DEVELOPMENTAL PROPERTIES OF MOUSE MUSCLE SATELLITE CELLS

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Satellite cell populations were isolated from the hind limb muscles of neonatal, rapidly growing, and young adult mice and allowed to proliferate in vitro. Satellite cell progeny from all three ages of mice fused to form multinucleated myotubes that underwent spontaneous contraction. Striations were evident on fragmented myofibrils prepared from these cultures. Demonstration of contraction indicated that myofibrillar proteins which are involved in the contractile mechanism must have been synthesized and assembled by satellite cell progeny.

As the extent of this myogenic cell population in muscle may be a factor in determining the potential for muscle accretion, an attempt was made to determine the concentration of these myogenic cells in postnatal muscle. Clonal analysis revealed that the concentration of myogenic cells declined with age; however, these same experiments suggested that satellite cells from mice of increasing age required less of a conditioned medium effect for fusion.

To determine if the rates of myofibrillar protein synthesis and total protein synthesis were influenced by the age of mice from which satellite

cells were isolated, cultures were labeled with ^{3}H-leucine. The radioactivity in slices of the myosin heavy chain band (200,000 daltons) on (SDS)-polyacrylamide gels was measured. An inhibitor of cell replication Ara-C (1-β-D-arabinosylcytosine) was administered to cultures to provide a model in which protein synthesis of myogenic nuclei could be more accurately assessed. During postnatal growth the decrease in concentration of satellite cells in hind limb muscles was accompanied by a slight decline in the protein synthetic capacity of these same myogenic cells in vitro.

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By

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DEDICATION

To my grandparents, Mr. and Mrs. William D. Miller, whose love, enthusiasm, determination, and appreciation for life served as an endless source of encouragement.

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LIST OF ABBREVIATIONS

Ach - acetylcholine

AchE - acetylcholinesterase

α-BGT - alpha-bungarotoxin

Ara-C - 1-β-D-arabinosylcytosine

ATP - adenosine 5'-triphosphate

BSS - buffered saline solution

BrdUrd - 5-bromodeoxyuridine

C^{o} - degrees celsius

CB - cytochalasin B

Cb - chondroblast

cm - centimeters

CPK - creatine phosphokinase

cpm - counts per minute

DNA - deoxyribonucleic acid

dpm - disintegrations per minute

EDTA - ethylenediaminetetraacetic acid

EE - embryo extract

EGTA - ethylene glycol-bis-(β-amino ethyl ether)N,N-tetraacetic acid

EMEM - Eagle's Minimum Essential Medium

Fb - fibroblast

FdUrd - flurodexyuridine

g - acceleration due to gravity

g - gram

G_1 - post-mitotic phase of the cell cycle

G_2 - phase between DNA synthesis and start of mitosis

^3H - tritium

HnRNA - heterogeneous nuclear RNA

hr - hour(s)

M - molar

M - mitosis

Mb - myoblast

MCF - myogenic colony forming

mg - milligram

MHC - myosin heavy chain

min - minute(s)

ml - milliliter

MLC - myosin light chain

mM - millimolar

mm - millimeter

mRNA - messenger RNA

Ms - mesenchyme

N - normality

nm - nanometer

~P - high energy phosphate

PPCbFb - presumptive chondroblast-fibroblast

PPMbFb - presumptive myoblast-fibroblast

PMb - presumptive myoblast

RNA - ribonucleic acid

rRNA - ribosomal RNA

S - DNA synthesizing phase of the cell cycle

SDS - sodium dodecyl sulfate

sec - second(s)

TCA - trichloroacetic acid

Tris - tris-(hydroxymethyl)-aminomethane

T-tubule - transverse tubule

uCi - microcurie

ug - microgram

ul - microliter

um - micrometer

I. INTRODUCTION

The means by which muscle tissue controls the qualitative and quantitative synthesis of the myofibrillar proteins is of primary importance in understanding the process of muscle accretion. Embryonic muscle growth is the result of the fusion of mononucleated myoblasts into multinucleated myotubes, followed by the bulk synthesis of the muscle proteins. During postnatal muscle growth there is an increase in the nucleic acid content of muscle even though myonuclei are incapable of mitotic activity. This increase can be attributed to satellite cells. The goal of this research project, therefore, was to study the developmental properties of the muscle satellite cell population. The results of this study, when analyzed with the results of previous studies by other investigators, show that satellite cells proliferate *in vitro* and are capable of myogenesis and myofibrillar protein synthesis. Therefore, satellite cells are similar to embryonic presumptive myoblasts and *in vivo* their progeny are capable of fusing with the differentiated myofiber resulting in the increase in myonuclei during muscle growth.

II. LITERATURE REVIEW

A. The Fetal Development of Striated Muscle

1. Determination of the Myogenic Cell Lineage During Embryonic Muscle Differentiation

Skeletal muscle is derived from the lateral plate and paraxial mesoderm of the embryo (Fischman, 1972). Although the precise derivation of the more proximal muscles of the main body mass is a controversial matter, limb muscle differentiates from the mesenchyme of the lateral plate (somatopleuric) mesoderm (Fischman, 1972). The primitive mesenchyme cells give rise to early compartments of cells ancestral to the chondrogenic, fibrogenic, and myogenic lineages (Abbot et al., 1974; Dienstman and Holtzer, 1975). Abbot et al. (1974) have proposed a model whereby mesenchymal cells pass through a series of binary divisions and as a result differentiate into distinct chondrogenic (Cb), fibrogenic (Fb), and myogenic (Mb) cell lineages. Eight day old chick embryo limb buds contain single, common precursor cells of both the myogenic and fibrogenic lineages. In vitro, therefore, the cells of this compartment (termed PPMbFb) possess a binary metabolic option (Holtzer and Bischoff, 1970; Abbot et al., 1974; Holtzer et al., 1975b). Cells of the PPMbFb compartment fail to yield chondrogenic clones as evidenced by lack of synthesis of metachromatic chondrotin sulfate or type II collagen chains (Dienstman and Holtzer, 1975). A cell in the Ms compartment may not yield a terminal myoblast, chondroblast, and fibroblast within one generation. Its options are to

yield a PPMbFb or a PPCbFb (progenitor cell of the chondrogenic and fibrogenic lineage) upon its first bifurcation from the Ms lineage (Dienstman and Holtzer, 1975).

Several investigators (Yaffe, 1968; Holtzer et al., 1975b; Dienstman et al., 1974) have stated that differentiation potential is maintained within the stem cell of a lineage and that diversification is determined by action of an endogenous, predetermined gene program rather than by exogenous molecules of the microenvironment. Diversification may also be independent of neural control (Fischman, 1972; Holtzer and Bischoff, 1970). Therefore, stem cell differentiation must involve a quantal mitosis which permits transcription of regions of the genome in the daughter cells that are not available in the stem cell. This could provide metabolic options in the daughter cell very different from those of the mother cell (Holtzer et al., 1973b). In order for precursor myogenic cells not to be depleted there must be progenitor cells capable of proliferative mitotic activity (Holtzer et al., 1975b). These cells enter the terminal compartments of their respective lineages at a rate corresponding to the demands of fetal muscle growth. Consistent with this theory are the findings of Dienstman et al. (1974) that the percentage of terminally differentiated cells increases in embryos of increasing age and fetal development. At all times, however, a compartment of cells ancestral to both the myogenic and fibrogenic lineages is present, even though the proportion of these precursor cells is very small.

Quantal mitotic activity results in two daughter nuclei of which one or both differ phenotypically from the mother cell. A daughter nucleus which differs can further differentiate and enter a subsequent compartment within the lineage (Holtzer et al., 1975b). There is circumstantial evidence

for a compartment of cells in muscle committed exclusively to embryonic myogenesis. These myogenic progeny are capable of both proliferative and quantal mitotic activity and are termed presumptive myoblasts (PMb). PMb are morphologically indistinguishable from other mesenchymal cells of the mesoderm. The cells resulting from a quantal mitosis of PMb are called myoblasts (Mb) and are the first morphologically distinguishable myogenic cells. Myoblasts (Mb) are mononucleated, spindle-shaped cells which can usually be distinguished from flattened, polygonal undifferentiated mesenchyme cells (Fischman, 1972; Holtzer et al., 1975b). Other investigators (Abbot et al., 1974) have noted, however, that both myogenic and nonmyogenic cells under certain conditions may appear spindle shaped, and Hauschka (1974a) has found flattened polygonal human myoblasts. Myoblasts contain an oval nucleus that will display one or more small nucleoli. These cells have an abundance of cytoplasm containing large spherical mitochondria, numerous polyribosomes, and some glycogen granules (Reznik, 1970).

Studies on myosin and actin synthesis and assembly suggest that replicating PMb differ biochemically from their daughter, postmitotic Mb (Holtzer et al., 1957; Okazaki and Holtzer, 1965). If PMb synthesize actin and myosin, they are not assembled into 5-7 nm (actin) or 15 nm (myosin) filaments, nor are they organized into hexagonal arrays (Bischoff and Holtzer, 1970; Stromer et al., 1974). Holtzer (1961, 1970) and Chi et al. (1975a) have shown that PMb myosin differs immunologically from the myosin of myoblasts and myotubes, and that PMb myosin is similar to that of nonmyogenic cells. Chi et al. (1975a) using fluorescein labeled antibodies to light meromyosin have also shown that myosin light chains (MLC) differ between replicating PMb and postmitotic Mb. In addition, PMb fail to

bind fluorescein labeled antibodies to skeletal muscle tropomyosin, whereas Mb will display this ability (Holtzer et al., 1973b). The molecular weights of MLC from Mb are indistinguishable from those of differentiated skeletal muscle myosin, and the molecular weights of MLC from PMb resemble those of MLC isolated from nonmyogenic cells (Chi et al., 1975a). This suggests that the structural gene for myosin in the PMb differs from the myosin structural gene in the Mb (Holtzer et al., 1957, 1973b, 1975b). The myosin synthesized by the PMb may be constitutive myosin which is common to all cell types (Chi et al., 1975a), and the switch in the types of myosin synthesized during myogenesis is consistent with the quantal mitotic theory of cell differentiation. Data on actin synthesis (Storti et al., 1976; Elzinga et al., 1976) and T-tubules (Ishikawa, Bischoff, and Holtzer, 1968) are also consistent with the notion that Mb have an ongoing synthesis program distinct from that of PMb.

Myoblasts are the terminal cell in the myogenic lineage, are incapable of mitotic activity, and are able to assemble the newly synthesized contractile proteins into hexagonal arrays (Bischoff and Holtzer, 1969; Fischman, 1967, 1972; Holtzer and Bischoff, 1970; Ishikawa, Bischoff and Holtzer, 1968). While these cells do not normally enter the S-phase of the cell cycle, alteration of the _in vitro_ environment of the myoblasts may in some instances induce them to undergo new mitotic activity (O'Neill and Stockdale, 1972; Doering and Fischman, 1974). O'Neill and Stockdale (1972), for example, demonstrated that a lower percentage of cells plated at low density would fuse without dividing than when cells from the same inoculum were plated at a higher density. In addition, Coleman and Coleman (1968) found that 33% of the cells in 11 day chicken embryo skeletal muscle were dividing, whereas 90% of these cells would divide in culture. These data

can be explained by environmentally induced stimulation of Mb replication. Cessation of mitotic activity cannot be used to distinguish myoblasts from presumptive myoblasts unless myoblasts have fused to form multinucleated syncitia (Stromer et al., 1974) or unless they contain thick and thin filaments in hexagonal arrays. Positive identification of terminal myoblasts can only be made, therefore, at the electron microscope level.

