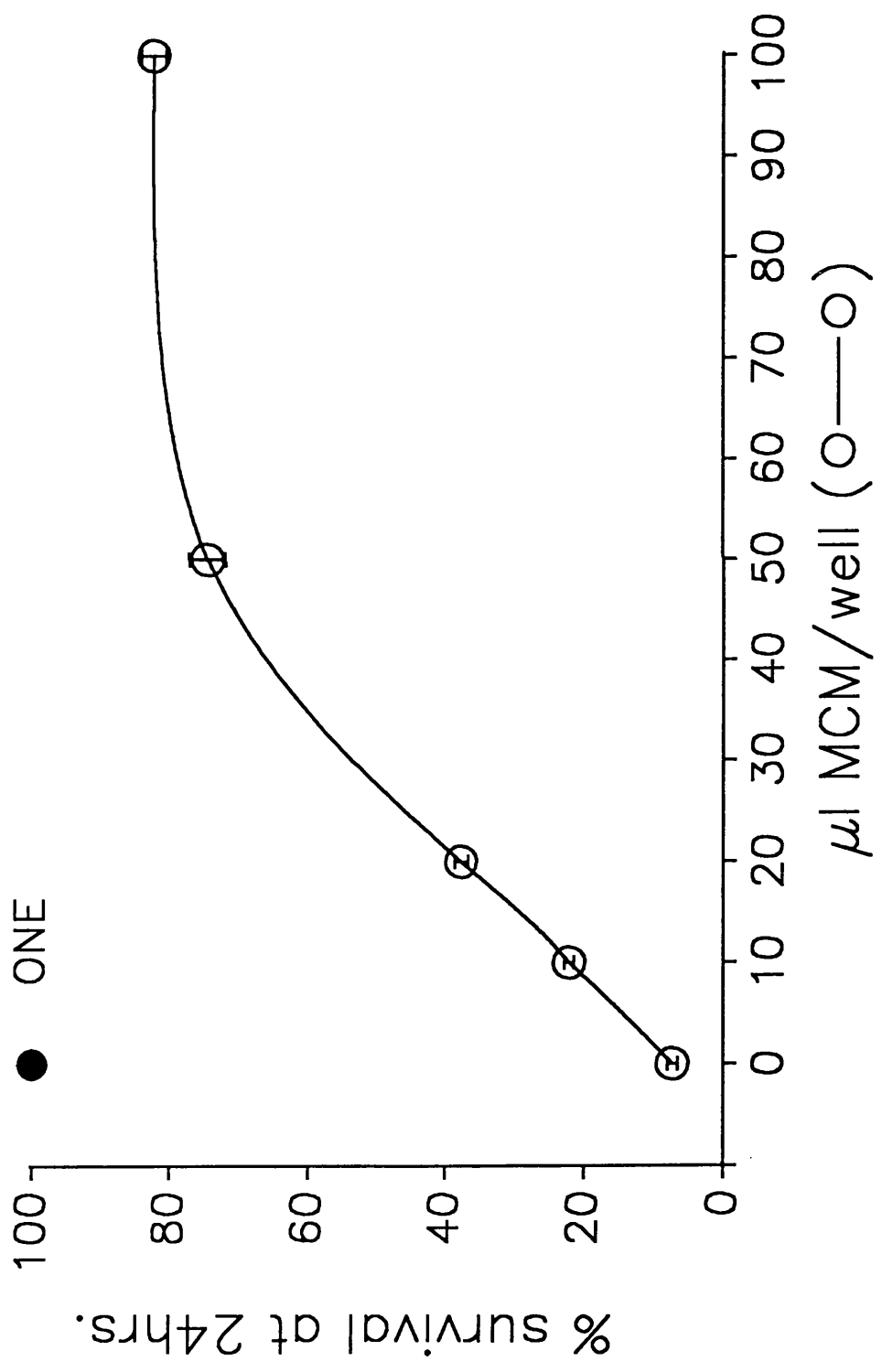% survival at 24hrs.

μl MCM/well (O—O)

● ONE

Fig.4.4   Activity of MCM depleted of IMP in the 24hr. ciliary survival assay

The following dilution curves are shown:

(i) the original MCM on which the depletion experiment was performed ("MCM"; circles)

(ii) MCM treated with control beads ("cont depl MCM"; triangles)

(iii) MCM depleted of IMP with antibody p14 beads ("p14 depl MCM"; squares).

Concentration of IMP with 100μl/well was as follows: original MCM - 2.6×10^{-10}M; cont depl MCM - 2.2×10^{-10}M; p14 depl MCM - $<5.0\times10^{-11}$M.

% survival at 24 hrs.

μl condition/well

○ MCM
△ cont depl MCM
□ p14 depl MCM
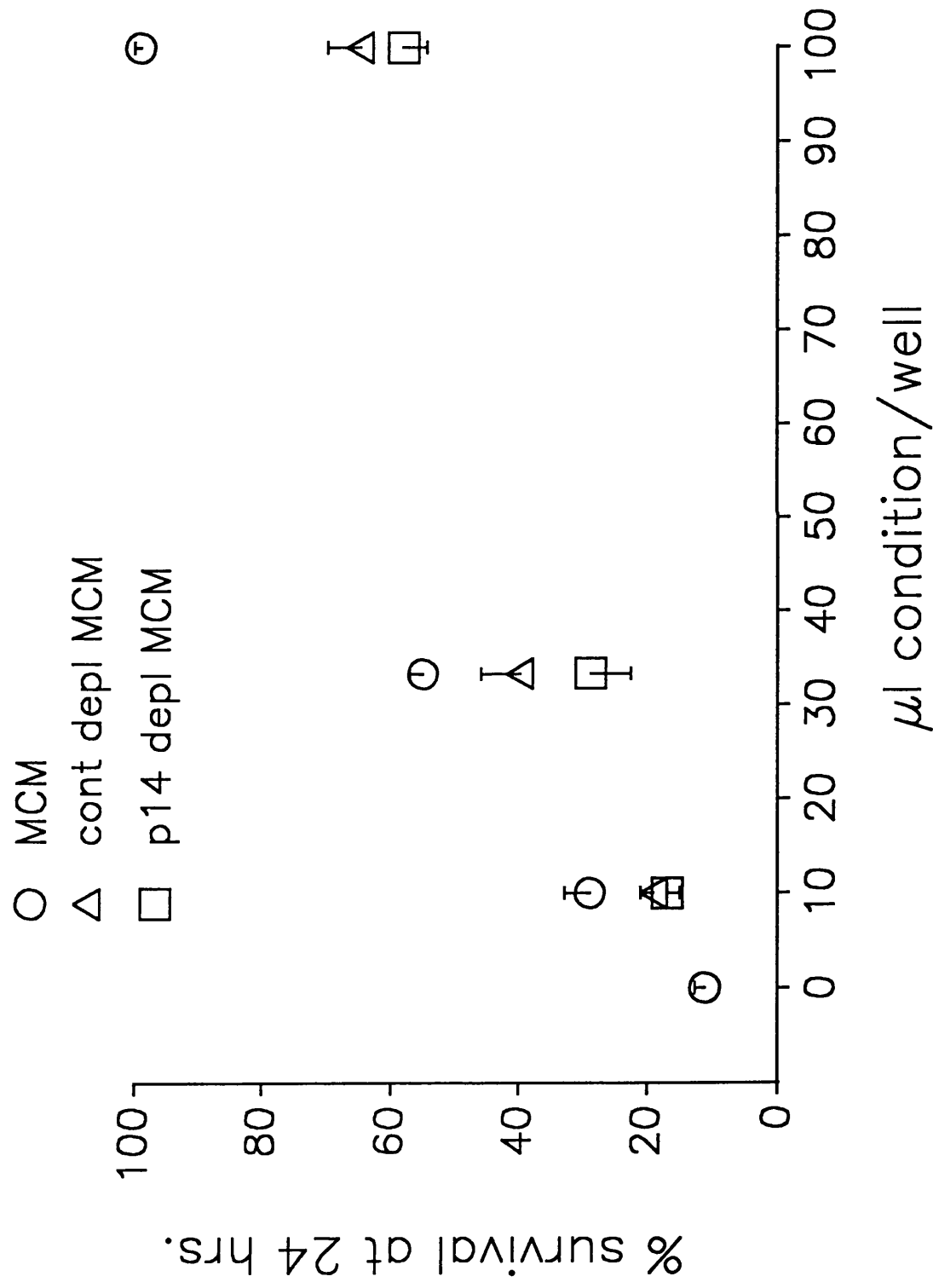
to be separated from IMP and (ii) the concentration of IMP needed to be increased. Out of known molecules, the most likely candidate for the active substance in MCM was bFGF, or another member of the bFGF family. These considerations led to the strategy of concentration of MCM and passage over a heparin-sepharose column which was described in the previous chapter.

Activity in concentrated MCM (cMCM)

After MCM was concentrated using PEG, the survival activity was also concentrated, although the total amount of bioactivity, measured by comparing the dilutions of MCM giving 50% survival before and after concentration, was considerably reduced (Fig.4.5).

An important control was to see whether the components of the defined medium used to produce the MCM were themselves active in the assay when concentrated. Insulin and conalbumin were tested alone and in combination at 20x their concentration in the defined medium i.e. 10^{-5}M insulin and 0.6% (w/v) conalbumin; and defined medium concentrated with PEG using the same protocol as that used for MCM was also tested. Insulin gave 30% survival in the assay but neither conalbumin nor concentrated defined medium had any effect. Thus the activity in cMCM is due to components produced by myotubes.

Activity in fractionated MCM
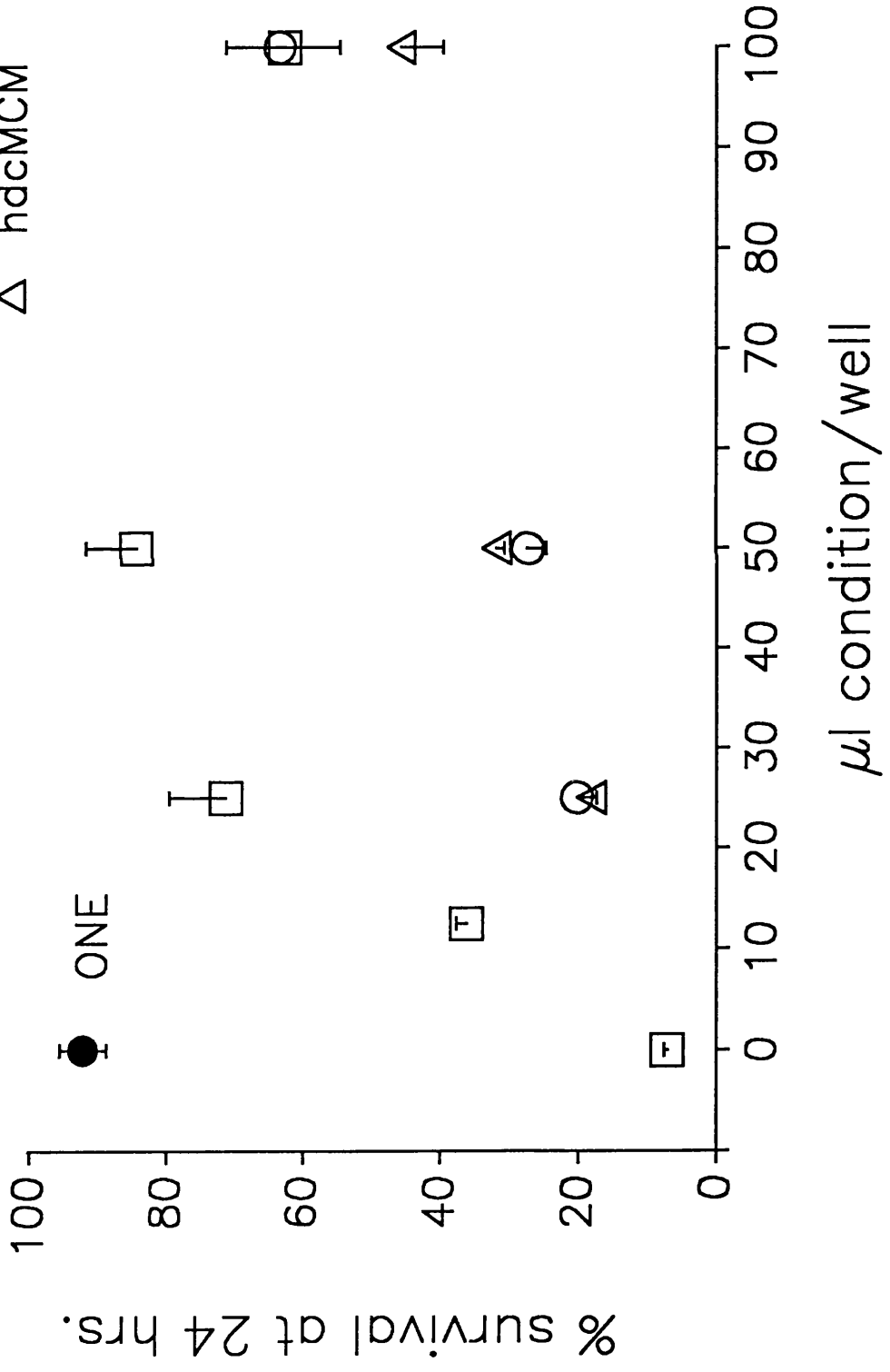
The three IMP-containing preparations of fractionated

Fig.4.5 Activity of concentrated MCM (cMCM) and heparin-depleted cMCM (hdcMCM) in the ciliary survival assay.