2. Expression of Muscle Differentiation Through Changes in Morphology and Ultrastructure

Analysis of myogenesis in embryonic growth is complicated by its asynchronous situation (Fischman, 1972). Therefore, the literature presented in the following section will include both in vivo and in vitro investigations.

Myogenesis in vivo is first marked by the establishment of myogenic cell lineages during the gastrula stage. A sequence of obligatory cell divisions follow, which lead to PMb and finally the terminal myogenic cell, the myoblast (Holtzer, 1970). One distinctive difference occurs at this point between in vivo and cell cultures of embryonic skeletal muscle. A preproliferation period occurs during in vitro studies. This period is marked by a lag in DNA polymerase activity and little cell replication (Stockdale and O'Neill, 1972). The length of the preproliferation period is probably subject to such variables as composition of the substrate coating the plate surface, cell viability after isolation, cell attachment, composition of the nutritional medium, and interaction of the cell membrane with its environment (Stockdale and O'Neill, 1972; Nameroff and Holtzer, 1969). Following proliferation and formation of myoblasts, fusion of myoblasts to form multinucleated myotubes occurs. Subsequent to fusion there is a period of bulk synthesis of the contractile proteins. This is

followed by assembly of synthesized proteins into myofilaments and organization into definitive sarcomeres displaying M, H, and Z bands (Holtzer, 1970; Fischman, 1967, 1972).

The myogenic cell populations isolated from the early embryo also exemplify an asynchronous situation. The cell populations isolated from 3 day embryos form very few muscle colonies in vitro (Bonner and Hauschka, 1974). As cells from embryos of increasing age (3, 6 and 8 days) were isolated, the percentage of muscle forming clones also increased. Embryos of these 3 ages contain cell populations which yield 10%, 60% and 90% myogenic clones, respectively. These findings are supported by those of White et al. (1975) and Dienstman et al. (1974) who found that cultures from embryos of increasing ages yielded increased percentages of cell populations capable of terminal myogenic differentiation. Therefore, the extent of in vitro myogenesis varies not only with the cell's micro- and macro-environment, but also with the developmental stage of the embryo.

During proliferation cells enter the mitotic cycle which consists of four distinct phases. The first phase is the gap phase (G_1) where synthesis of the machinery necessary for the mechanics of cell replication occurs. The second phase (S) is where the cell's compliment of DNA is synthesized. The third phase (G_2) is where RNA and protein synthesis again occur and the fourth phase is where actual cell mitosis occurs (M) (Holtzer and Bischoff, 1970). The duration of each of the phases for replicating myogenic cells have been determined by various investigators and are as follows: S = 4.1-4.3 hr, G_1 = 2-3 hr, G_2 = 2.5 hr, and M = 0.8 hr (Bischoff and Holtzer, 1969, 1970; Lough and Bischoff, 1976).

Okazaki and Holtzer (1966) have established that a myoblast must be in G_1 for fusion to occur. These investigators exposed cultures to

^{3}H-thymidine for 4 hr. Since the longest portion of the cell cycle is the S phase, a majority of the cells incorporated ^{3}H-thymidine. At the end of 4 hr no labeled nuclei were found (Okazaki and Holtzer, 1966). Therefore, cells in the S, G_2, and M phases cannot fuse.

Okazaki and Holtzer (1966) labeled cells for 0.5 hr with ^{3}H-thymidine and then observed fixed cultures by microscopy and autoradiography at various time intervals. These investigators discovered that a minimum of 5-8 hr elapse between the end of mitosis and the completion of fusion. This finding is also in accordance with that of Doering and Fischman (1974) and Lough and Bischoff (1976). Slater (1975) using cultures of 10-12 day old chick embryos and ^{3}H-thymidine found that 50% of the cell population completed DNA synthesis by 31.5-35 hr after plating, and 50% of the cell population fused by 43-48 hr. This suggests a post-mitotic phase of approximately 11.5 hr in duration. At the opposite extreme Marchok and Herrmann (1967) found the post-synthetic DNA period possibly exceeds one week in the embryo. As post-mitotic myoblasts withdraw from the cell cycle both in culture and in vivo, there is an increase in the average length of G_1 for a particular culture population (Konigsberg, 1971), and there is a greater distribution of G_1 times with increasing developmental age (Konigsberg, 1971; Marchok and Herrmann, 1967). Marchok and Herrmann, for example, found the G_1 period increased from an average of 3.9 hr to 8.9 hr during in vitro studies of chicken embryos between 9-16 days of age.

Bischoff and Holtzer (1969) have proposed that myogenic cells committed to fusion arise from a quantal mitosis. This mitosis is a critical mitosis yielding daughter cells with a pattern of synthetic activities different from that of the mother cell (Ishikawa, Bischoff and Holtzer, 1968). Under certain environmental conditions postmitotic cells arrested in G_1

appear capable of re-entering the cell cycle (O'Neill and Stockdale, 1972).

These findings contradict those of Dienstman and Holtzer (1975) who cultured cells in the presence of EGTA which blocks fusion yet allows cells to undergo mitotic activity and enter G_1. After 4 days the EGTA containing medium was replaced with ^{3}H-thymidine containing medium. In another 4 days autoradiographs revealed over 95% of the myonuclei within multinucleated syncitia unlabeled. Therefore, the number of cell cycles early myogenic cells undergo may be defined by environmental restraints or by intrinsic factors (Stockdale and O'Neill, 1972). These events dictate the composition of cell populations responsible for myogenesis (Holtzer and Bischoff, 1970); consequently, much emphasis should be focused on understanding these processes.

Mitotic activity of precursor cells is obligatory for maximum muscle differentiation in vitro (Nameroff and Holtzer, 1969; Yaffe, 1971; Bischoff and Holtzer, 1970). Supportive to this theory are investigations showing that the mitotic inhibitor, cytosine arabinoside chloride, will prevent the quantal mitosis between the PMb compartment and Mb compartment. Conditioned medium will not promote fusion in these AraC-treated cultures. Even though the in vitro or in vivo microenvironment may promote replication within a compartment (or permit terminal differentiation from one compartment to the other), it is apparent that some DNA synthesis is required for fusion (Bischoff and Holtzer, 1970). Supportive to this notion is the fact that 5-bromodeoxyuridine, which is incorporated into DNA, forces cells to stay in the cell cycle and prevents them from undergoing a final quantal mitosis (Bischoff and Holtzer, 1970; Lough and Bischoff, 1976; Turner et al., 1976). The BrdUrd sensitive target is replicated very early during the S phase (Lough and Bischoff, 1976; O'Neill and

Stockdale, 1974); yet after 6 weeks in BrdUrd, cells still differentiate when placed in fresh medium (Abbot et al., 1974). Therefore, the ability to differentiate and enter a quantal cell cycle is maintained as stored information.

Dienstman and Holtzer (1975) have postulated two schemes of which one or both may result in a quantal cell cycle. At the level of DNA replication, a regulator gene at a particular locus (which is associated with the program of synthesis for the next compartment) may be altered. The second scheme involves individual programming of a cell by a trivial or specific cytoplasmic factor. Here a nucleus would respond to an inducer, either during the cell cycle or in a particular compartment of a lineage. Either scheme is related to specific cell cycle events. The activation of one program in a compartment and the cessation of another in a previous compartment is subject to nuclear-cytoplasmic interaction (Dienstman and Holtzer, 1975; Holtzer et al., 1973b).

Mitotic figures have never been found within the sarcolemma of the skeletal muscle cell--the myofiber. Nuclei of cells containing striations do not incorporate ^{3}H-thymidine into their DNA (Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; Bischoff and Holtzer, 1968). When cell populations from embryos of increasing age are cloned *in vitro* the percent fusion in the muscle clones increases (Bonner and Hauschka, 1974). *In vivo* fusion of post-mitotic myoblasts starts after 4.5-5.0 days and continues after birth (Hilfer et al., 1973). The extent of myotube formation in embryonic muscle is determined by the proportion of replicating PMb and post-mitotic Mb. Post-mitotic myoblasts exist in the embryo (Doering and Fischman, 1974; Bischoff and Holtzer, 1969; Slater, 1975). The percentage of nuclei synthesizing DNA decreases with increasing

age of the embryo. For example, Marchok and Herrmann (1967) showed that the percentage of replicating cells was 69% in the 7 day embryo, 15% in the 18 day embryo and 3% for embryos 8 days post-hatching. The percentage of cells synthesizing DNA was 38% in the 7 day, 21% in the 9 day, 5% in the 18 day embryos and 1% in leg muscle from 5 day old chicks. Therefore, in the 9 day embryo there is a large portion of post-mitotic myoblasts not yet incorporated into myotubes. In the day nine to eighteen embryo, the percentage of nuclei in myotubes increases dramatically. This is a result of myoblast fusion. The myogenic potential of a post-mitotic population is exemplified by the results of Slater (1975). Embryonic cultures were exposed to ^{3}H-thymidine with 95% of the unlabeled nuclei fusing without division; whereas, the maximum percent fusion for nuclei which replicated was only 79%. Therefore, the fusion potential of post-mitotic myoblasts in vivo was greater than that of post-mitotic myoblasts which entered the Mb compartment in vitro. This lower percentage was probably due to cells which had remained in the penultimate, PMb compartment and whose progeny would fuse at a later time in culture (Bischoff and Holtzer, 1969). Hauschka (1974a) has found the percentage of myogenic colony forming (MCF) cells in human embryo muscle to be 14% at the 36th day of development, increasing to 90% by the 100th day. This level is maintained until the 172nd day of development. A level of 80-90% MCF cells may exist in a 78 year old man (Hauschka, 1974b); consequently, attention should be given to possible influences upon the stability of the Mb compartment in the myogenic lineage.

Muscle differentiation is characterized by repression of DNA synthesis and gene expression (Vertel and Fischman, 1976). By examining the ultrastructure of post-mitotic, mononucleated myoblasts, assembly of the

contractile proteins can be observed. Numerous investigators have found closely associated thick and thin filaments in the cytoplasm of post-mitotic myoblasts (Holtzer et al., 1975a; Fambrough and Rash, 1972; Okazaki and Holtzer, 1966; Holtzer et al., 1975b; Fischman, 1972). The proteins of interdigitating thick and thin filaments contained in fusion inhibited Mb contain both myosin heavy and light chains (Rubenstein et al., 1976; Chi et al., 1975a). Therefore, it has been suggested that myosin is not made throughout the cytoplasm but in proximity to the site of incorporation into the myofibril, with actin, myosin, and tropomyosin synthesis coupled in the terminal myoblast (Holtzer et al., 1975b). Whether the myofibrils of post-mitotic myoblasts are carried over intact into multinucleated myotubes or whether the fusion process requires breakdown of the pre-existing filaments remains unanswered (Trotter and Nameroff, 1976). Also, sarcoplasmic reticulum (Trotter and Nameroff, 1976) and T-tubule formation (Ishikawa, Bischoff and Holtzer, 1968) have been found in cells of the Mb compartment. Therefore, quantitative instead of qualitative changes accompany terminal muscle differentiation in vivo or in vitro, since muscle protein synthesis, polymerization, and assembly may occur even in the post-mitotic myoblast (Schubert et al., 1973; Holtzer et al., 1975a).

The first event which is ultrastructurally detectable and unique to myogenesis is the fusion of the post-mitotic myoblasts (Stromer et al., 1974; Fischman, 1972). Terminally differentiated myoblasts line up in an end-to-end fashion so their membranes touch and fuse. Fusion leads to the formation of multinucleated, skeletal muscle fibers (Holtzer et al., 1957; Okazaki and Holtzer, 1966). The uniqueness of cell fusion to myogenesis was shown by pulse-labeling liver, kidney, and cardiac cells and adding these nonmyogenic cells to unlabeled myogenic cells in the process

of fusing. These nonmyogenic cells were excluded from the multinucleated syncitia (Okazaki and Holtzer, 1965). Fusion has been studied closely so as to discover the sequence of morphological, behavioral, and biochemical events which occur. The exclusion of nonmyogenic cells indicates a recognition system, operating between surfaces of cells with the ability to fuse (Holtzer, 1970). The recognition of committed myoblasts may be the first required step in a timetable of cell fusion events.

After alignment the surface membranes of myoblasts gradually fuse and disappear, with the confluence of the two cytoplasms following immediately (Fischman, 1972; Stromer et al., 1974). Myoblasts may fuse with other myoblasts or myotubes and myotubes may fuse with each other (Holtzer, 1970; Okazaki and Holtzer, 1965, 1966; Fischman, 1972). Yet, Bischoff and Holtzer (1970) have demonstrated that labeled nuclei will not be incorporated into myotubes older than 4 days in vitro. The failure of myoblasts to become incorporated into older myotubes creates the possibility that the block to fusion resides either in the myotube membrane itself (plasmalemma) or in some extracellular deposit (Bischoff and Holtzer, 1969). Investigators have discovered in adult skeletal muscle a 30 nm amorphus layer separated from the plasma membrane by a 10 nm gap (Muir et al., 1965). This membrane is the basement lamina and can be detected in myotubes at about 4 days in culture (Bischoff and Holtzer, 1970). During embryogenesis in vivo, myoblast fusion starts after 4.5-5 days and continues until after birth (Bonner and Hauschka, 1974). Abbot et al. (1974) have demonstrated that myotubes synthesize glycosaminoglycans and collagen-like chains in cultures where cytosine arabinoside was used to kill replicating mononucleated fibroblasts, and the collagen synthesized by embryonic chick skeletal muscle was not different than the type I

collagen synthesized by fibrogenic cells (Ketley et al., 1976; Mayne et al., 1972). It is not known if this type collagen is used to construct the filamentous material of the basement lamina (Abbot et al., 1974). Yet, Nameroff and Holtzer (1969) have demonstrated that interstitial matrices interfere with the movement of muscle precursor cells and differentiation.

The second step in fusion involves changes in the continuous cell membrane of the newly formed myotube. As cells fuse, the myotube elongates. Holtzer (1970) stated that if fusion solely involves cell surface interactions, the resulting multinucleated syncitia would be round sacs. Bischoff and Holtzer (1968) demonstrated that colchicine, an antimitotic drug, would cause fragmentation of elongated myotubes with the cytoplasmic (sarcoplasmic) bridges being pulled apart and the myonuclei scattered throughout the cytoplasm within a myosac. Subsequent work of de la Haba and Amundsen (1972) provided evidence for the separation of cell fusion and myotube formation. A filtrable factor was separated from the embryo extract component of culture medium and found to initiate myotube elongation. Without this factor only myosacs are formed; elongation to form myotubes did not occur. These findings support the idea that fusion is a two-step process.

In another approach toward separating the fusion process into identifiable steps, Turner et al. (1976) found that when EGTA-treated cultures were switched to BrdUrd containing medium, they fused rapidly. When these inhibitors were used in the reverse order, fusion did not occur. Therefore, at least two sensitive, sequential processes associated with cell fusion exist--one BrdUrd sensitive and the other EGTA sensitive (Turner et al., 1976). Holtzer and Bischoff (1968) added colchicine (which arrests cells in metaphase) to labeled cells 6 hr after the cells

incorporated ^{3}H-thymidine and witnessed the formation of myosacs. When the medium was replaced with fresh medium and labeled mononucleated cells were added, the myosacs elongated; yet, new nuclei did not fuse with myotubes. Therefore, it appears that colchicine alters cell surface properties (Holtzer and Bischoff, 1968). Colchicine and colcemid may fragment myotubes by breaking down microtubules (Ishikawa, Bischoff and Holtzer, 1968) which may be involved in myotube elongation.

The properties of the medium surrounding myogenic cell cultures change with the age of the culture and play a role in mediating the end of cell proliferation. Numerous investigators have studied the effects of "conditioned" medium upon cellular differentiation, especially fusion. It is apparent that the conditioning of nutritional medium is a result of metabolic processing caused by cellular functions (Holtzer et al., 1975b; Doering and Fischman, 1974). The metabolic processing of medium may involve the release of a specific cell metabolite(s) into the medium. This effect may be dependent upon the diffusion and final concentration of the metabolite in the medium (Konigsberg, 1971). White et al. (1975) demonstrated that this factor is capable of attaching to a substrate coating, as a culture plate covered with conditioned medium, which was then poured off, still displayed an increased percent cell fusion. This factor is neither cell nor species specific (Slater, 1975; Hauschka, 1974a). The cell metabolite(s) in conditioned medium may affect cell survival, replication or migration (Holtzer et al., 1975b), but will not induce the onset of fusion (Holtzer et al., 1975b; White et al., 1975). Present evidence supports the possibility that the factors in conditioned medium depress cell proliferation (Konigsberg et al., 1971; Hauschka, 1974a). Yet, whether these factors are division inhibitors or antagonists of fusion

inhibitors remains to be answered. White et al. (1975) have revealed that conditioned medium effects are involved with differential growth rates of myogenic cells isolated from early embryos. Myoblasts from 3-4 day embryos required conditioned medium for fusion in vitro, whereas, cells from day 5 embryos would fuse in fresh medium (same investigators). Isolation of the factors from conditioned medium could provide for a means of control over cellular differentiation and have great implications upon the control of muscle growth and development.

3. Bulk Synthesis of the Contractile Proteins and Myofibrillogenesis

As the completion of membrane fusion approaches, early myotubes initiate other facets of muscle differentiation. The fused nuclei occupy the central axis of the cytoplasm of the newly-formed myotube and are similar to the nuclei of the original myoblast (Mauro et al., 1970). Almost immediately after fusion, the bulk synthesis of the contractile proteins begins. Ishikawa, Bischoff and Holtzer (1968) state that 5-7 nm (thin) and 15 nm (thick) filaments appear simultaneously, although scattered in the myotube. The thick and thin myofilaments align with the long axis of the myotube in a subsarcolemmal position (Fischman, 1967). At the periphery, these newly-formed thick and thin filaments self-assemble to form hexagonal myofibrillar arrays which become the initiating structures of developing myofibrils (Fischman, 1967, 1972). The regularity of packing is least perfect at the outermost layer of filaments in each myofibril (Fischman, 1967). In mature myofibrils three transverse structures exist which might position the myofilaments in hexagonal arrays (Fischman, 1967). These are the Z disc or Z band, the M band cross-bridges between thick filaments and the cross-bridges between the thick and thin filaments in the A band-I band

overlap (Fischman, 1967). Fischman (1972, 1967) states that Z-band development does not necessarily precede the formation of other areas of the sarcomere. The Z-bands do not appear until filaments appear in individual arrays (Fischman, 1972). Holtzer (1970) mentions that H bands are not evident in early myofibrils as they depend on the formation of the Z band.

The hexagonal arrays that are first formed at the periphery of the myotube serve as nucleation sites for further assembly of filaments (Holtzer et al., 1973b). The development of myofibrils is asynchronous from one myotube to another and varies within the same myotube (Mauro et al., 1970). With development, mitochondrial proliferation occurs along with the deposition of glycogen (Fischman, 1972), and additional myofibrils are formed until the entire interior of the myotube is filled, leaving only space for the myonuclei in the center (Fischman, 1967; Holtzer et al., 1973). Then the nuclei migrate to the periphery and take up a subsarcolemmal position, with the myofibrils becoming more organized and evenly distributed in the sarcoplasm (Stromer et al., 1974). With the myonuclei at the periphery, the differentiated myotube is now a skeletal muscle cell, the multinucleated myofiber (Fischman, 1972).

4. Changes in Muscle Enzyme Levels in Differentiating Muscle Cells

Myogenic differentiation includes the initiation of synthesis of required enzymes. These enzymes provide sufficient amounts of ATP for the contractile mechanism. The glycolytic pathway is one of the metabolic pathways which produces energy, in the form of ATP, for muscle contraction. There is evidence for the presence and activity of glycolytic enzymes, and enzymes involved with the synthesis of creatine phosphate

(the energy reserve of muscle) in mononucleated, myogenic cells. Wahrmann et al. (1976) demonstrated that blocking myogenic cells from fusion with cytochalasin B did not effect the expression of phosphorylase, phosphorylase kinase or glycogen synthetase. It has been demonstrated with the use of EGTA that the accumulation of fructose diphosphate aldolase activity indicates that increases of terminal muscle differentiation begin prior to fusion (Turner et al., 1976). Also, increases in the specific activity of adenylate kinase are independent of cell fusion (Tarikas et al., 1974). Another investigation reveals that myokinase activity is as high in low Ca^{2+} concentration-fusion-inhibited cells as in control cultures (Adams et al., 1976). The adenylate cyclase and acid phosphatase activity levels are higher in the plasma membranes of replicating myoblasts than in fused cultures (Winand et al., 1975). Prives and Paterson (1974) found adenylate cyclase activity to occur at increasing levels in fusion arrested myoblasts. Therefore, the levels of glycolytic and glycolytic associated enzymes in vitro, indicates that the increases characteristic of terminal muscle differentiation begin prior to the onset of fusion.

Creatine phosphate, synthesized by creatine phosphokinase (CPK), is the storage form of ~P in muscle. In vitro investigations have shown CPK activity prior to the onset of fusion (Turner et al., 1976; Wahrmann et al., 1976; Morris et al., 1976), yet CPK accumulation is inhibited by conditions which favor rapid cell division (Morris et al., 1976). Therefore, once PMb enter a final quantal mitosis and become fusion capable Mb, the specific activity of CPK can be as high as in fusing cultures (Adams et al., 1976; Tarikas et al., 1974; Morris et al., 1976). Although the fusion of myoblasts is accompanied by an increase in CPK accumulation (Gearheart and Mintz, 1975; Rogers et al., 1975; Turner et al., 1976),

fusion is not required for the presence of CPK activity.

Thus, based upon studies concerning enzymes, which play a key role in supplying the necessary energy for muscle contraction, cell fusion is not a prerequisite for the expression of myogenic differentiation.

5. Membrane Structure and Function During Differentiation

The action potential which causes myofibrils to contract is transfered from the nerve fiber to the myofiber at the myoneural junction. Here the motor nerve terminates into several branches at invaginations of the sarcolemma. These terminal endings which adhere tightly to the membrane of the myofiber are called the motor end plate. As the action potential arrives at the end plate, a chemical messenger, acetylcholine, is released. Acetylcholine causes the sarcolemma to become more permeable to Na^+ and K^+ ions and its polarity reverses with a potential being propagated along its length. Since acetylcholinesterase activity (which destroys acetylcholine) and acetylcholine sensitivity are native to muscle, they have been used as a distinguishing characteristic of muscle differentiation (Fambrough and Rash, 1971; Tennyson et al., 1973).