The following are shown:

(i) The MCM prior to concentration ("MCM"; open circles)

(ii) Concentrated MCM ("cMCM"; open squares)

(iii) Heparin-depleted cMCM ("hdcMCM"; open triangles)

(iv) ONE (filled circle)

In this example, the MCM was concentrated 26-fold, and the IMP was concentrated 11-fold. 30% of the IMP in the cMCM was depleted on the heparin column. Concentration of IMP with 100μl/well was as follows: MCM - 4.9x10^{-10}M; cMCM - 5.4x10^{-9}M; hdcMCM - 3.8x10^{-9}M.

% survival at 24 hrs.

μl condition/well

ONE

○ MCM
□ cMCM
△ hdcMCM

MCM to be considered are (i) cMCM that was depleted over a heparin column (hdcMCM), (ii) the 0.5M salt elution from the heparin column (hecMCM), and (iii) the IMP pool from an anion exchange run of MCM (ieMCM).

hdcMCM had activity in the survival assay, but in contrast to MCM and cMCM, the maximum survival obtained with different preparations at 100μl/well was 50% (see Fig.4.1. and Fig.4.5). The results of experiments in which hdcMCM had been depleted of IMP are shown in Fig.4.6. The % survival was generally similar in control-depleted and p14-depleted samples; a lower survival with the p14-depleted sample in one assay was not repeatable.

The maximum survival with either bFGF or hdcMCM alone was 50% and so it was of interest to see the result when both were added at the same time (see Fig.4.7a). The % survival was greater with bFGF + hdcMCM than with either bFGF or hdcMCM alone, and was roughly additive. When hdcMCM depleted of IMP was used, the additive effect on survival with bFGF remained (Fig.4.7b).

Both hecMCM and ieMCM had survival activity, and in both cases the levels of survival seen with ONE or pSNE were achieved. The results of experiments in which hecMCM and ieMCM had been depleted of 90% of their IMP by antibody p14 are shown in Figs.4.8 and 4.9 respectively. There was less activity in p14-depleted than control-depleted hecMCM at the higher but not the lower concentrations; this difference was not statistically significant. The dilution curves obtained with p14- and control-depleted ieMCM were

Fig.4.6 Activity of heparin-depleted cMCM depleted of IMP in the 24hr. ciliary survival assay

4 experiments are shown in which 100μl/well of control-depleted hdcMCM or 100μl/well of hdcMCM depleted by antibody p14 were added.

In experiments 1 and 2, using one preparation of hdcMCM, the final IMP concentration in the cultures with p14-depleted hdcMCM was 0.9x10^{-10}M, and 1.2x10^{-9}M in the cultures with control-depleted hdcMCM. In experiments 3 and 4, a different preparation of hdcMCM was used. The final IMP concentration in cultures with p14-depleted hdcMCM was 2.0x10^{-10}M, and 1.42x10^{-9}M in cultures with control-depleted hdcMCM.
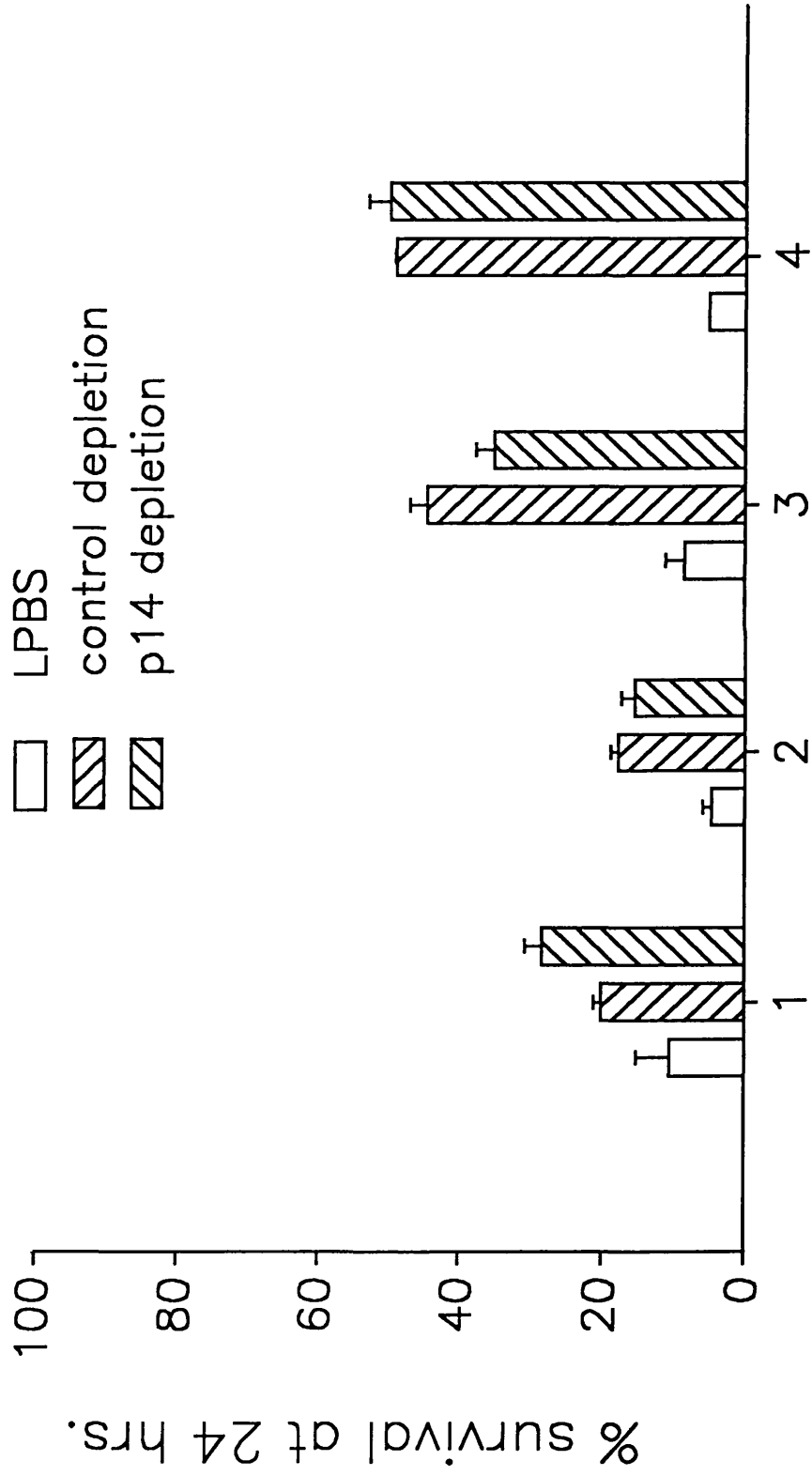
Fig.4.7 Effect of hdcMCM in combination with bFGF in the 24hr. ciliary survival assay.

(a) Effects of 100μl/well of hdcMCM and 5ng/ml bFGF alone and in combination.

(b) Two experiments in which the effects of hdcMCM depleted of IMP in combination with bFGF were examined. p14-depleted hdcMCM ("p14 depl") and control-depleted hdcMCM ("cont depl") were used at 100μl/well alone and in combination with 5ng/ml bFGF. The final concentration of IMP was 2×10^{-10}M with p14-depleted hdcMCM and 1.42×10^{-9} with control-depleted hdcMCM.

Note that some of the data in (b) have already been included in Fig.4.6.

(a)

% survival at 24 hrs.