All myotubes exhibit ACh sensitivity (Fambrough and Rash, 1971); yet, small bipolar cells do not exhibit detectable responses to applied ACh, whereas, elongated mononucleated cells with myofilaments are sensitive to ACh. Fambrough and Rash (1971) investigated if ACh receptors might play a role in cell recognition or fusion. When they blocked the ACh receptors with tubocurare or excess ACh, no interference with myogenesis was noted. Therefore, these mechanisms are not obligatorily associated with cell fusion (Fambrough and Rash, 1971).

Hartzell and Fambrough (1973), utilizing labeled α-bungarotoxin

(α-BGT), which binds irreversibly to ACh receptors, found that ACh receptor density increases more slowly after fusion than the incorporation of new receptors into the membrane. This occurs due to the increase in surface area of the myotube.

Acetylcholine receptors are not incorporated into the cell membrane immediately after synthesis, since the intracellular pool of receptors (Teng and Fiszman, 1976) can supply the cell surface with new receptors for about 2-3 hr in the absence of protein synthesis (presence of cyclohexamide) (Devreotes and Fambrough, 1976). Also, the inhibition of ATP synthesis with dinitrophenol suppresses the appearance of new receptor sites suggesting that ATP is required for their incorporation into the cell membrane (Hartzell and Fambrough, 1973).

Acetylcholinesterase activity has been studied in vitro and in vivo, both biochemically and cytochemically. AChE activity is highest during myotube formation (Tennyson et al., 1973; Fluck and Strohman, 1973; Gearhart and Mintz, 1975); yet, Fluck and Strohman (1973) found AChE activity in PMb and Mb cells. AChE activity was associated predominantly with cells arrested in the G_1 phase of the cell cycle. Acetylcholinesterase activity is restricted to myogenic cells since fibroblastic cells always show a negative response when tested for AChE.

6. Myosin Synthesis in Differentiating Muscle Cells

Myofibrils constitute over 50% of the protein in mature skeletal muscle. The most prevalent structural protein of the myofibril is myosin. Although the chemistry, morphology, and functional properties of myosin have been known for some time, only recently have investigators begun to study its synthesis in differentiating muscle. The presence of skeletal

muscle myosin coincidental with changes in myogenic cell ultrastructure has been used as evidence for skeletal muscle differentiation. Myosin is synthesized on polyribosomal clusters assembled on the messenger RNA molecule coding for the heavy subunit of myosin. The relative ease by which polysomes synthesizing myosin heavy chain (200,000 dalton subunit) can be isolated, and the presence of myosin as 55% of the myofibrillar proteins, has led investigators to study it in detail.

The synthesis of myosin is not conclusive evidence that a particular cell is myogenic, as myosin has been found in fibroblasts, chondrocytes and other nonmyogenic cells (Adelstein et al., 1972). This myosin is believed to be constitutive myosin synthesized by all cell types, and may participate in cell movement, mitotic activity, or changes in cell shape (Shizuta, Davies, Olden, and Pastan, 1976). Chi et al. (1975b) have discovered the presence of 3 different light chains (LC) for myosin. Pure myotube cultures yielded myosin light chains one (LC_1, 25,000 daltons) and two (LC_2, 18,000 daltons) in approximately equal ratios. Replicating fibroblast cultures yield a trace of LC_1, along with LC_2, and LC_3 (16,000 daltons). The presence of LC_3 appears dispensible for the structure and function of the myosin filament since pure myotube cultures exhibited contraction. Standard muscle cultures yielded LC_1, LC_2, and LC_3 in the ratio of 1.0:1.9:0.5, respectively, with LC_3 being contributed by contaminating fibroblasts. Therefore, the light chains of myoblasts, myotubes, and mature muscle are similar to each other, yet differ from nonmyogenic and PMb cells. Consequently, there appears to be more than one structural gene regulating their synthesis (Holtzer et al., 1975a; Dienstman and Holtzer, 1975; Chi et al., 1975b). With the light chains of PMb indistinguishable from those of nonmyogenic cells, the synthesis of modest

amounts of myosin by PMb is believed to be coordinated with actin synthesis and to be constitutive myosin. This type of myosin is prevalent in the plasmalemma of single cells (Rubenstein, Chi, and Holtzer, 1976; Chi et al., 1975a). Further differences exist between the two types of myosin based upon the use of in vitro single label, pulse-chase experiments. Here, myosin of PMb was labeled and chased until cells ceased replicating and fused. The myosin extracted from these myotubes displayed a half life of 3 days, the same half life for myosin from cultures of replicating chondrocytes and fibroblasts (Rubenstein et al., 1976). The myosin labeled in fused myoblast cultures was shown to have a half life of 6 days. Evidence exists for a difference in the rate of myosin synthesis based upon the myosin to actin ratio, 2.8 for myotubes and 0.6-0.9 for non-muscle cells (Rubenstein et al., 1976). Myosin synthesis and accumulation in fibroblasts does not increase drastically on a per cell basis (Moss and Strohman, 1976), remaining a constant percentage of total cell protein during 96 hr in vitro (Vertel and Fischman, 1976).

A number of investigators have found myosin mRNA in replicating PMb (Buckingham et al., 1974; Yaffe and Dym, 1972; Chi et al., 1975a). Based on myosin light chain composition and immunological studies a switch in the kinds of myosin synthesized in mother PMb versus daughter Mb may occur (Holtzer, 1970; Chi et al., 1975a). This is consistent with the quantal cell cycle theory, with a quantal mitosis allowing for the definitive myofibrillar myosin heavy and light genes to become available for transcription. Although PMb have transcribed myosin mRNA, they may lack the initiation factor for its translation (Heywood, Kennedy, and Bester, 1975a). Eukaryotic cells contain positive and negative factors which can initiate or prevent translation of a specific mRNA (Heywood, Kennedy, and Bester, 1975b). Negative control inhibits translation by blocking the binding of mRNA to ribosomes during initiation of protein synthesis. Buckingham

et al. (1974) have shown that PMb have a high level of mRNA synthesis, yet translation does not occur until the mRNA is stabilized by the morphological changes which Mb undergo with fusion. Heywood, Kennedy, and Bester (1975a) have shown that BrdUrd-treated myoblasts synthesize myosin and thus express differentiated phenotypes. These findings are in accordance with those of Emerson and Beckner (1975), who detected myosin synthesis in mononucleated quail muscle cells in vitro, provided the cells were not active in DNA synthesis. Reporter (1974) found myosin synthesis not inhibited when fusion was prevented by lysolecithin. These findings contradict those of Pryzbyla and Strohman (1974) who did not find MHC mRNA in mononucleated myoblasts and those of Paterson and Strohman (1972) who did not detect increasing myosin synthesis in EGTA cultures. However, mononucleated muscle cells have demonstrated myosin synthesis (Young et al., 1975; Rubenstein et al., 1976). Since fusion arrested myoblasts display myofilaments, fusion is not a prerequisite for the expression of muscle differentiation, nor is it necessary for the accumulation of myosin (Chi et al., 1975a).

An increase in myosin synthesis is not closely associated with cell fusion as a 2 day lag occurs between maximum percent fusion and maximum myosin synthesis as a percentage of total incorporation (Morris et al., 1972). Initiation of the bulk synthesis of proteins may be coincidental with fusion, yet some transcription of myosin mRNA must occur prior to fusion. Yaffe and Dym (1972) halted 97% of the RNA synthesis just prior to fusion by adding actinomycin D to cultures. This, however, did not prevent the appearance of newly-synthesized myosin after fusion. Therefore, the transition to the differentiated state is a result of the activation of pre-transcribed, stored messenger RNA (Yaffe and Dym, 1972). Holtzer

et al. (1975b) said that PMb do not simply synthesize less myosin than myoblasts, as a qualitative difference exists in the types of myosin synthesized. Therefore, gene expression may be triggered by a repression in DNA synthesis rather than cell fusion (Vertel and Fischman, 1976).

B. Postnatal Muscle Growth and the Role of the Muscle Satellite Cell

1. Characterization of the Satellite Cell Population and its Capacity for Regenerating Muscle

A population of extra-myofiber cells was first observed by Mauro (1961) in electron microscope sections of frog striated muscle. These cells were small, mononucleated, and fusiform shaped and were found lying beneath the basement membrane and exterior to the plasmalemma of the multinucleated myofiber. Mauro called this population of cells satellite cells. Since then, numerous investigators (Muir et al., 1965; Church, 1969; Kahn and Simpson, 1974; Aloisi, 1970; Trupin, 1976; Konigsberg et al., 1975) have found populations of satellite cells in the white mouse, fruit bat, lizard, rat, amphibian, and Japanese quail, respectively.

Improved resolution in electron microscopy has made positive identification of satellite cell nuclei more readily attainable, as satellite cells lie in close proximity to the myonuclei of the myofiber with the distance separating the plasma membrane of the satellite cell and the sarcolemma being only 15 nm (Muir et al., 1965).

The greatest increase in striated muscle mass occurs during postnatal growth, yet the cell population responsible for growth in young animals remains obscure (MacConnachie, Enesco, and LeBlond, 1964; Moss and LeBlond, 1970). The satellite cell has been implicated in various physiologic and pathologic processes involving striated muscle growth and development.

Information on its location and morphology may be essential to understanding any possible role the satellite cell may play in muscle growth. The size of the satellite cell has been found to vary among species. In the regenerating lizard tail (Kahn and Simpson, 1974), it is 150-200 um long, 4-5 um wide, and 1-2 um thick. The dimensions of the satellite cell nucleus in two species of frogs are 12.4-12.8 um in length, 4.4-4.7 um in width and 1.1-1.2 um in thickness. Muir et al. (1965) found the satellite cell in the bat wing to be 25 um in length, 5 um wide, and 4 um in height. The width of the satellite cell is only slightly larger than the width of its nucleus (Mauro, 1961; Ontell, 1973; Muir et al., 1965). The basement lamina, which overlies the satellite cell, consists of irregular filamentous material and is surrounded by the endomysium. The myofiber associated basal lamina and endomysium compose the endomysial tube. Therefore, a muscle fiber bundle would consist of a number of endomysial tubes surrounded by perimysium. Thus, a muscle satellite cell is in close proximity to other skeletal muscle fibers. A satellite cell may either rest on top of its associated myofiber, or in a depression within the sarcolemma (Kahn and Simpson, 1974; Mauro, Shafiq, and Milhorat, 1970).

The size and dimensions of nuclei are useful in distinguishing satellite cell nuclei from subsarcolemmal myonuclei. Myonuclei are usually larger than satellite cell nuclei (Muir et al., 1965; Trupin, 1976), have a lower nucleocytoplasmic ratio, and display less condensed chromatin (Mauro, Shafiq, and Milhorat, 1970; Muir et al., 1965; Trupin, 1976; Church, 1969; Ontell, 1973, 1975; Konigsberg et al., 1975). The genetic material of the myonucleus is a delicate mesh of fine filaments and granules and is much more diffuse than in a satellite cell nucleus (MacConnachie, Enesco, and LeBlond, 1971). The clumped, dense chromatin of the satellite cell nucleus is

usually distributed adjacent to the inner membrane of the nuclear envelope. It appears to form thick bands around the periphery of the nucleus, whereas, myonuclei display a prominent nucleolus and large amounts of less electron-dense nucleoplasm (Kahn and Simpson, 1974; Ontell, 1973). Satellite cell nuclei do not display an easily distinguishable nucleolus (Trupin, 1976). The cytoplasm associated with myonuclei, called sarcoplasm, has glycogen granules, an abundance of large spherical mitochondria, bundles of myofilaments, and polyribosomes. There are few organelles within the thin rim of perinuclear cytoplasm of the satellite cell. The mitochondria are smaller with fewer internal cristae, there are fewer ribosomes, and no elaborate rough endoplasmic reticulum is present (Trupin, 1976; Church, 1969; Mauro, Shafiq, and Milhorat, 1970). Ishikawa (1970) found satellite cells do not contain detectable glycogen granules. The cytoplasm of satellite cells does display centrioles near the nucleus, organelles which have never been seen in the sarcoplasm of the multinucleated myofiber (Muir et al., 1965). Therefore, satellite cells do contain some of the machinery necessary for mitotic activity and construction of the mitotic spindle.

The total number of nuclei within each myofiber increases during postnatal muscle growth (Enesco and Puddy 1964; Moss, 1968; Moss and LeBlond, 1970; Church, 1970b); yet, once myoblasts are incorporated into myotubes, they lose their mitotic ability. Enesco and Puddy (1964) found the number of nuclei in rat muscle fibers to increase three fold between the suckling stage (15-18 day) and young adulthood (79-94 days). A number of *in vivo* studies (MacConnachie, Enesco, LeBlond, 1964; Church, 1969; Moss and LeBlond, 1970) have shown that satellite cells are capable of incorporating labeled thymidine into their nuclei, indicating that they

are undergoing DNA synthesis and probably mitotic activity. These labeled nuclei within the basement membrane will fuse with the fiber proper (Moss and LeBlond, 1970), while the number of labeled nuclei increased with time, even though myonuclei are postmitotic (MacConnachie et al., 1964; Moss and LeBlond, 1971).

Holtzer (1970) has suggested that satellite cells are an adult form of "myoblast" which has been arrested in a postmitotic phase, yet is fully capable of mitosis during postnatal muscle growth and regeneration. Once satellite cells become activated, their nuclei enlarge, and the numbers of ribosomes and the amount of cytoplasm increase. These changes are typical of non-cycling cells which have been stimulated to leave the G_1 phase of the cell cycle and enter the G_2 and S phases (Ontell, 1975; Bischoff, 1974, 1975). A satellite cell is a myogenic stem cell or reserve myoblast capable of giving rise to postmitotic daughter myoblasts (Church, 1969, 1970a). Evidence also exists for the proliferative ability and myogenic capacity of satellite cells based upon the presence of "twin satellite cells" beneath the basement membrane and the presence of myofibril formation in cells occupying the satellite cell position in both embryonic and postnatal muscle (Ishikawa, 1970). Moss and LeBlond (1971) labeled nuclei within the basement membrane and noticed that after 72 hr not all the nuclei labeled were incorporated into the myofiber. This is consistant with the <u>in vitro</u> findings which suggest that a population of replicating myogenic cells will always be present even after fusion levels off (Bischoff and Holtzer, 1969). The setting aside of myogenic stem cells for muscle regeneration and growth helps to explain observations made during earlier studies of muscle regeneration and tissue culture (Muir et al., 1965).

Why are presumptive myoblasts (PMb) which have ceased dividing set aside during embryonic differentiation or postnatal growth? Konigsberg et al. (1975) suggest that myogenic cells may simply become trapped beneath the basement membrane during embryonic development, and Church (1969, 1970a) states that satellite cells are recognizable in bat wing muscle from the time the basement membrane becomes visible during the eighth week of implantation (22 weeks elapse between implantation and birth). Satellite cells can be witnessed beneath the basement membrane in a 15-week human fetus (Ishikawa, 1966). In vitro the basement membrane becomes visible after single cells have been in culture seven days (Mauro, Shafiq and Milhorat, 1970).

Myogenic cells may cease to divide due to the extracellular matrix produced by several types of tissues which have been shown to inhibit proliferation of myogenic cells (Nameroff and Holtzer, 1969). The basal lamina, a connective tissue membrane, may serve this purpose.

Consistent with the above theories are the findings of Bischoff (1974), who used enzymatic digestion to free satellite cells from their connective tissue membrane. These cells were then cultured in vitro. Enzymatic liberation of this cell population resulted in the isolation of cells capable of in vitro myogenesis. They exhibited a preproliferation period, a doubling time of 22 hr, and multinucleated myotube formation (Bischoff, 1974).

It has been shown that cultured fragments of adult muscle tissue will give rise to a population of myogenic cells capable of the neoformation of differentiating muscle fibers. Bischoff (1975) and Konigsberg et al. (1975) both isolated single skeletal muscle fibers and placed them in culture. Both experiments involved an injury-triggered proliferation

of the satellite cell population, fusion, and the formation of long, cross-striated, multinucleated cells. Therefore, satellite cells are a myogenic population similar to the PMb of embryonic muscle (Bischoff, 1975). Trupin (1976), in regeneration studies of minced frog gastrocnemius muscle, suggests that all the nuclei in newly-formed myotubes could arise from a single presumptive myogenic cell.

Morphological differences are evident in satellite cells from mice of various ages (Schultz, 1976). Electron micrographs show the satellite cells of very young muscle (7-14 days of age) have abundant cytoplasm and are rich in ribosomal associated rough endoplasmic reticulum. With increasing age, there is a qualitative and quantitative decrease in the number of organelles. Satellite cells appear to become more dormant as age increases, and in adult muscle (1-10 months of age) they contain clumped chromatin in a nucleus surrounded by a small amount of cytoplasm.

Corresponding to the apparent decrease in metabolic activity of the satellite cells with age is a decrease in their frequency in muscle (Bischoff, 1974; Allbrook et al., 1971; Ishikawa, 1970; Church, 1969, 1970b; Trupin, 1976; Muir et al., 1965; Schmalbruch and Hellhammer, 1976). For example, the percentage of total muscle nuclei which are satellite cells in 25, 200, and 800 g rats has been found to be 12, 2, and 0.7% (Ontell, 1973; Moss and LeBlond, 1971). There is an apparent species difference in satellite cell populations as adult bat wing muscle, which is capable of extensive and rapid regeneration, contains 10-12.5% of its cell population as satellite cells (Muir et al., 1965; Church, 1970b).

Although the frequency of satellite cells declines as postnatal muscle growth progresses (Bischoff and Holtzer, 1969; Marchok and Herrmann, 1967), the yield of myogenic cells can be increased by inducing injury

to the muscle to create a regenerative response (Bischoff, 1974). Ontell (1973) denervated rat muscle and one week later found the satellite cell population to increase from 2% to 6% in a 200 g rat. Two weeks later the percentage had increased to 12%. In an 800 g rat, the percentage increased to 3% and 5% after 2 and 3 weeks following denervation. Church (1970b) has shown the percentage of satellite cells within the basement membrane of adult bat wing muscle to increase from 12.5% to 35% or more in regenerating muscle. Hanzlikova et al. (1975) demonstrated that a combination of hypertrophy and denervation would increase satellite cell numbers from 5.8% to 29.9% of all muscle nuclei in 150-170 g rats.

Sufficient numbers of satellite cells are present to account for regeneration (Trupin, 1976), and Church (1969) indicates that satellite cells would only have to divide once or twice from birth to maturity to account for the number of satellite cells in adult muscle. Therefore, most evidence suggests that the rate and quantity of muscle formation during embryogenesis and postnatal growth depends upon the mitotic activity of reserve myogenic stem cells.

When postnatal muscle is injured, satellite cells undergo proliferative activity, and many of their daughter cells enter the terminal myogenic compartment. Muscle regeneration in adults simply recapituates myogenesis in the embryo (Dienstman and Holtzer, 1975), with satellite cells serving as mitotically quiescent presumptive myoblasts.

2. Changes in Muscle Mass During Postnatal Muscle Growth

Postnatal muscle growth occurs almost exclusively by an increase in length and diameter of the myofiber (Carlson, 1974). The postembryonic increase in muscle girth is due almost entirely to the hypertrophy of

the existing myofibers. Hypertrophy involves an increase in number and size of individual myofibrils (Goldspink, 1970, 1972). The limbs of most mammals approximately double in length during postnatal growth; therefore, an increase in myofiber length may occur (Goldspink, 1970). The mechanisms by which muscle accretion occurs will be explored in the following paragraphs.

Myofibrils possess the ability to lengthen in one of two ways; individual sarcomeres may lengthen, or the number of sarcomeres in a single myofibril may increase (Stromer et al., 1974). During neonatal myogenesis the average relaxed sarcomere length is shorter than in the adult enabling the formation of more actin-myosin crossbridges. This may stabilize newly-formed hexagonal arrays of thin and thick filaments (Goldspink, 1970). As the animal ages, the average resting sarcomere length increases and is a result of a decrease in the amount of overlap of interdigitating thick and thin filaments (Stromer et al., 1974). The addition of a number of sarcomeres in series to a myofibril is the main contributing factor to the increase in myofiber length (Goldspink, 1972). Goldspink (1972) found the number of sarcomeres per myofibril to increase substantially in the mouse soleus muscle during postnatal growth. Most of this increase occurred during the first three weeks after birth. The point at which new sarcomeres add to a myofibril has been investigated by Goldspink (1972). Young rats were injected with labeled adenosine, and the label appeared at the muscle-tendon junction where the myofibers terminate. Also, the sarcomere length is shorter in the terminal sarcomeres which is a region of active protein synthesis, containing ribosomes and unorganized myofilaments (Goldspink, 1972).

The mode of myofibril proliferation during growth involves an increase

in the number of actin and myosin filaments. Once the myofibril reaches a certain diameter, it has been postulated to split longitudinally (Goldspink, 1972). Goldspink (1970) has shown evidence for this theory with electron micrographs of Z-disks splitting. This theory is also substantiated by the presence of two general categories of myofibrils. Both 40 um and 15-20 um diameter myofibrils are present in the mouse biceps brachii. There is also a concurrent 15 fold increase in the number of myofibrils during postnatal growth.

Myofibrils probably grow in diameter by adding myofilaments to the outside of the existing myofibril (Fischman, 1972), based upon the presence of loose myofilaments around the periphery of a myofibril. Hexagonal arrays are so tightly organized that it is doubtful that de novo synthesis of new myofibrillar protein subunits could occur (Burleigh, 1974). Venable (1969) administered radioactive labeled amino acids to muscle in vivo and noticed incorporation of the label into the peripheral myofilaments of the myofibril. If myofibril formation was initiated by addition of filaments to Z-disk proteins to form a new sarcomere, short segments of myofibrils should be witnessed in muscle. Such segments of sarcomeres have not been seen in electron micrographs of postnatal skeletal muscle (Stromer et al., 1974).

The degree of postnatal muscle differentiation varies for different species and is a function of the degree of embryological development at birth. The small postnatal increase in the number of myofibers (hyperplasia) can be considered an extension of embryonic differentiation (Stromer et al., 1974).

3. Changes in Nucleic Acid Metabolism During Postnatal Muscle Growth and the Control of Myofibrillar Protein Synthesis

Accompanying postnatal myofiber hypertrophy there is an increase in the amount of sarcoplasmic material, including a pronounced increase in the number of myonuclei (Carlson, 1974). The DNA content of muscle increases during postnatal growth (Enesco and Puddy, 1964; Moss, 1968); yet, this excess DNA cannot be accounted for by the existing nuclei of the myofiber (Enesco and Puddy, 1964). Moss (1968) found that the number of myonuclei increases beyond the stage where fiber length ceases to increase and that larger diameter fibers possess more myonuclei. Similarly, Burleigh (1974) says that the number of muscle nuclei is a primary determinant of muscle mass, and Allbrook et al. (1971) and Enesco and Puddy (1964) both demonstrated an increase in the number of myonuclei during postnatal growth. Therefore, a postnatal increase in the number of fiber nuclei is associated with fiber hypertrophy and fiber lengthening (Goldspink, 1972).