control hdcMCM   bFGF   bFGF +
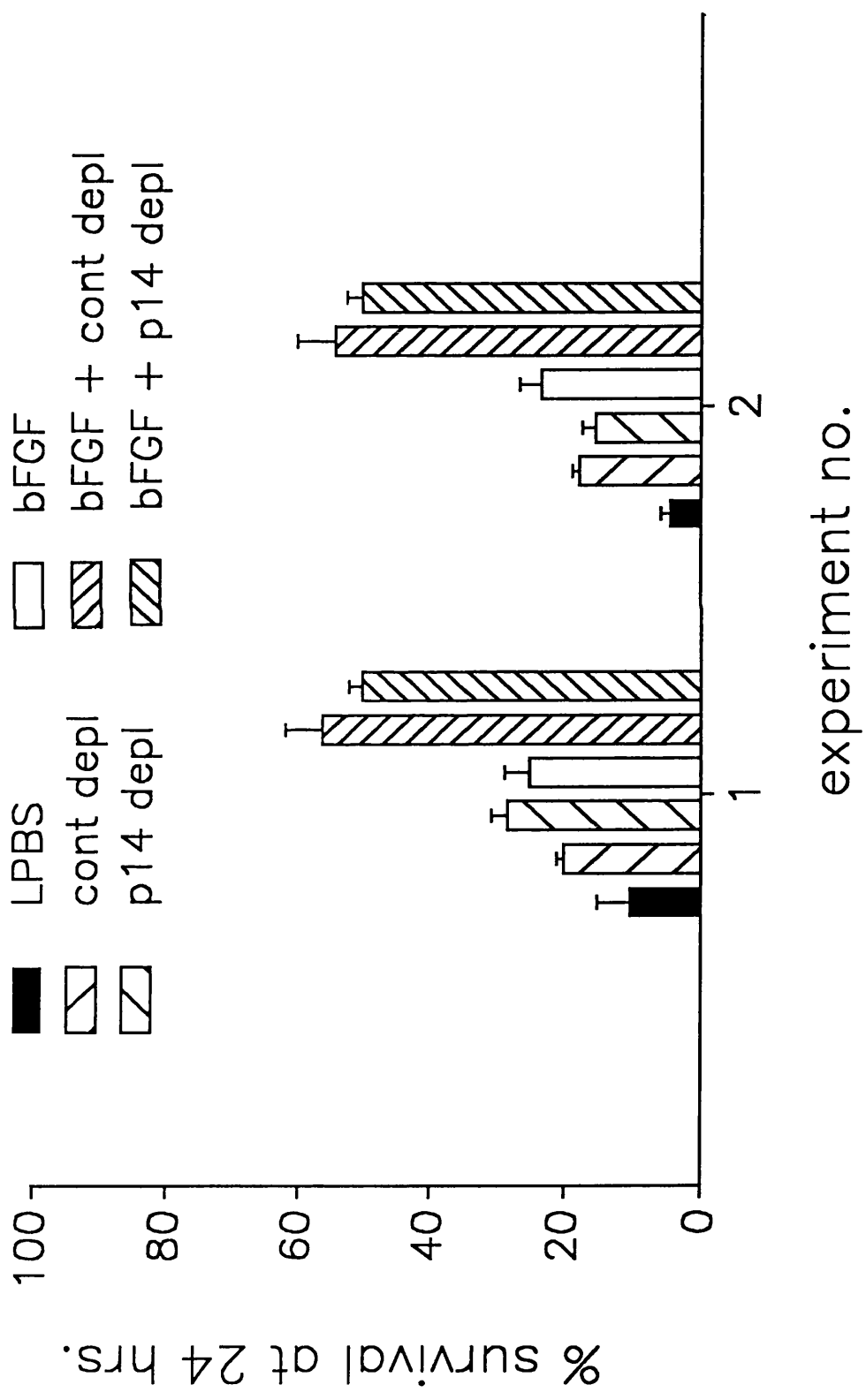   hdcMCM
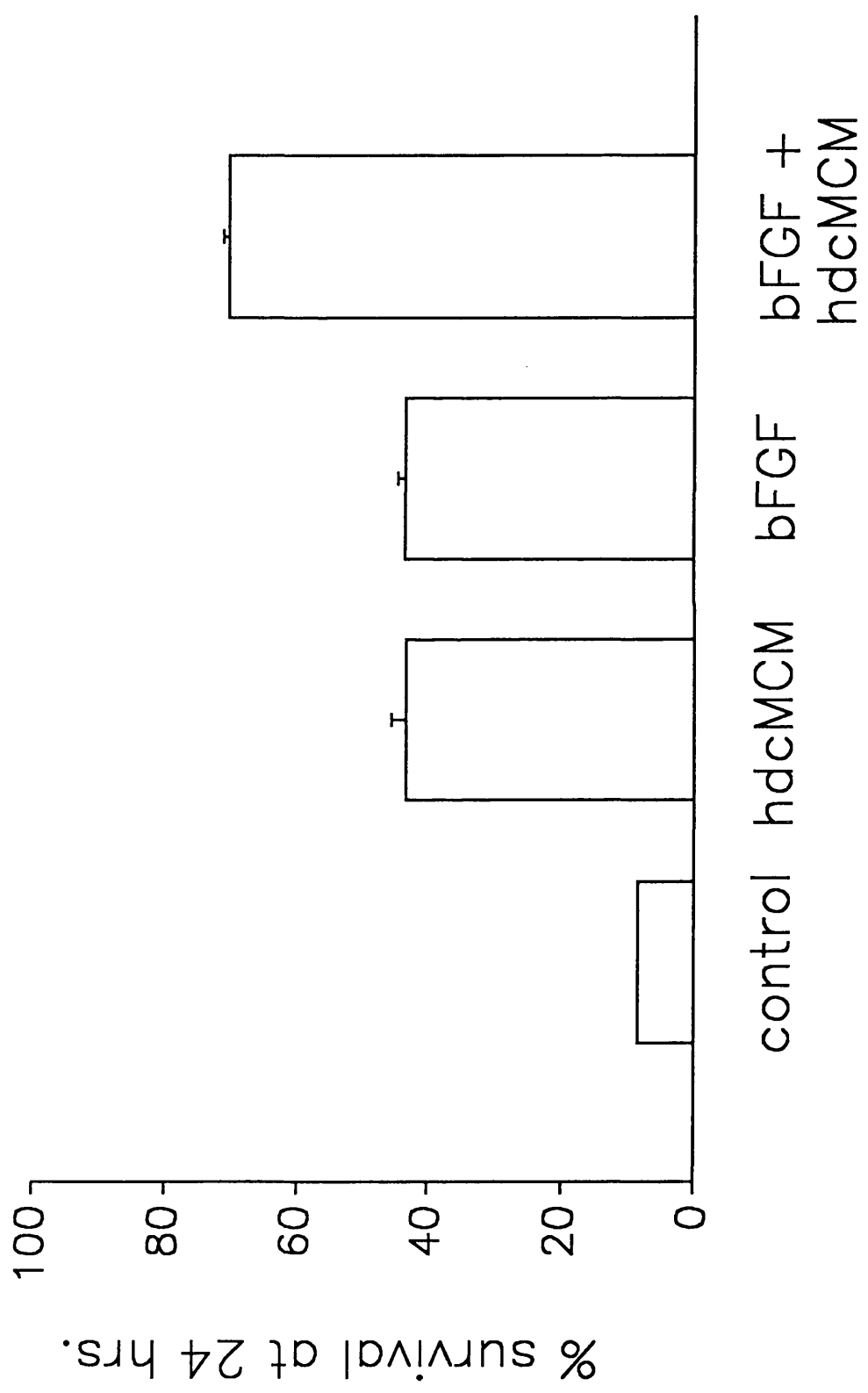
(b)

Fig.4.8 Activity of IMP-depleted hecMCM in the 24hr. ciliary survival assay.

Dose response curves are shown for hecMCM depleted of IMP by p14 ("p14 depl hecMCM", squares) and control-depleted hecMCM ("cont depl hecMCM", triangles).

In this experiment, the concentration of IMP at $100\mu l$/well was 1.4×10^{-10}M with IMP-depleted hecMCM and 1.3×10^{-9}M with control-depleted hecMCM.

% survival at 24 hrs.

μl condition/well

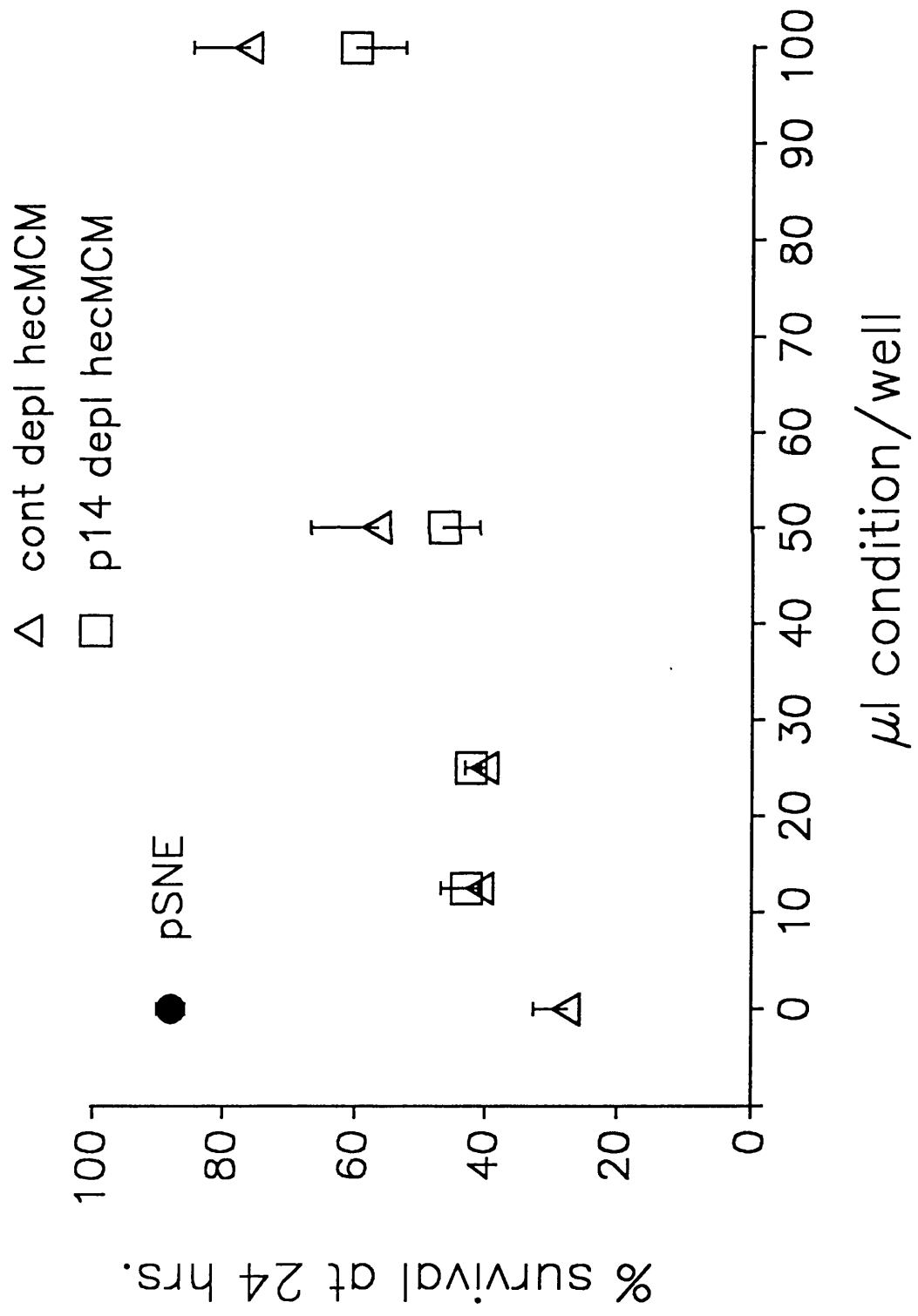△ cont depl hecMCM
□ p14 depl hecMCM

pSNE

Fig.4.9 Activity of IMP-depleted ieMCM in the 24hr. ciliary survival assay.

Dose response curves are shown for ieMCM (open circles), ieMCM depleted of IMP by p14 ("p14 depl ieMCM", squares) and control-depleted iecMCM ("cont depl ieMCM",triangles).

The concentration of IMP at 100μl/well was 7x10^{-11}M with IMP-depleted ieMCM and 5x10^{-10}M with control-depleted ieMCM.
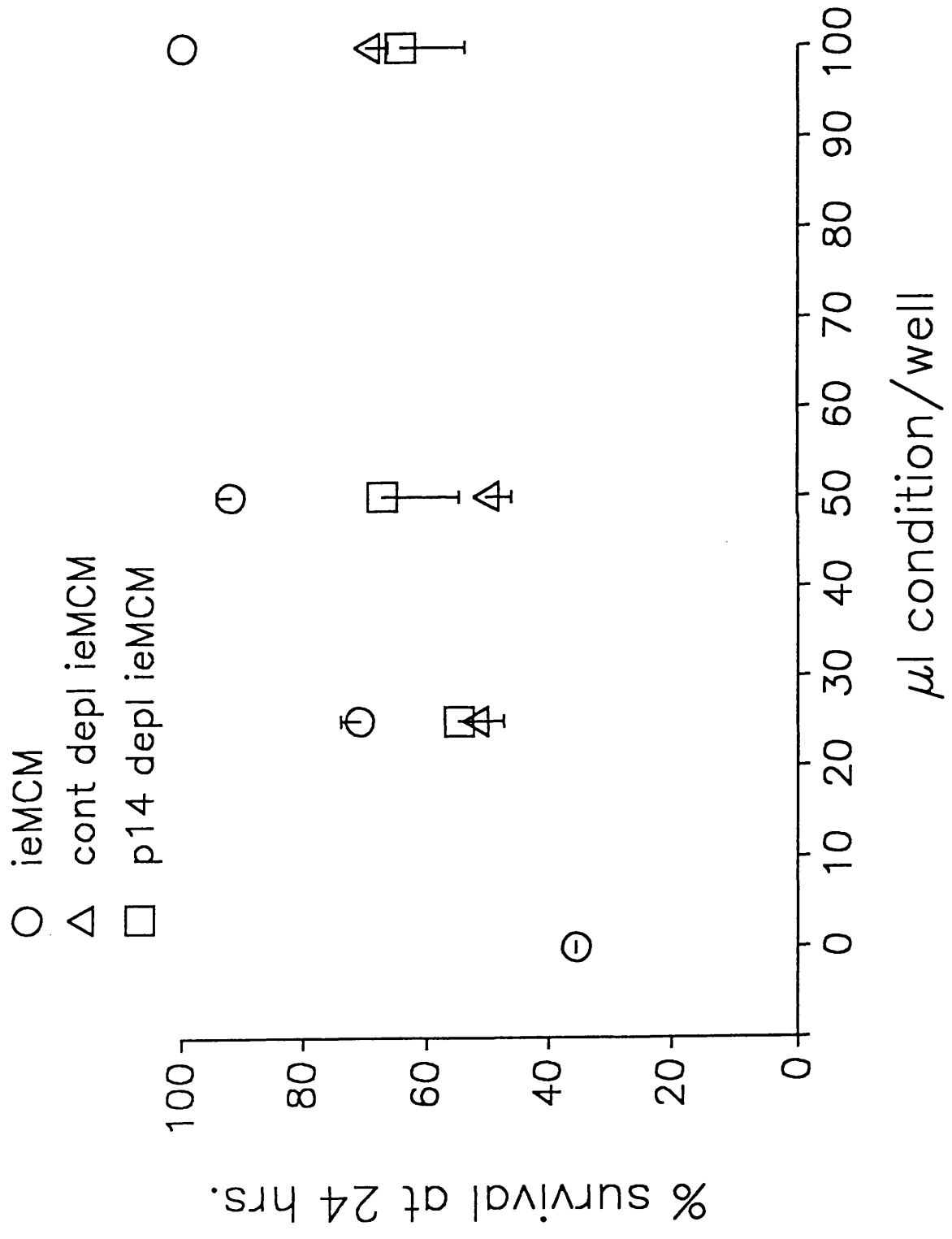
quite flat and a lot of activity had been lost in both antibody- and control-depleted fractions in comparison with the starting material. Activity in p14-depleted fractions was never less than in the corresponding control-depleted fractions.

Longer term survival

It was of interest to look at longer term survival in the ciliary assay, and long term survival was also a prerequisite for studies of ChAT activity. In cultures treated with ONE, percentage survival with time was as follows: 1 day ca.90%, 2 days ca.50%, 5 days ca.25% (see Fig.4.10a). In cultures treated with MCM, cMCM or hdcMCM, percentage survival decreased more rapidly with time: very few neurons were seen by 3 days, and none remained by 5 days.

When both ONE and hdcMCM were added to the same culture, the survival at times greater than 30hrs. was greater than with either condition alone, such that survival at 5 days was increased to 50% (see Fig.4.10b). This increased survival was roughly additive at 2 days, but in some experiments there was evidence that at later times the effect was synergistic, the % survival with ONE + hdcMCM being greater than the sum of the % survivals with either condition alone. This effect was seen at 3 days in 2 out of 3 experiments (see Fig.4.10a) and also in an experiment at 5 and 7 days, where a difference between ONE and ONE + hdcMCM on survival was seen past a time when any

Fig.4.10 Interaction between ONE and hdcMCM in the ciliary survival assay over several days.

(a) Experiment in which the interaction between ONE and hdcMCM was examined up to 3 days. hdcMCM depleted of IMP by p14 ("p14 depl") and control-depleted hdcMCM ("cont depl") were added at $100\mu l$/well alone and in the presence of ONE at the start of the culture period. The final concentration of IMP with p14-depleted hdcMCM was 2×10^{-10}M and with control-depleted hdcMCM it was 1.4×10^{-9}M.
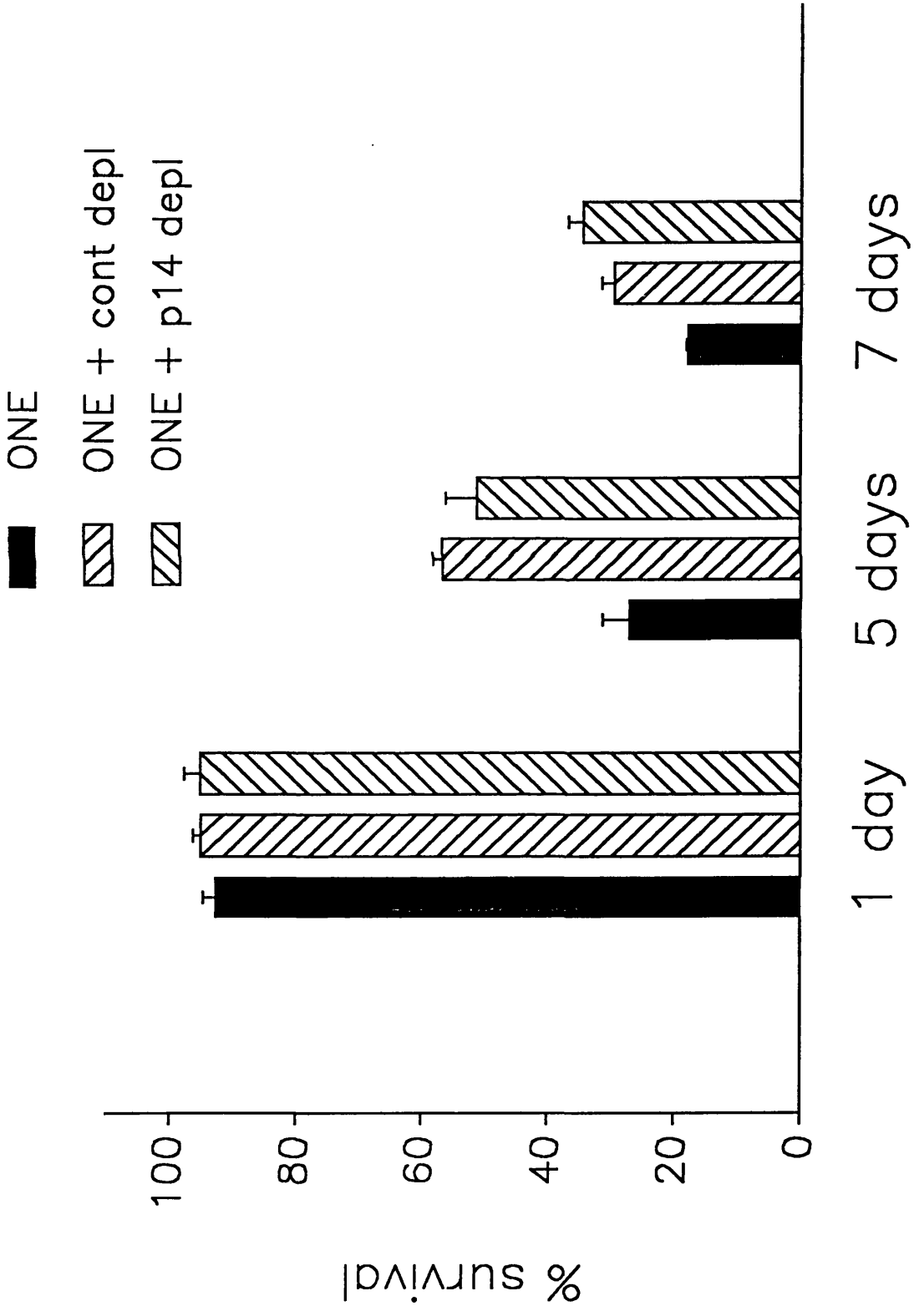
(Note that the % survival at 3 days in the presence of p14-depleted and control-depleted hdcMCM alone were estimated by visual inspection).

(b) Experiment in which the interaction between ONE and hdcMCM was examined at 5 and 7 days. IMP concentrations in control- and p14- depleted hdcMCM as in (a).

(a)
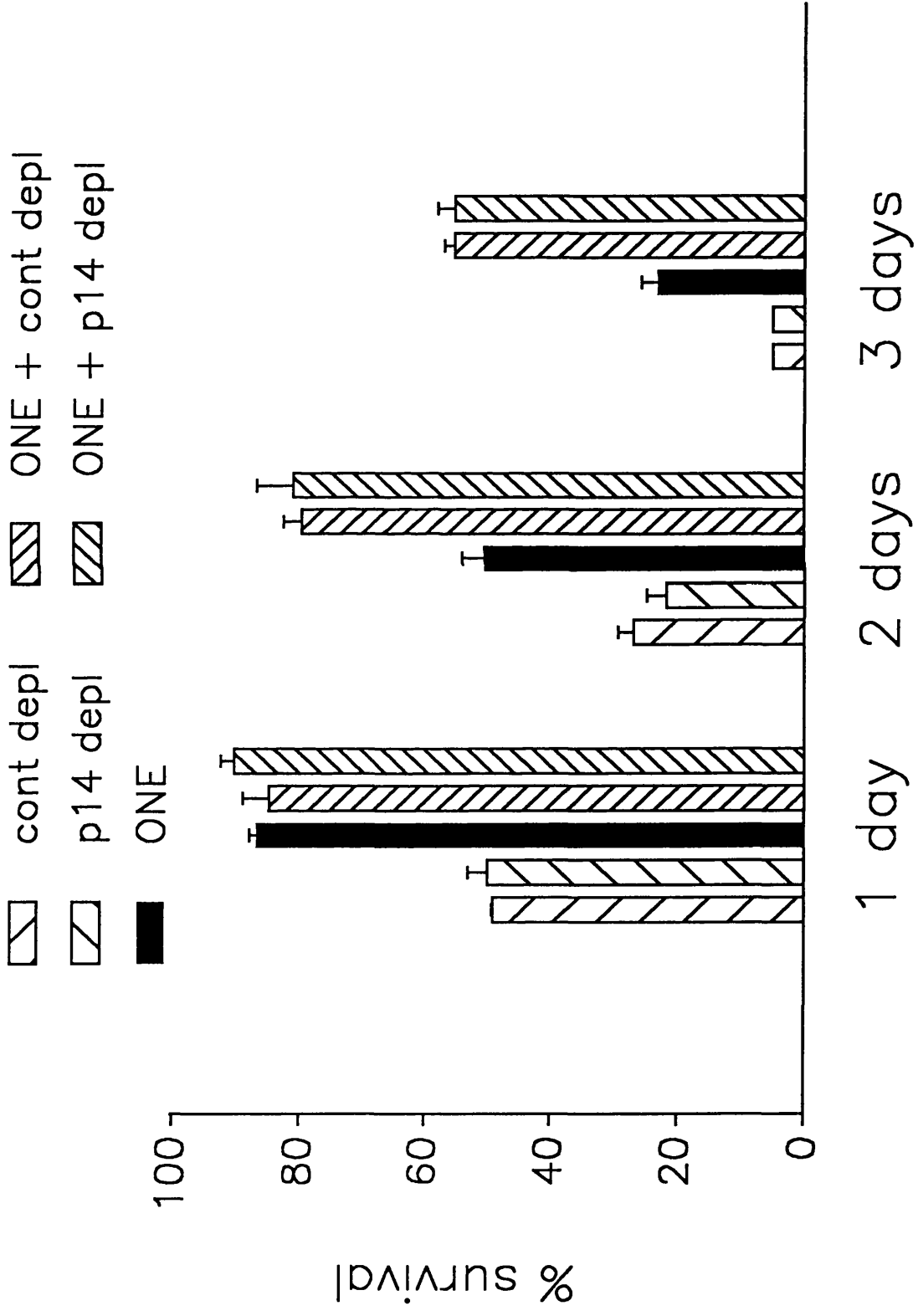
(b)

neurons survived in the presence of hdcMCM alone in other assays (Fig.4.10b).

The possible rôle of IMP in this interaction with ONE on long term survival was assessed using hdcMCM depleted of IMP (Figs.4.10a and 4.10b). Survival in the presence of ONE + p14-depleted hdcMCM and ONE + control-depleted hdcMCM were similar at all times examined.


ChAT activity

As MCM fractions did not support long term survival alone, their effect on ChAT activity was measured in cultures to which ONE was also added. Neuronal survival in these cultures is described above.