Muscle satellite cells are capable of fusing with the adult myofiber (Moss and LeBlond, 1970, 1971) and to be capable of regenerating injured skeletal muscle (Bischoff, 1975; Hanzlikova et al., 1975; Ontell, 1975; Konigsberg et al., 1975). Therefore, satellite cells are likely the mode of DNA increase in postnatal muscle.

Moss (1968) has suggested that the cross-sectional area of a myofiber is directly proportional to the number of myonuclei. Evidence that muscle satellite cells are myogenic suggests that transcribed segments of newly-fused satellite cell genome may contribute to fiber hypertrophy by directing myofibrillar protein synthesis. Informational proteins (i.e., mRNA, tRNA, etc.) may in turn be responsible for the increase in

the amount of sarcoplasmic material. The muscle satellite cell may be a dormant myoblast (Mauro, 1961; Church, 1969, 1970a; Muir et al., 1965; Bischoff, 1974); yet, evidence has been presented which shows muscle satellite cells become "activated" and proliferate during muscle injury, trauma, or hypertrophy. Satellite cells present at the time of injury give rise to myoblasts capable of restoring the number of myonuclei (Church, 1970a,b). Some of their descendents remain as satellite cells, thereby providing a potential supply of myoblasts in the event of repeated injury or trauma. This population of cells is capable of proliferation so as to replenish their numbers beneath the basal lamina while still replacing degenerating myonuclei (Church, 1970a,b). A model which allows for the myogenic capacity mentioned above would designate a satellite cell as a reserve presumptive myoblast (reserve PMb). The ability to enter the cell cycle would exclude a satellite cell from being defined as a terminal postmitotic myoblast, yet the ability to yield fusion capable progeny would mean that some of the satellite cell mitosis must be quantal, with at least one daughter nuclei differing from the mother cell (Church, 1970a). If satellite cell nuclei undergo quantal mitotic activity where both daughter cells are fusion-capable, then eventually depletion of the satellite cell population would occur. This situation does not always occur as satellite cells have been found to exist throughout the life time of an individual (Schultz, 1976; Bischoff, 1974, 1975; Church, 1969). Also, satellite cells are defined as reserve presumptive myoblasts and not terminal Mb, as electron micrographs of satellite cells fail to display organized arrays of myofilaments in their cytoplasm.

The reason why PMb are set aside may arise from influences of the environment (Nameroff and Holtzer, 1969) or the inability of newly-formed

Mb to fuse with differentiated myotubes (Bischoff and Holtzer, 1969). Once the basement membrane appears during early embryonic differentiation, cells which have been set aside are recognizable in the satellite cell position (Ishikawa, 1970). Whatever causes myogenic cells to cease division, then after extended periods become activated and re-enter the cell cycle, has great implications upon muscle growth.

Since satellite cells are capable of fully regenerating skeletal muscle (Konigsberg et al., 1975; Bischoff, 1975), cell populations derived from these populations must be qualitatively similar to myoblasts of embryonic and perinatal muscle. Therefore, satellite cell progeny must possess the ability to transcribe and translate muscle protein synthetic machinery.

With the use of radioactive labeled thymidine and the appearance of labeled nuclei at the ends of the myofibers, it is evident that satellite cells provide the necessary additional nuclei to allow an increase in myofiber length and diameter (Goldspink, 1972). As myofibrillogenesis occurs, myonuclei decrease in size, chromatin becomes more condensed, and nucleoli lose prominance (Carlson, 1974). These type of nuclei are not indicative of nuclei which are actively transcribing RNA molecules for the translation of the myofibrillar proteins. The absolute amount of nucleic acids increases during growth (Enesco and Puddy, 1964; Burleigh, 1974; Millward et al., 1975; Goldberg, 1969b; Moss, 1968). Therefore, myogenic populations derived from satellite cells contribute nucleic acids to the sarcoplasm of myofibers. These nucleic acids are mostly in the form of DNA as satellite cells display little cytoplasm with few ribosomes (Trupin, 1976; Ishikawa, 1966). This DNA is the source of programmed genetic information which can transcribe protein synthesizing

machinery (Munro, 1976).

The mechanism of transcription involves the formation of large heterogeneous nuclear RNA (HnRNA) which is excised into lower molecular weight RNA. Although over 80% of muscle RNA is ribosomal (Millward et al., 1973), a portion of the remaining RNA transcribed is messenger RNA (mRNA) and is combined with a protein to yield an informosome. This is the form in which mRNA leaves the nucleus for the sarcoplasm (Munro, 1976) and for the translation of the myofibrillar proteins.

Even though there is an increase in the amount of RNA during muscle hypertrophy (Goldberg, 1969b), the concentration of RNA decreases due to the large increase in the myofibrillar content. Millward et al. (1973) state that if the control of protein synthesis is related to a change in ribosomal content, then there is a change in the ribosomal capacity for protein synthesis. Yet, if changes occur in synthesis which alter the factors modulating each phase of translation, then these changes involve ribosomal efficiency (expressed as mg of protein synthesized per unit of RNA). Young et al. (1971) have reported a decrease in the frequency and the protein synthetic capacity of polyribosomes in muscle with increasing age.

The production of mRNA may become the limiting factor in myofibrillar protein synthesis as the percentage of ribosomes in polysomes reflects a reduction in mRNA synthesis during growth (Goldspink, 1972). Yet, recently, it has been found that polysomes of young muscles synthesize a much higher percentage of myofibrillar protein than polysomes from adult muscle (Goldspink, 1972); consequently, it appears to be the activity of the RNA which is responsible for the slow down in postnatal muscle growth.

The basic concept behind muscle growth is that protein accretion is

equal to protein synthesis minus protein catabolism (Millward et al., 1975). Myofibrils are in a continuous state of growth and remodeling in response to metabolic and physical demands upon the myofiber (Fischman, 1972). With work induced hypertrophy protein synthesis is increased while protein catabolism is decreased (Goldberg, 1969b); and, therefore, rapid accumulation of muscle protein will occur (Young et al., 1971). Apparently, maximum muscle growth rates are limited by a high rate of protein turnover, yet increased protein turnover may be necessary for muscle hypertrophy (Millward et al., 1975). Since myofiber hypertrophy is accompanied by longitudinal splitting of large diameter myofibrils (Goldspink, 1972), this process may result in the loss of myofilaments or myofibrillar portions. Therefore, rapid growth and the concurrent increased splitting of myofibrils will result in increased protein turnover.

4. Conclusion

The eventual slow down of postnatal muscle growth appears to result from a decrease in RNA activity (Goldspink, 1972; Burleigh, 1974; Millward, 1975), as increased rates of breakdown seem to be a necessary accompanyment to muscle growth. If muscle growth slows down due to decreased rates of translation of mRNA molecules, it may be possible to enhance muscle accretion by supplying new protein synthesizing machinery to the myofiber. A source of new machinery lies within the nuclei of the muscle satellite cell.

It is not known why myogenic cells cease to divide or what causes them to re-enter the cell cycle, yet the determining element in muscle growth may be the control of the balance between a proliferative and

quantal mitosis of a cell in the myogenic lineage (Holtzer and Bischoff, 1970).

III. MATERIALS AND METHODS

Unless otherwise noted, preparations were done at temperatures of 0-4°C and precooled solutions were used. Double-deionized, glass distilled water stored in polyethylene containers was used to make all solutions. All reagents were of reagent grade.

A. Methods of Preparation

1. Procurement of the Animal Model

White mice, Outbred Spartan HA (ICR), were obtained from Spartan Research Animals, Inc., Haslett, Michigan. Foundation breeding females were purchased and maintained in laboratory animal facilities at M.S.U. on standard laboratory mouse diet (Peerless Laboratory Animal Diet). These females were mated to purchased males to provide neonatal and three-week old mice of both sexes. Young adults used in these studies were all purchased from Spartan Research Animals, Inc. immediately before use. All mice were anaesthesized with ether immediately prior to excision of their hind limbs.

2. Preparation of Cells for Muscle Culture

The procedure used for isolating myogenic cells from the fresh hind limb muscle of mice was patterned after that of Bischoff (1974). Muscle was excised from the hind limbs of mice and immediately placed in an uncoated culture dish containing buffered saline solution (0.137 M NaCl,

0.0027 M KCl, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, 0.00015 M NaH_2PO_4, 0.00136 M Na_3HPO_4, 0.006 M $NaHCO_3$, 0.0055 M glucose, 37°C, pH 7.4). The muscle was trimmed free of excess connective tissue and placed in a clean uncoated culture dish containing 10 ml of fresh buffered saline solution (BSS). The muscle then was minced into fragments approximately 1 mm^3 in size. To this suspension pronase (Calbiochem, B grade) was added to yield a final concentration of 1 mg/ml. This suspension was incubated at 37°C for 60 min while undergoing mild agitation in a water bath. The suspension was centrifuged at 1,500 x g for 1.0 min, and the pellet was resuspended in 15 ml of complete medium. Complete medium contained 85% Eagle's Minimum Essential Medium, 10% horse serum, 5% chicken embryo extract prepared as described below, 125 units/ml of penicillin, 3.44 ug/ml of Fungizone, 125 ug/ml streptomycin. Suspended tissue was agitated with a Vortex mixer for 30 sec. This suspension was centrifuged at 700 x g for 1 min and the supernatant saved. The pellet from this centrifugation was resuspended and recentrifuged two more times exactly as above, and the supernatants from the three centrifugations were combined. These combined supernatants were centrifuged at 1,500 x g for 3 min and the supernatant discarded. The pellet from this centrifugation contained cells and large debris while most of the small myofibrils were left in suspension. The pellet was resuspended in complete medium by aspiration with a disposable pipette. To approximate the number of cells available for plating, an aliquot of the cell suspension was counted with a hemocytometer. Cells were plated at a cell density of 2.25 x 10^4 cells/cm^2 in tissue culture dishes (Falcon and Corning), which had previously been coated with approximately 2 ug/cm^2 of sterile collagen and then air-dried. Cultures were incubated in 15 ml of complete medium at 37°C in the presence of 5%

CO_2 and 95% air. Medium changes were made every 24 hr with 15 ml of fresh medium prewarmed to 37°C.

3. Preplating

Yaffe (1968) discovered that when a heterogeneous cell population was plated on an uncoated culture dish, most of the fibroblastic and epithelial cells began to attach to the surface in a short time, while myogenic cells remained floating in the medium. This principle has been used to increase the percentage of myogenic cells in a cell suspension. The initial heterogeneous cell suspension was plated onto uncoated culture dishes and incubated at 37°C in a 95% air, 5% CO_2 atmosphere. After 40 min the culture medium was recollected and the cells which remained floating were counted and plated at a density of 1.25×10^4 cells/cm^2 on collagen-coated dishes containing 15 ml of complete medium. These cultures were then incubated at 37°C in a 95% air, 5% CO_2 atmosphere.