The results of experiments to look at the effect of hdcMCM on ChAT activity, and the rôle of IMP in this effect are shown in Fig.4.11. At 3 and 5 days, the ChAT activity/surviving neuron was greater with hdcMCM, whereas at 7 days the ChAT activity/neuron was similar with and without hdcMCM. In no case was a difference seen between cultures containing control-depleted and p14-depleted hdcMCM.

ChAT activity of control-depleted and p14-depleted hecMCM and iecMCM were tested in a 3 day assay in the presence of ONE in a pilot experiment in which neuronal survival was assessed by visual inspection. Neuronal survival was good, and apparently similar in all conditions, and no differences in ChAT activity were seen between control-depleted and p14-depleted conditions.

Fig.4.11 ChAT activity of CG neurons: effect of hdcMCM and hdcMCM depleted of IMP in the presence of ONE.

ChAT activity is expressed in microunits per neuron. p14-depleted hdcMCM and control-depleted hdcMCM were added at 100μl/well on day 0, with a further 50μl/well added after 5 days to the 7 day cultures. Final concentration of IMP with p14-depleted hdcMCM was 2.0×10^{-10}M and with control-depleted hdcMCM it was 1.4×10^{-9}M.
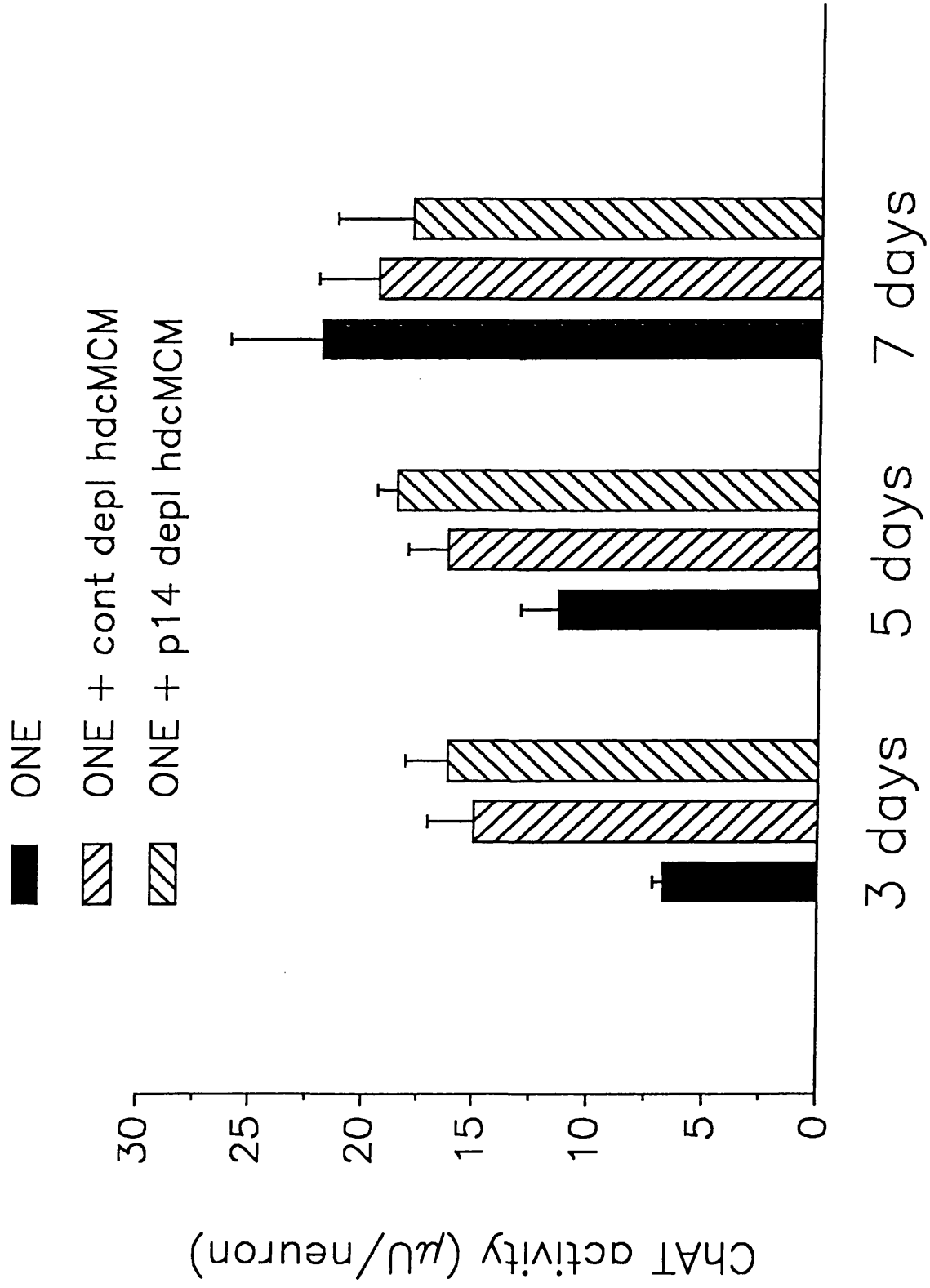
II. VENTRAL SPINAL CORD NEURONS

Retrograde labelling of motoneurons

After dissociation of spinal cords from backfilled embryos, a proportion of the larger cells contained numerous brightly fluorescent dots in their cell bodies (Fig.4.12). After attachment, the fluorescently labelled cells could be identified as neurons; fluorescence in the smallest neurons or non-neuronal cells was never seen. Sections of backfilled cords confirmed that only motoneurons in the lateral motor column had been labelled (Fig.4.12).

The yield of both total cells and fluorescently labelled cells were both quite consistent, with the total yield of cells being about 220,000/ventral lumbar cord and the yield of fluorescent cells being about 15,000/ventral lumbar cord (in which both hindlimbs had been injected), or about 7.5% of total cells.

By 3 days of culture, the fluorescence had faded but was still clearly visible. By 5 days very few neurons were fluorescently labelled in any conditions.

Motoneuron survival in bulk cultures of ventral spinal cord

Fluorescent motoneurons rapidly extended neurites in culture and surviving motoneurons always had long neurites and large cell bodies. Under control conditions (LPBS), the % of post-attachment fluorescent cells remaining was ca.35% after 2 days in culture, ca.15% after 3 days, and

Fig.4.12. Retrograde labelling of motoneurons using WGA-FITC

(A) Transverse section of lumbosacral cord of St 29 (E6) chick whose hindlimbs were injected with 2μl WGA-FITC at St 26 (E5), viewed under Nomarski optics. Scale bar = 100μm.

(B) Same field as in (A) viewed under fluorescence optics, showing intensely fluorescent motoneurons in the lateral motor columns.

(C) Dissociated ventral lumbosacral cord cells in fraction I from a metrizamide gradient viewed under fluorescence optics, showing a fluorescently labelled motoneuron.

(D) Same field as in (C) viewed under phase contrast. Scale bar = 25μm.
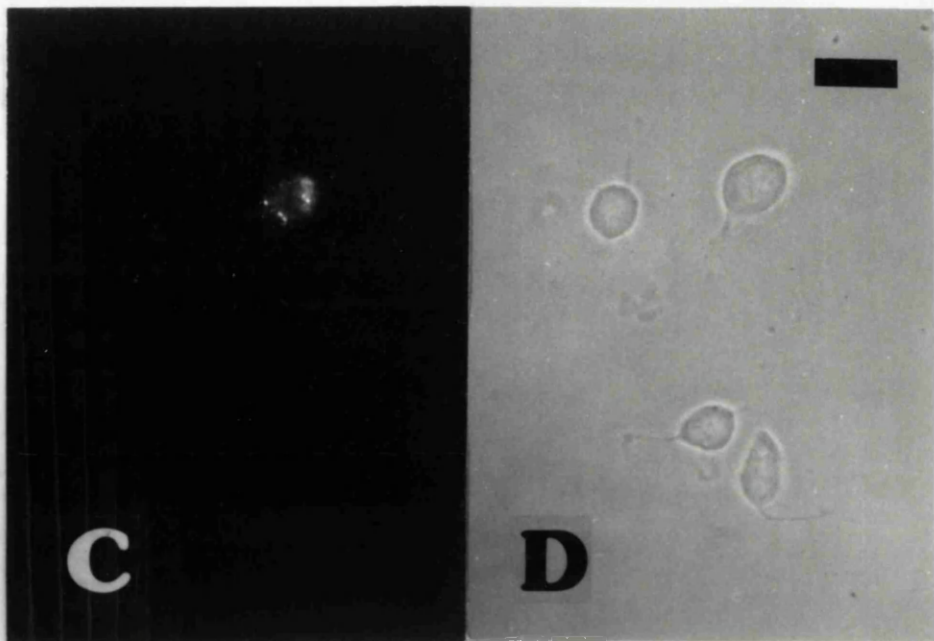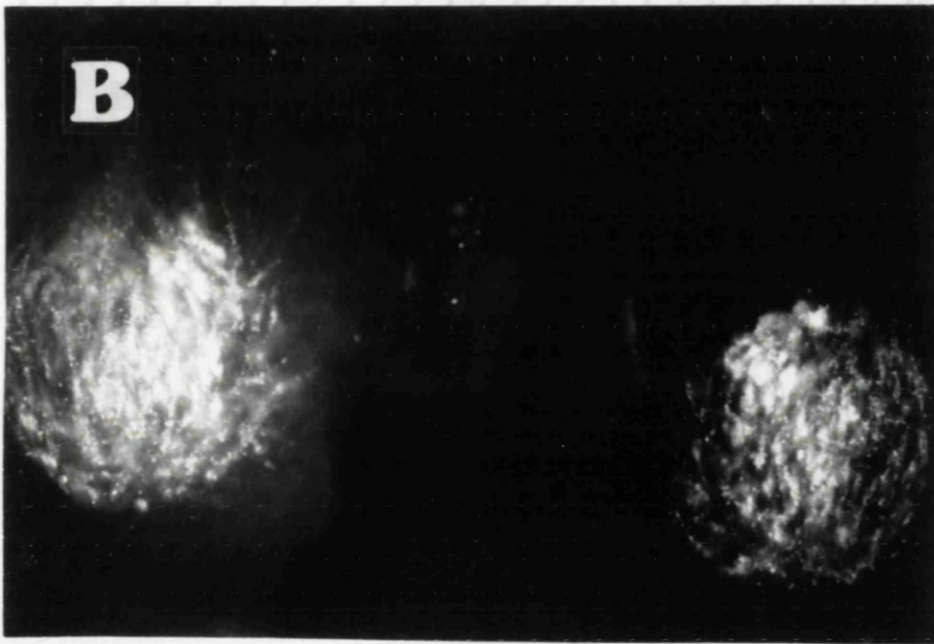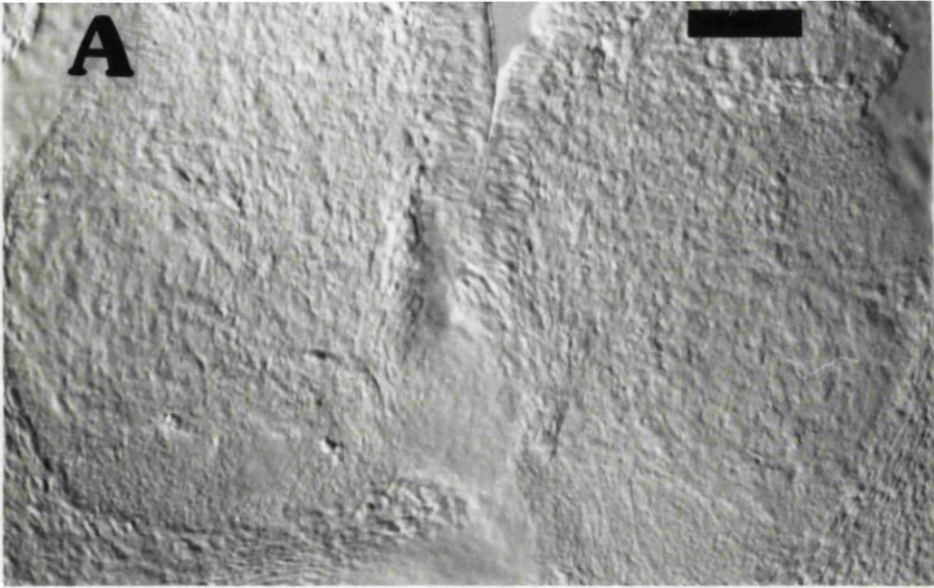
negligible after 4 days. These percentages were greater when either bFGF or conc MCM were added to the cultures, and pilot experiments showed that the greatest effect compared to the controls was after 3 days; most experiments were therefore assayed at this time point.

bFGF at 10ng/ml gave a mean count of fluorescent cells at 3 days of 26% the initial count or between 1.5-2x the number in control conditions. When the results from different assays were pooled, the survival with bFGF was significantly different from the survival in control conditions (Student's t-test; n_1=21, n_2=16; t=-3.73; P<0.001). A 10-fold greater concentration of bFGF did not give a greater effect (data not shown).

cMCM that was concentrated on the Minitan system had a greater effect in the assay, with a mean % of initial fluorescent cells at 3 days of 43% (s.e.m.=4.1%,n=6) or 3x the number in control conditions. However, defined medium that had been concentrated 20x on the Minitan system had a similar effect, so the effect of cMCM might be explained by medium components alone, although the comparison is not straightforward since the medium components themselves might be altered by myotubes.

The relatively small effect in experimental conditions over controls, the large amount of scatter and the uncertainty over the activity of components secreted by myotubes all set limits on the usefulness of this assay and led to the alternative strategy of enriching for motoneurons before placing them in culture.

Fractionated ventral spinal cord cultures

The results of the yields of cells in the fractions from the metrizamide gradient are shown in Table 4.1. The proportion of total cells in fraction I was variable from assay to assay, but in all cases fraction I was enriched for both large and fluorescent cells, with an average 4-fold enrichment for fluorescent cells. The proportion of motoneurons in fraction I could be estimated by comparing the proportion of fluorescent cells in fraction I to the estimated proportion of motoneurons that were backfilled, based on the number of motoneurons present in the lumbar cord at that stage. This estimate was generally around 50%. All the bioassay results described were on fraction I cells.

Non-neuronal cells were very rare in cultures of fraction I cells, making the addition of mitotic inhibitors unnecessary. Under control conditions, the total number of neurons had fallen to ca.5% of post-attachment values after 3 or 4 days. With 10 or 100ng/ml bFGF, total survival was 10% after these times and this effect was statistically significant (Mann-Whitney U test; n_1=7, n_2=7; U=46; P<0.001).

cMCM had a much greater effect on survival, between 45-55% total neurons surviving after 4 days (see Fig.4.13); concentrated defined medium did not have an effect in this assay (data not shown). All IMP-containing cMCM fractions (hdcMCM, hecMCM and iecMCM) were tested and found to have

Table 4.1   Fractionation of ventral spinal cord cells on
a   metrizamide gradient.

Results from 5 metrizamide gradient experiments are
shown.

(a) shows the yield of total and fluorescently
labelled ventral cord cells after dissociation and their
recovery over the metrizamide gradient (i.e. cells
recovered in fractions I and II combined).

(b) is self-explanatory

(c) shows the % large and % fluorescently labelled
cells before and after fractionation.

156

(a)

	Total	Fluorescent
Cell yield/ ventral cord x 10^3	236 ± 15	14.8 ± 1.5
Total % recovery over metrizamide	74.7 ± 6.0	73.2 ± 7.0

(b)   % cells in fraction I after metrizamide gradient

   = 16.2 ± 4.0 %

(c)

	% large	% fluorescent
Before fractionation	27.4 ± 2.2	6.5 ± 0.8
Fraction I	79.9 ± 6.6	32.9 ± 10.3
Fraction II	20.4 ± 1.8	6.4 ± 1.0

Fig.4.13. Survival of neurons in fraction I from a metrizamide gradient.

(a) Cells in control conditions (100μl LPBS/well) viewed under phase contrast. Scale bar = 50μm.

(b) Cells in the presence of 100μl/well cMCM.

(a)
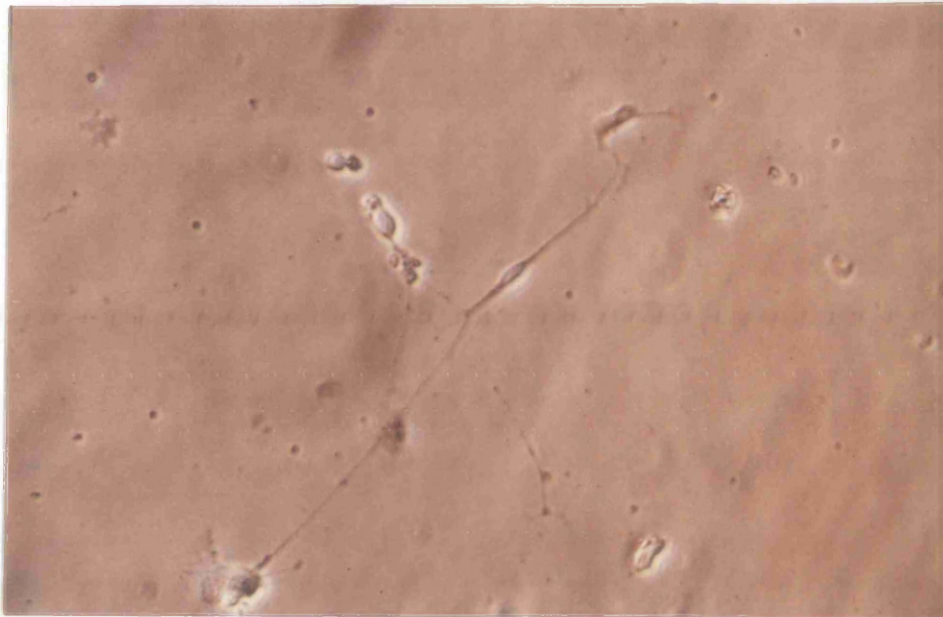
(b)
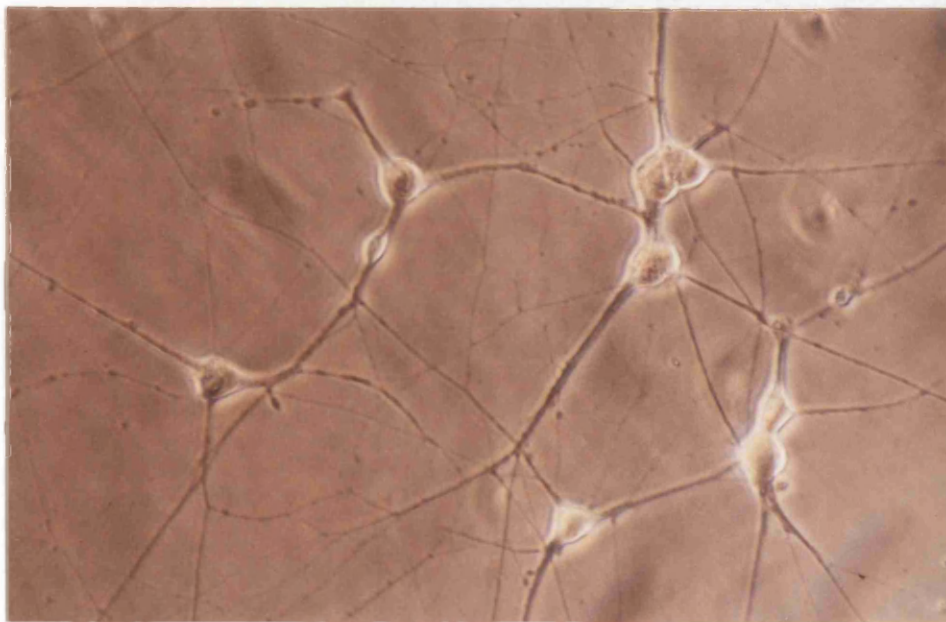
Fig.4.14   Survival of fractionated ventral spinal cord
neurons with hdcMCM depleted of IMP

Survival of total fraction I neurons was measured
after 3 days. The concentration of IMP at 100μl\well was
$2.0\text{x}10^{-10}$M with p14-depleted hdcMCM and $1.4\text{x}10^{-9}$M with
control-depleted hdcMCM. Note that the cMCM used in this
assay was used as a positive control and was not from the
same batch of cMCM as that used to make the hdcMCM.
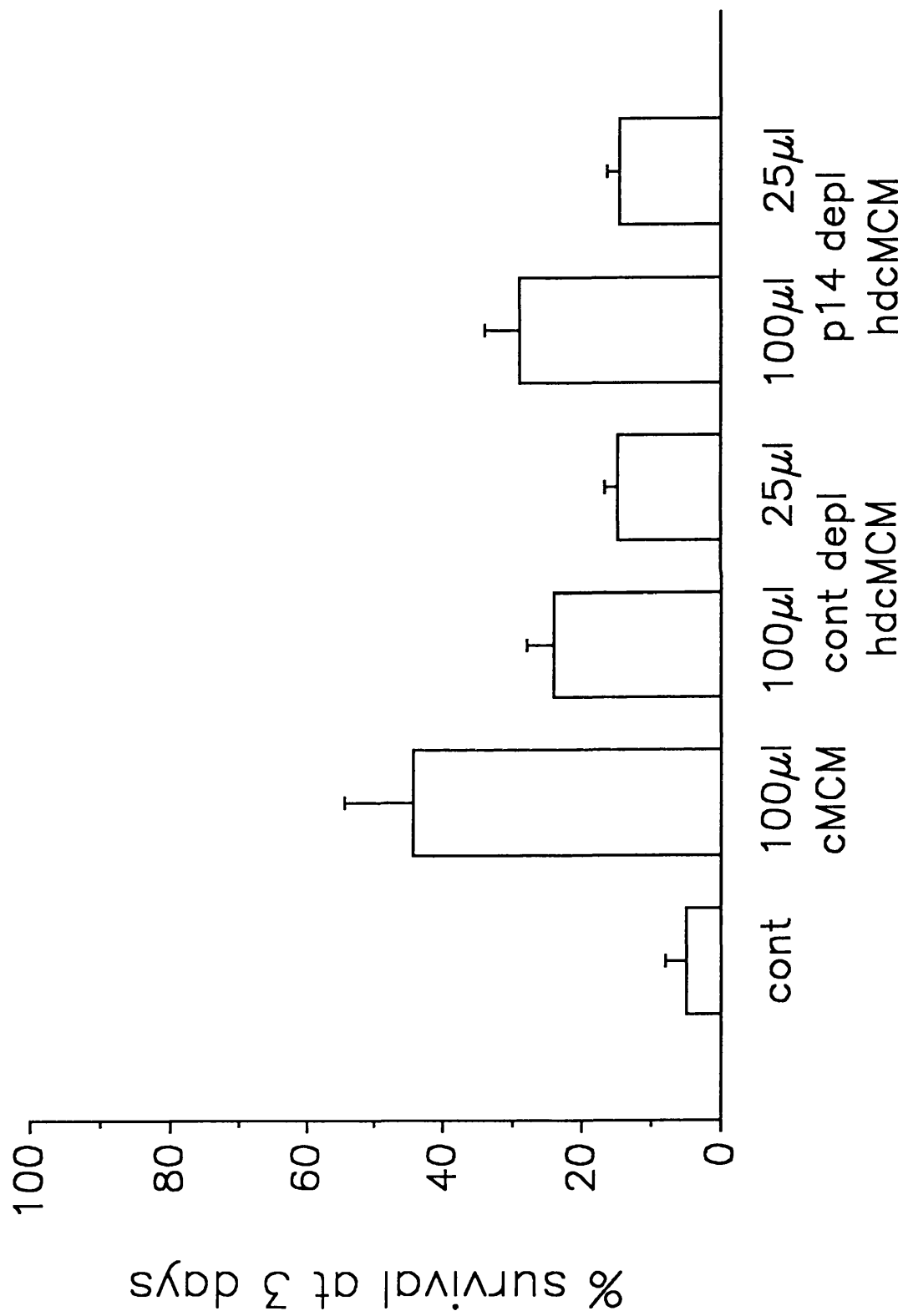
Fig.4.15 Effects on both total and fluorescent fraction I spinal cord neurons of bFGF and cMCM.