4. Chicken Embryo Extract

Twelve-day chicken embryos were decapitated and forced through a 50 ml disposable syringe. An equal volume of BSS was added. This mixture was left at room temperature for 0.5 hr and was then centrifuged at 1,500 x g for 15 min. The supernatant from this centrifugation was saved and held at -8°C for up to 4 months. After the extract had been thawed for use in complete medium, it was again centrifuged at 1,500 x g for 10 min to remove insoluble material.

5. Preparation of Collagen

Collagen (calf skin) was dissolved in 0.1% acetic acid to a final concentration of 0.2 mg/ml and then steam autoclaved. Collagen was

stored at -5°C.

6. Preparation of Myosin

Connective tissue and fat were trimmed away from 30 g of mouse hind limb muscle. Then the muscle was placed in 3.33 volumes of extraction buffer (0.3 M KCl, 0.15 M K_2HPO_4, pH 6.5) and homogenized on a Virtis. This suspension was allowed to set at 2-3°C for 15 min. Cold water (13.3 volumes) was added while stirring and the suspension strained through gauze. An additional 23.3 volumes of cold water were added slowly and the suspension allowed to set at 2-3°C for 2 hr. The white precipitant was collected and centrifuged at 2,000 x g for 30 min. The myosin precipitant was then dissolved in 24.5 ml of 0.3 M KCl and 0.5 ml of 1.0 M Tris, pH 7.5, added. This solution was homogenized with a 40 ml Dounce homogenizer (B-pestle) and diluted to 32 ml with cold water, then centrifuged at 44,000 x g for 1 hr. The supernatant was filtered through glass wool and diluted to 0.03 M KCl with cold water. The myosin precipitant was collected by centrifugation at 2,000 x g for 0.5 hr and dissolved in 2.0 ml of 3.0 M KCl and 0.75 ml of 1.0 M Tris-acetate, pH 7.0, added. The volume was brought to 12 ml with cold water and then the solution was homogenized (Dounce, B-pestle) and centrifuged at 44,000 x g for 1.0 hr. The supernatant was filtered through glass wool, diluted to 26 ml with cold water, and centrifuged at 44,000 x g for 1.0 hr. The supernatant was diluted 7.7 fold with cold water, and the myosin precipitant was collected by centrifugation at 2,000 x g for 0.75 hr, then dissolved in 1.66 ml of 3.0 M KCl and 0.62 ml of 1.0 M Tris-acetate, pH 7.0. This solution was diluted to 10 ml with cold water, centrifuged at 44,000 x g for 1.0 hr and filtered as before. This solution was diluted 2.174 times

with cold water, centrifuged at 44,000 x g for 1.0 hr, and the supernatant filtered. The supernatant was then diluted 7.7 fold and the myosin precipitant collected via centrifugation at 2,000 x g for 0.5 hr. The myosin pellet was dissolved in 1.0 ml of 2 M KCl (pH 7.0) and diluted to 4.0 ml with cold water. Glycerol was added to a final concentration of 40%. The purified myosin was stored at -20°C.

7. Sucrose Preparation of Myofibrils from Culture

Culture plates were rinsed twice with cold BSS, drained thoroughly, and covered with 4.5 ml of 0.25 M sucrose, 0.05 M Tris, 1 mM EDTA, pH 7.6. Triton X-100 (5%) was added to the buffer to yield a final concentration of 0.5%. After 15 min, material was scraped off the plates and homogenized with a polytron using 4-5 sec blasts in 15 sec intervals. The suspension was centrifuged at 165 x g for 5 min and the pellet resuspended in 5 volumes of 0.25 M sucrose, 0.05 M Tris, 1 mM EDTA, pH 7.6, and centrifuged at 255 x g for 3 min. The pellet was then resuspended in 5 volumes of 0.05 M Tris, 1 mM EDTA, pH 7.6, and centrifuged at 165 x g for 3 min. The pellet was resuspended in 5 volumes of 0.15 M NaCl, mixed, and centrifuged at 165 x g for 3 min. The myofibrils were resuspended in 5 volumes of 1 mM EDTA, pH 7.0-7.6, mixed, and collected by centrifugation at 165 x g for 3 min. The supernatant was decanted, and the pellet was placed in 5 volumes of cold water and mixed. This suspension was centrifuged at 370 x g for 5 min, the supernatant decanted, and the myofibrils resuspended in an equal volume of 0.15 M NaCl.

8. Preparation of Muscle Clones

Myogenic cell suspensions were isolated as described previously from all three ages of mice. An aliquot of the cell suspension was counted

with a hemotocytometer and cells were plated into 15 cm collagen-coated culture dishes at densities of 100 (1 x), 250 (2.5 x), and 500 (5 x) cells per dish containing 15 ml of complete medium. Cultures were incubated in 95% air, 5% CO_2 at 37°C. Nutritional medium changes were made every 24 hr until the number of cells within clones reached approximately 100. At this point, conditioned medium (12 ml), which had been prepared as described below, was placed on clonal cultures for a period of 48 hr. Cultures were then prepared for light microscope analysis.

9. Preparation of Conditioned Medium

Conditioned medium was prepared similar to the procedure of White and Hauschka (1971). Regular complete medium was prepared as stated previously in these procedures, with 60 ml placed on cultures in 15 cm collagen-coated polystyrene culture dishes. These cultures were prepared by plating a total of 1.5×10^7 cells per culture dish. These cells were derived from preplated cell suspensions of neonatal mice and isolated according to the method of Bischoff (1974). Complete medium was conditioned for a 24 hr period between 1 and 2 days in culture while cells were rapidly fusing. Conditioned medium was transferred directly to clonal cultures.

B. Analytical Procedures

1. Determinations of Total Nuclei, Fused Nuclei, and Percent Fusion

In order to prepare muscle cell cultures for counting nuclei, cultures were rinsed twice with BSS at 37°C, fixed in absolute methanol for 5 min, and stained with Giemsa stain for 20 min at room temperature. Nuclei were counted in randomly-selected fields, and the percentage of nuclei

within myotubes was calculated by dividing the total number of fused nuclei by total nuclei and multiplying by 100. The area of individual culture dishes and diameter of the field of vision of the inverted phase microscope (Opton by Zeiss) were known. Therefore, the number of cells per plate could be calculated.

2. Quantitation of Cells Per Gram Based upon Cell Counts at 5.0 hr In Vitro

Cells were prepared by the method of Bischoff (1974) and resuspended in complete medium. The cell suspension was plated into the collagen-coated wells of a Falcon Microtest II dish. Wells were Giemsa stained after 5.0 hr and 20 randomly-selected fields counted in each well. The average number of cells/field was multiplied by the number of fields/well to yield the number of cells/well. The total number of cells was determined from the number of wells used for plating the cell suspension. From the number of grams of hind limb tissue used to prepare the cell pellet, the concentration of cells in the muscle could be approximated and expressed as cells/gram of muscle.

3. Percentage of Muscle Colony Forming Cells Based upon Cloning Experiments

Clonal cultures were fixed for counting as mentioned above. Colonies were observed with an inverted light microscope and scored as nonfusing or fusing clones (Bonner and Hauschka, 1974). Colonies were designated as myogenic clones only if they contained clearly discernible multinucleated myotubes. All other colonies were scored as nonmyogenic. The percentage of myogenic clones was determined by dividing the number of clones exhibiting fusion by the total number of clones in a culture dish, and multiplying by 100.

4. Cells Per Gram Based upon Clonal Analysis

From clonal analysis the total number of clones per culture was known. Since each clone was derived from a single cell, the number of clones per plate reflected the number of cells contained in the aliquot of cell suspension that was plated. By multiplying the number of cells/aliquot times the number of aliquots available from the cell suspension, the total number of cells available was determined. The number of cells/gram was determined by dividing the total number of cells available in the suspension by the number of grams of muscle used to prepare the suspension.

5. Pulse-Labeling of Cultures

Muscle cell cultures were evaluated for rate of myosin heavy-chain (MHC) synthesis at various degrees of cell fusion by pulse-labeling cultures with (^{3}H)-L-leucine (Paterson and Strohman, 1972). Cultures in either 6 or 10 cm plates were labeled at 37°C for 4.0 hr with 2.0 or 4.0 ml of complete medium containing 20 uCi of (^{3}H)-L-leucine/ml (New England Nuclear). At the end of the labeling period, dishes were rinsed twice with cold 0.25 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4, and the cells were scraped from the surface into 0.5 ml of 0.25 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4. Culture tissue was homogenized with 20 strokes of a 7 ml Dounce homogenizer (B-pestle), and the homogenate was allowed to set for 15 min at 2°C. The homogenate was then centrifuged at 1,600 x g for 20 min at 2°C. The supernatant was removed, and the KCl concentration lowered to 0.025 M by adding 4.5 ml of cold water; 20 ug of carrier myosin, prepared and purified as stated previously were added. Tubes were left at 2°C for at least 8 hr, then the myosin-containing

material was pelleted at 1,600 x g for 50 min. The pellet was dissolved and the precipitated protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described below.

6. SDS-polyacrylamide Gel Electrophoresis of Proteins

Myosin-containing pellets, and myofibrils prepared from culture, were dissolved by heating at 80°C for 20 min in 75-100 ul of a solution containing 1.0% SDS, 0.05 M Tris-HCl, pH 7.1, 20% glycerol, 0.01% Pyronin Y with the pH adjusted to 7.2 with 6 N HCl. The dissolved samples were loaded quantitatively onto 8 cm, 10.0% polyacrylamide gels and were electrophoresed in a chamber containing 0.1% SDS, 0.20 M Tris-glycine, pH 8.8., buffer at 0.5 milliamps per gel tube. Gels were stained with .033% coomassie blue for at least 4 hr and then destained in a H_2O:glacial acetic acid:methanol mixture (87.5:7.5:5 v/v).

7. Measurement of Radioactivity in 200,000 Dalton Subunit of Myosin in SDS-polyacrylamide Gels

Destained gels were frozen in dry ice and consecutive 0.5 mm slices were taken through the 200,000-dalton subunit of the myosin band. Slices were dissolved in 0.2 ml of 30% hydrogen peroxide by heating at 55°C for 8 hr in polyethylene bio-vials (Beckman), 2.2 ml of Aquasol (New England Nuclear) were added to each vial and vials vortexed mildly. Vials were stored in the dark for at least 24 hr at room temperature before being counted in a Beckman 3133P liquid scintillation spectrometer. Counts per min (cpm) were converted into disintegrations per min (dpm) using the external standard channels ratio method with chloroform as the quenching agent.

8. Measurement of Radioactivity in Remaining SDS-polyacrylamide Gel

All gel slices prior to the MHC band and the remainder of the gel below the band were placed in a 10 ml volumetric flask. The gel material was dissolved by adding 2.0 ml of 30% H_2O_2 and heating for 8.0 hr at 55°C. This solution was brought to a final volume of 10.0 ml by adding water, then mixed by inversion. A 0.200 ml aliquot was placed in a polyethylene Biovial (Beckman), 2.2 ml of Aquasol were added, and vials were vortexed, stored, and counted as previously described.

9. Total Radioactivity Incorporated into TCA Precipitable Protein

The pellet from the first centrifugation of the pulse-labeled homogenate and the supernatant from above the myosin containing precipitant were combined. This material was vortexed, 0.5 ml of 50% TCA (trichloroacetic acid) added, and vortexed again. This suspension was allowed to set at 2°C for 2 hr, then centrifuged at 700 x g. The supernatant was discarded and 4.0 ml of cold 10% TCA added to the pellet. This was vortexed, then centrifuged at 700 x g for 10 min. The previous step was repeated and the supernatant carefully removed. The pellet was dissolved in 10.0 ml of 20% NaOH and mixed. A 0.200 ml aliquot was placed in a polyethylene bio-vial, 2.2 ml of Aquasol added, and vortexed; 1.4 ml of water were added so as to form a stable gel phase. Vials were stored and counted as previously stated.