Counts of % total cells remaining and % fluorescent cells remaining after 4 days were made in control conditions (100μl LPBS), with 10ng/ml bFGF and with 100μl/well cMCM. Note the change in the vertical scale from previous Figs.
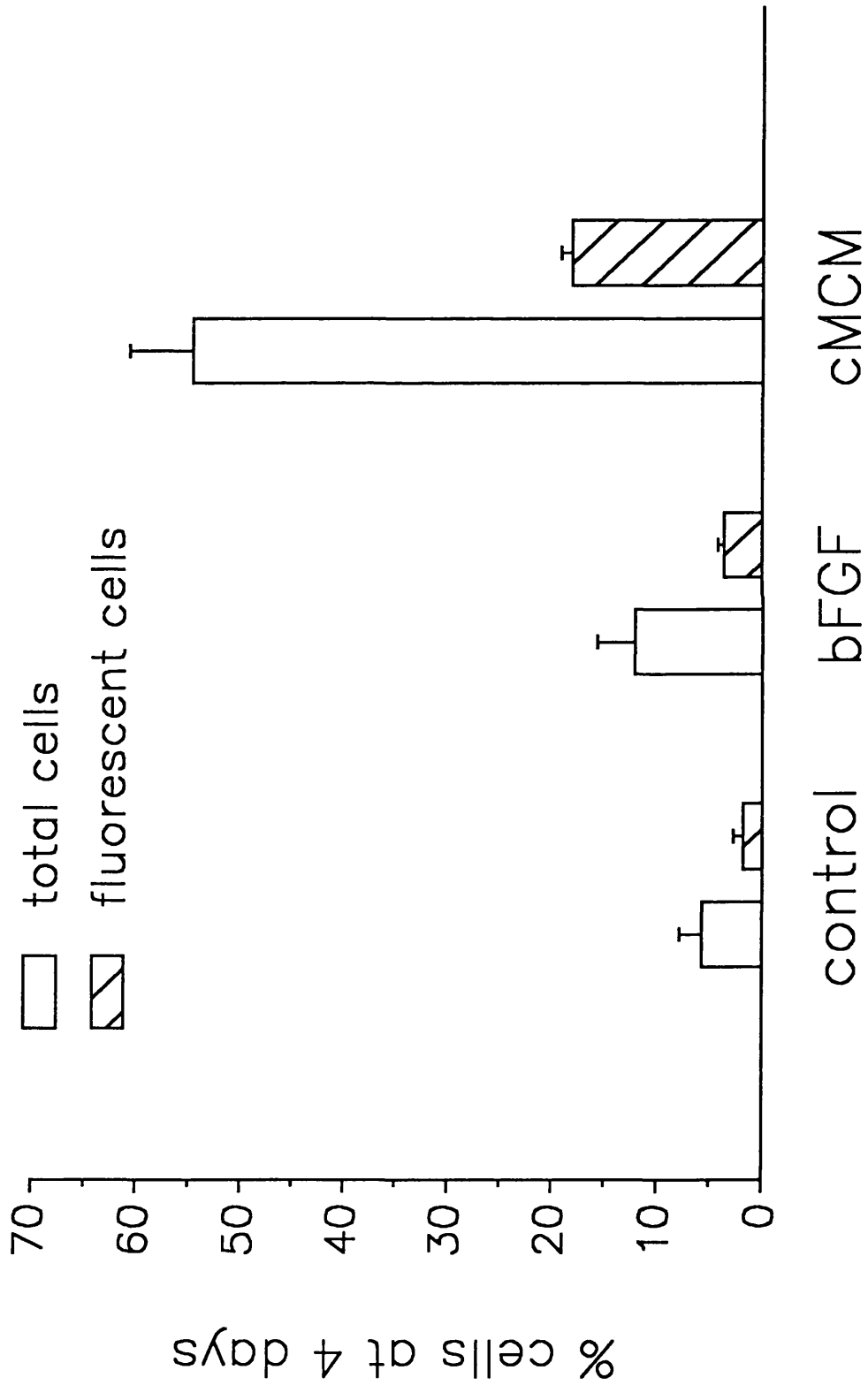
survival activity. The only available fraction which had been depleted of IMP by p14 was hdcMCM, and the results of the bioassay are shown in fig.4.14. There was no difference in survival of total cells between control-depleted and p14-depleted hdcMCM. The remaining MCM fractions could not be depleted of IMP by p14-affinity at this time.

In one assay, fluorescent as well as total cells were counted (Fig.4.15). With cMCM, the % fluorescent cells remaining after 4 days was 18%, compared to 55% of total cells. The number of both fluorescent and total cells with cMCM was 10 times that in control conditions and 5 times that with bFGF. Thus the fluorescently labelled motoneurons were behaving in an identical way to the non-labelled neurons in these cultures.

DISCUSSION

The main findings of the experiments described in this chapter are (i) that various fractions of MCM and muscle extract have activity in several different neuronal assays and (ii) that where tested these activities are not due to IMP. The individual results in different assays will be considered before returning to a broader discussion of the implications for the bioactivity of IMP, in the conclusion of this chapter.

CILIARY ASSAYS

General

The number of neurons obtained per ganglion agrees well with the total number of neurons in a stage 34 (E8) CG counted in tissue sections (Landmesser and Pilar, 1974b). The substantial difference between the maximum and minimum survival obtained at 24 hrs. in different conditions (i.e. 15-90%) is comparable to that of some other previously reported short term survival assays (Bonyhady et al., 1980; Ebendal et al., 1985; Wallace and Johnson, 1987). Comparison of long term survival with other assays is difficult, as the conditions in different assays are variable; it is surprising that the long term survival activity of CNTF has not reported in the literature. ChAT activity at 3 days was comparable to other reports, but at longer times it did not increase as much as is usually found (e.g. Nishi and Berg, 1981).

Various fractions of MCM and muscle extract had survival activity in the 24 hr. survival assay; and hdcMCM was shown to promote longer term survival and to enhance ChAT activity. An effect of chick skeletal MCM on CG neuron survival has been demonstrated previously (Nishi and Berg, 1977; Bennett and Nurcombe, 1979) but although adult rat skeletal muscle extract has been shown to have an effect on survival (Ebendal, 1987) and ChAT activity (McLennan and Hendry, 1978), this is the first report of an effect of embryonic skeletal muscle extract on survival of

CG neurons. More interestingly, the synergistic interaction found between ONE and hdcMCM on CG neuron survival is the first example of a synergy of factors on CG neurons.

Role of IMP in CG neuron bioactivities

Many different IMP-containing fractions had activity in the 24 hr. CG neuron survival assay: ieME, MCM, cMCM, hdcMCM, hecMCM, and ieMCM. In contrast, neither acidic muscle extract nor the eluate from the antibody affinity depletions of purified extract or MCM had activity in this assay. Four of the active fractions (MCM, hdcMCM, hecMCM and ieMCM) were substantially depleted of IMP by p14 antibody and subsequently tested for survival activity: in no case was survival activity clearly affected compared to fractions run over a control column. The only hint of a difference with depleted fractions was in the case of ieMCM, but the effect was only seen at high concentrations and is thus difficult to interpret. This experiment could not be performed for the muscle extract fraction as only a small percentage depletion of IMP by p14 was obtained. Thus at least the majority of the 24 hr. survival activity present in MCM fractions is not due to IMP.

The rôle of IMP in the activity of hdcMCM on long term survival and ChAT activity was also investigated and no difference was found in activity when IMP was depleted. Thus again at least the majority of these effects are not due to IMP.

Nature of the CG neuron bioactivities

As the majority of the bioactivity described is not due to IMP, we should consider what molecule(s) it might be. A list of the molecules and partially purified factors known to have bioactivity for CG neurons is given in Table 4.2. Except for the FGFs, none of these factors has been purified from either skeletal muscle extract or skeletal MCM and data on their presence or absence in skeletal muscle are very limited.

As mentioned in chapter 1, there is evidence that a molecule antigenically related to bFGF is found in embryonic muscle. In addition, biologically active molecules that may well be bFGF have been isolated from adult muscle: a heparin-binding factor which has the spectrum of mitogenic activity of bFGF has been isolated from adult chicken skeletal muscle (Kardami et al.,1985) and a heparin-binding activity which stimulates ChAT activity of CG neurons and which is immunoprecipitated by anti-bFGF antibodies has been reported in human muscle (Vaca et al.,1989). Thus bFGF, or a molecule antigenically related to it, is clearly a candidate for the activity in muscle extract reported here. Whether bFGF is expected to be present in MCM is not so clear: the presence of bFGF in MCM has not yet been demonstrated, and, as it lacks a signal sequence, it may not be secreted.

However, there are grounds for thinking that bFGF cannot account for all the bioactivity described. Being highly basic, bFGF would not be expected to be present in

Table 4.2   Trophic factors for ciliary ganglion neurons

	Source	MW (kD)	Biological activity		References
			Survival	ChAT	
CNTF	Embryonic chick eye	20.4	+	+	Adler et al.,(1979)
CNTF	Adult rat sciatic nerve	24	+	+	Manthorpe et al.,(1986)
bFGF	Bovine brain	18	+	?	Unsicker et al.,(1987) Schubert et al.,(1987)
aFGF	Bovine pituitary	16	+	?	Unsicker et al.,(1987)
Insulin		6	+	?	Skaper et al.,(1984)
Purpurin	Embryonic chick retinal CM	20	+	?	Schubert and LaCorbière,(1985)
-	Chick eye extract	50	-	+	Nishi and Berg,(1981)
-	Ox heart	20	+	?	Bonyhady et al.,(1980)
-	Pig lung	3	+	+	Wallace and Johnson,(1986)

the same fractions as IMP following ion-exchange chromatography (i.e. in ieME or ieMCM), and, as it binds to heparin with high affinity, it should not be present in hdcMCM or hecMCM. Also, hdcMCM when used in combination with bFGF gave a % survival in the 24hr. assay greater than the largest effect seen with bFGF alone (the dose of bFGF used, 5ng/ml, was larger than the dose needed to elicit maximal response in neuronal bioassays, e.g. Schubert et al.,1987). However, not all members of the FGF family are ruled out by the above criteria and so could account for the bioactivities seen. For example, the activity in hdcMCM could be a member of the FGF family which does not bind to heparin and which acts through a different receptor to bFGF.

There are not enough comparative data between the activities identified here and the other factors in Table 4.2 to allow a discussion of their possible identity.

VENTRAL SPINAL CORD CULTURES

Culture methods

The yields of total cells/spinal cord and fluorescent cells/spinal cord obtained are comparable to those of O'Brien and Fischbach (1986) and Dohrmann et al.(1986). The results of the metrizamide gradient separation are also similar to those of Dohrmann, except that the overall yield of cells was somewhat lower.

An important point to consider is what proportion of

cells in fraction I were motoneurons. The method used to estimate the % of fraction I cells that were motoneurons (50%) is very approximate, and assumes that survival of fluorescently-labelled neurons is similar to unlabelled neurons from the time of labelling to the time they are placed in culture, which may well not be true. A better indication comes from the bioassay result in which fluorescent cells behaved in the same way in relation to the total population in all conditions. This strongly suggests that the fluorescent cells are the same as the unlabelled cells, and thus that all fraction I cells, or a large majority of them, are motoneurons.

Activity of bFGF

It was of interest to test the bioactivity of bFGF on motoneurons, as although an effect of bFGF on motoneuron survival had previously been reported (Unsicker et al.,1987), the size and significance of this effect was not explicitly stated in the paper.

The results reported here support the view that bFGF does have a small effect on motoneuron survival: in unfractionated cultures the number of fluorescent cells was 1.5-2x greater at 3 days in the presence of bFGF than in control cultures, and in fractionated cultures the survival of fraction I cells was two-fold greater in the presence of bFGF than in control cultures; in both cases the effect of bFGF was statistically significant.

Activity of MCM and IMP

cMCM and several cMCM fractions (hdcMCM, hecMCM, ieMCM) had substantial survival activity in both unfractionated and fractionated ventral spinal cord cultures, although the significance of the activity in unfractionated cultures is unclear since concentrated defined medium itself had similar activity in the assay. The time course of survival of cells in fraction I in the presence of MCM fractions was similar to that obtained by Dohrmann et al.(1986) using a fraction of muscle extract. The activity of cMCM confirms previous reports that skeletal MCM has trophic activity for motoneurons (Calof and Reichardt,1984; O'Brien and Fischbach,1986).

The rôle of IMP in the bioactivity could only be tested for hdcMCM and in that case it was clear that the survival activity was not attributable to IMP. The list of known and partially purified trophic factors for motoneurons which we have already seen in Table 1.1 (chapter 1) includes many factors which are found in skeletal muscle. Again ,except for bFGF, there is little data to allow an assessment of whether any of these factors is the same as the activity identified here. The much greater bioactivity of cMCM than bFGF means that bFGF cannot alone account for the bioactivity, unless the purified bovine bFGF used has different activity to chick bFGF.

<u>CONCLUSION</u>

In summary, various IMP-containing fractions have been shown to have activity in a variety of neuronal bioassays, but in no case where the rôle of IMP has been assessed has evidence been found that IMP is bioactive in these assays. This is true for four different assays: (i) 24hr survival of CG neurons (ii) longer term survival of CG neurons in the presence of CNTF (iii) ChAT activity of CG neurons and (iv) survival of motoneurons. Thus two main points emerge: firstly, that neurotrophic activities due to possibly novel neurotrophic agents have been identified, and, secondly, that no evidence for an <u>in</u> <u>vitro</u> neurotrophic action of IMP has been found.

However, it cannot be concluded that IMP is definitely not a neurotrophic factor for the following reasons: (i) antibody depletion experiments could not be carried out in all cases and, in particular, the rôle of IMP in the activity in purified muscle extract could not be determined; (ii) there was only a small amount of data relating to the activity of IMP on motoneurons; (iii) the activity of IMP may be too small compared to the other activity present to be able to measure it in an antibody-depletion experiment; (iv) IMP may only be active on a subpopulation of neurons; (v) the concentration of bioactive IMP in the assays may be insufficient; (vi) IMP in MCM is heterogeneous, and this might affect bioactivity; (vii) the trophic activity of IMP might not be measurable in such assay systems. Of these, (i) - (iii) are self-

explanatory, but (iv)-(vii) require further elaboration.

The possibility of IMP only being active on a subpopulation of neurons is particularly pertinent with regard to CG neurons. In chapter 2, IMP was shown to be present in the targets of ciliary and not choroid neurons and may therefore only be bioactive on the former. No markers for the 2 subpopulations exist at present, neither the size difference nor the expression of somatostatin by the choroid neurons (Epstein et al.,1988) being consistently maintained in culture. However, as the yield of CG neurons was quantitative, the initial composition of the cultures was 50% ciliary and 50% choroid neurons and so all fractions with >50% survival activity are active on the ciliary subpopulation. Of IMP-containing fractions, only hdcMCM never had greater than 50% survival activity so it is possible that only choroid neurons were involved, although this seems extremely unlikely. In general, the results of the antibody depletion experiments are such that if even IMP was only acting on 50% of the neurons then a clear difference between control and p14 depleted fractions would have been expected. However, if IMP was acting on a smaller proportion of neurons, e.g. only the ciliary neurons that project to the ciliary muscle, then a difference may not have been seen.

With regard to point (v) above (that bioactive IMP may be present in insufficient concentrations in the assays), since the estimated concentration of IMP in the bioassays as measured in the RIA (ca.10^{-10}-10^{-9}M) should easily be

large enough for growth factor activity, the major consideration is the possibility that only a proportion of the immunoassayable IMP is bioactive. Efforts were made to keep purification procedures mild and rapid, using protease inhibitors where possible, but loss of bioactivity did occur during various purification procedures, and this could have included loss of bioactive IMP. If IMP is bioactive then this activity is destroyed by low pH, as witnessed by the lack of activity of acidic muscle extract and IMP eluted from an antibody affinity column.

The heterogeneity of IMP in MCM [point (vi)] is another complicating factor: the bioactivity of the forms of IMP that eluted at different salt concentrations from the DEAE column or the forms of IMP that could or could not be affinity-depleted by antiserum p14 may be different. The most pessimistic, but plausible, scenario is that antiserum p14 only recognises denatured (inactive) IMP and so all affinity-depletion experiments with that antiserum are destined to yield no information about IMP bioactivity.

Finally, it is possible that the assay conditions are not adequate for the measurement of the biological activity of IMP [point (vii)]. In particular, it may be that IMP is only active in the presence of some other permissive influence, such as another soluble factor or a matrix-derived factor. Although the activity of other neurotrophic factors can be modified by the presence of other molecules (e.g. the effect of laminin on the activity of NGF, which has already been mentioned), no neurotrophic factor to date

is absolutely dependent on another factor for activity.

In conclusion, the present study has tested whether IMP acts as a survival factor for ciliary ganglion or spinal motor neurons. The bioassay systems I have described demonstrated clear neurotrophic activity that was _not_ due to IMP, and all attempts to show any survival activity of IMP have been unsuccessful. On this evidence, therefore, it seems unlikely that IMP is a survival factor for CG neurons or motoneurons.

CONCLUSIONS

What is IMP?

At present there is little knowledge about either the structure of IMP or the relationship between the different forms of IMP. At the amino acid level, the only data are from the specificities of the antisera which do or do not recognize material in muscle. As we have seen, the epitope on IMP recognized by antisera p14 and L25 is related to but not necessarily identical to VIP_{4-10}; the RIA data suggest that all forms of IMP may have the same epitope.

Further progress on the elucidation of the structure of IMP will require further purification of IMP and/or molecular cloning of the IMP gene(s). In fact, attempts have already been made to clone the IMP gene by a variety of different strategies based on the expression of IMP and its recognition by antisera p14 and L25 (I.Mason, pers.comm.). None of these attempts have been successful, however, the main reasons being the apparent very low abundance of IMP mRNA and the inability of the antisera to recognize IMP in all situations (e.g. antiserum p14 does not recognize IMP on a Western blot).

Cloning of the IMP gene will therefore require a different antibody or amino acid sequence data. The most promising route to the latter will be the purification of IMP using an antibody-affinity column.

What does IMP do?

Although, as discussed in the conclusion to chapter 4, the possibility that IMP is a neurotrophic factor still remains, the lack of data to support this hypothesis obviously means that the net should be cast wider in search of a function for IMP. The two biggest clues in this search are the restriction of IMP _in vivo_ to the primary myotubes and its secretion by myotubes. The former strongly suggests that IMP is not involved in any general aspect of muscle development, such as synaptogenesis, which is common to both primary and secondary myotubes, and the latter suggests that IMP acts extracellularly. One complication is that the myotubes that secrete IMP _in vitro_ form at a time equivalent to the development of secondary myotubes _in vivo_. So, either the myotubes that form _in vitro_ have more in common with primary than secondary myotubes, or else the production of IMP by secondary myotubes is under negative control _in vivo_.

If IMP is a cell-cell signalling molecule, then other obvious targets for IMP would be the primary and/or secondary myoblasts. IMP is present around the time of fusion of primary myoblasts. Fusion of myoblasts is very specific, i.e. primary myoblasts only fuse with primary myoblasts or myotubes, and so one possibility is that IMP could be involved in mediating this specificity by, for example, stimulating fusion of primary myoblasts and/or inhibiting fusion of secondary myoblasts to the myotube secreting IMP. The secondary myoblasts, which are in close

association with IMP-positive primary myotubes for many days, seem particularly good candidates as targets for IMP action. Apart from the possible inhibitory effect on fusion just mentioned, possible actions of IMP on these myoblasts include a chemotactic action, an inhibitory effect on mitosis or an effect on their state of determination or differentiation. There are clearly many possibilities for the function of IMP other than a neurotrophic one.

Progress in elucidating the function of IMP would be greatly aided by its purification and/or cloning. As well as enabling the production of pure IMP for studies of bioactivity, this would allow generation of further anti-IMP antisera, with a good chance of producing an antiserum that could neutralize the biological activity of IMP.

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APPENDIX: CALCULATION OF IMP CONCENTRATIONS

I am indebted to Ed Mroz, (Massachusetts Eye and Ear Hospital, Boston), for the following method.

Firstly, an equation was derived with the relevant parameters based on the law of mass action.

Abbreviations: Ab_0=total antibody concentration, H^*_0=total tracer concentration, G_0=total cold ligand (VIP or IMP), K^*=affinity constant of tracer, K_G=affinity constant of cold ligand, R^*=ratio of bound to free tracer, R_G=ratio of bound to free cold ligand.

R^* is given by:

$$R^* = \frac{[H^*Ab]}{[H^*]} = K^* \{ Ab_0 - [H^*Ab] - [GAb] \}$$

$$= K^* \{ Ab_0 - \frac{R^*}{1 + R^*} \cdot H^*_0 - \frac{R_G}{1 + R_G} \cdot G_0 \}$$

Substituting for $R_G = (K_G/K^*)R^*$:

$$R^* = K^* \{ Ab_0 - \frac{R^*}{1 + R^*} \cdot H^*_0 - \frac{R^*}{\frac{K^*}{K_G} + R^*} \cdot G_0 \} \qquad (1)$$

To apply this equation to the VIP radioimmunoassay, the following steps were taken:

(1) The range of Ab_0 and K^* for L25 which give a reasonable B_0 and 50% displacement value with VIP were calculated. This range was from $K^*=10^{12}M$, $Ab_0=2 \times 10^{-12}M$ to $K^*=6 \times 10^{10}M$, $Ab_0=10^{-11}M$.

(2) Over this range, the effect of changing the ratio K^*/K_G on the slope of the curve in the logit-log plot was

investigated empirically. The results were as follows:

K^*/K_G			0.1	0.2	1	10	100	1000
Slope	A	$K^*=10^{12}M$ $Ab_0=2\times10^{-12}M$	3.6	2.9	2.35	2.25	2.25	2.25
	B	$K^*=6\times10^{10}M$ $Ab_0=10^{-11}M$	3.7	3.3	2.55	2.40	2.40	2.40

Note that with increasing K^*/K_G the slope gets shallower and reaches a minimum. The actual slopes obtained in the RIA with IMP samples were consistently in the range 2.1-2.2 and so it is likely that the conditions in the RIA are close to condition A (the minimum slope possible in condition B is 2.4), and that the affinity of L25 for IMP is at least 10 times lower than its affinity for hot VIP. The slope for cold VIP obtained in the RIA was 2.8, and so, assuming that the RIA is close to condition A, this means that the affinity of L25 for cold VIP is approximately 5 times that for radiolabelled VIP. Thus it is very likely that the affinity of L25 for cold VIP is at least 10 times its affinity for IMP.

Having estimated a K^*/K_G for IMP, equation (1) can then be used to calculate molar IMP concentrations from a standard curve obtained with a sample of IMP. The value obtained will represent a minimum.