10. Radioactive Complete Medium

Radioactive complete medium was prepared similar to regular complete medium with the exception that EMEM without leucine (Grand Island Biological Company) was used in place of regular EMEM. Leucine (L-leucine, Sigma

Chemical Company) was added to the complete medium to a concentration of 15.0 mg/l. Normal EMEM contains 52 mg leucine/l. Therefore, the specific radioactivity of leucine was increased by using EMEM with a lower concentration of leucine.

11. Measurement of Protein Concentration

Protein concentration was determined by the method of Lowry et al. (1951).

C. Microscopy

1. Light Microscopy of Cultures

Light microscopy was performed on live and Giemsa stained cultures with the use of a Zeiss Opton phase and bright-field inverted microscope. Both phase and bright-field light micrographs were made to provide evidence for various degrees of muscle differentiation which developed in an in vitro model. A Yashica Pentamatic attached to an eyepiece of the Zeiss inverted microscope was used for photography.

2. Light Microscopy of Sucrose Myofibrils

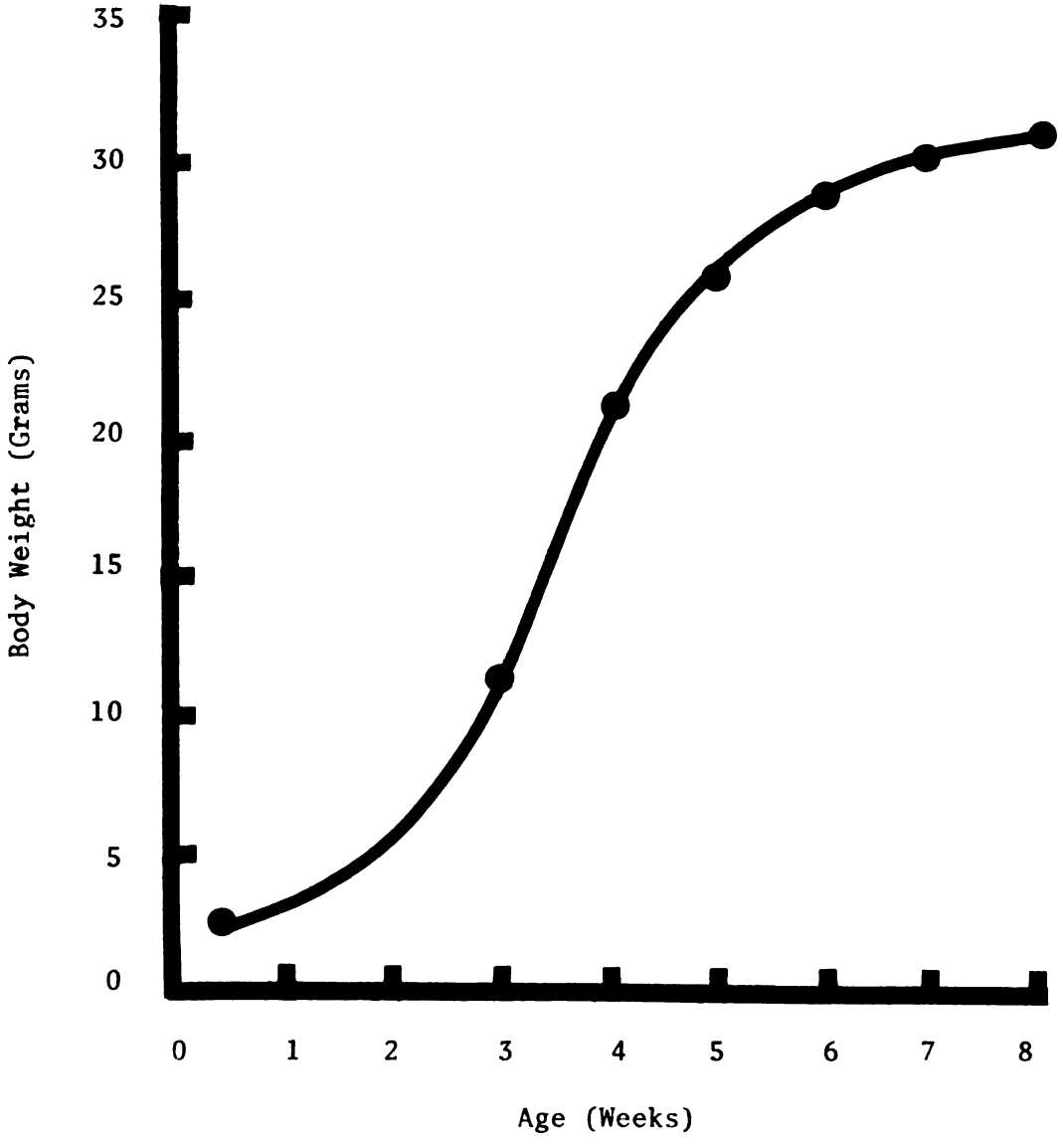
Slides of myofibrils isolated from culture were evaluated with phase optics on a Zeiss Photo-Microscope III. Myofibrils in 0.15 M NaCl were used to prepare slides, and phase micrographs were taken with the use of an Automatic Exposure Control.

IV. RESULTS AND DISCUSSION

The initial goal of this project was to evaluate the *in vitro* growth potential of skeletal muscle satellite cells. When preliminary experiments revealed that satellite cell populations from mice of various ages were capable of proliferation and fusion in cell culture, answers to two questions of primary importance were sought: (1) Can satellite cells synthesize the myofibrillar proteins and assemble them into functional myofibrillar arrays? and (2) Are the rates of myofibrillar protein synthesis, total protein synthesis and the degree of differentiation influenced by the age of the animal from which satellite cells are isolated?

A rapidly growing strain of white mice (Spartan Research Animals, Inc.) was used for the animal model. The short generation interval of this strain of mice allowed experiments to be carried out in a reasonable amount of time. The three ages chosen for these experiments were neonatal, rapidly growing (3 weeks), and young adult (7 weeks). Neonatal mice are very immature compared to the newborn of other species; consequently, neonatal mice were considered to be embryonic for the purpose of the experiments reported here. Mice at three weeks of age were chosen because they were making the most rapid weight gains (Figure 1), and seven week mice were used because their rate of weight gain approaches zero (Figure 1).

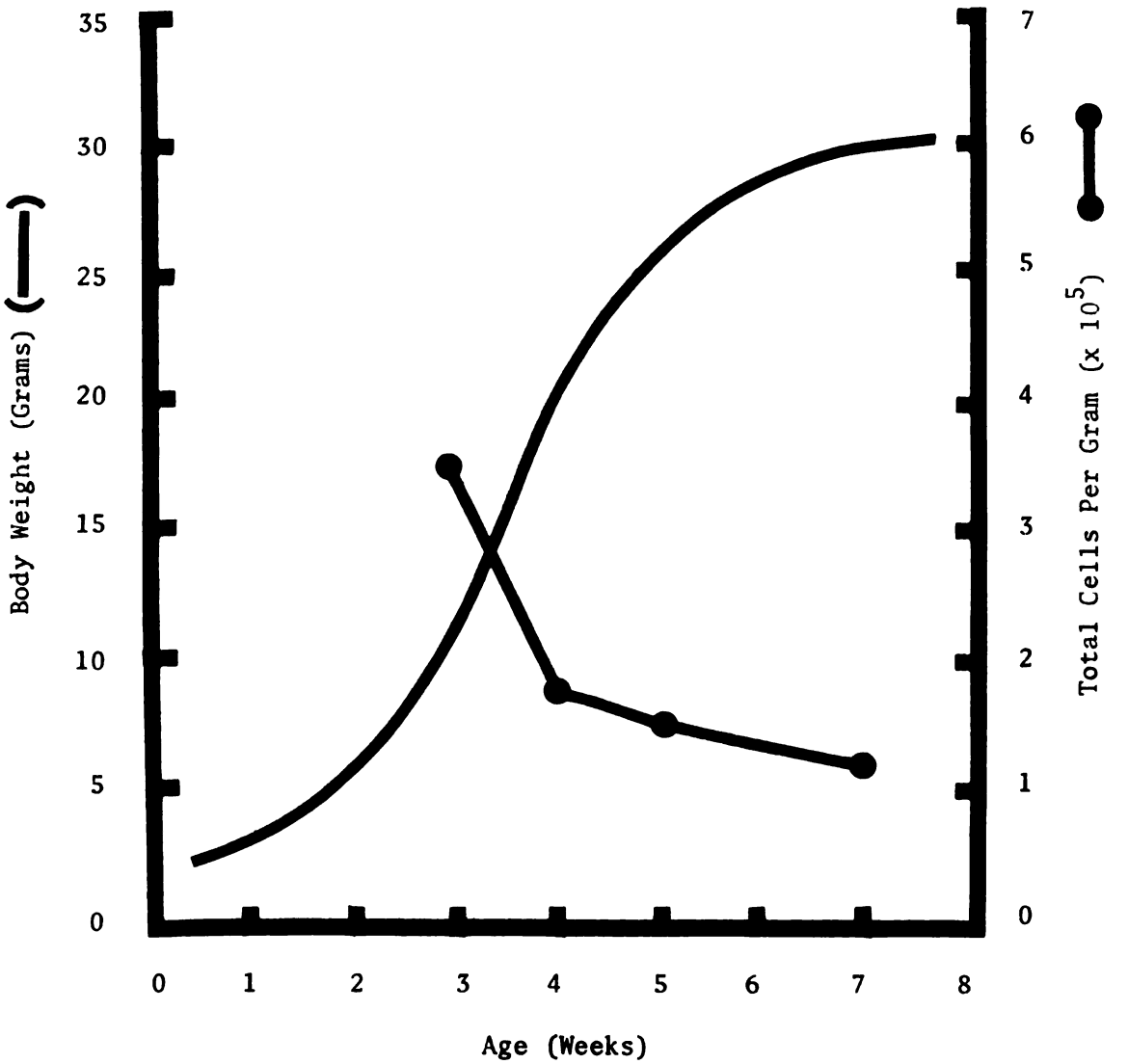
Figure 1. Growth curve of Outbred Spartan white mice. Data were provided by Spartan Research Animals, Inc. Each point is the mean of separate determinations made for female and male mice.


35
30
25
20
15
10
5
0
Body Weight (Grams)
0
1
2
3
4
5
6
7
8
Age (Weeks)

Evidence from in vitro investigations (Bischoff, 1975; Konigsberg et al., 1975) has shown that satellite cell populations capable of muscle regeneration are present in normal adult skeletal muscle. Therefore, the extent to which this myogenic population exists in muscle may be a factor in determining the potential for postnatal muscle accretion. In order to estimate the number of these myogenic cells, they were isolated from the three ages of mice according to the procedure of Bischoff (1974) as outlined in Materials and Methods. The cell pellets were resuspended in a known volume of complete medium, and aliquots were placed in collagen-coated wells of a Falcon Microtest II dish. Cells in the wells were Giemsa stained 5 hr after plating, the number of cells per well was counted, and the total number of cells per gram of muscle used for the isolation was calculated as outlined in Materials and Methods. Results of the experiments with 3, 4, 5 and 6 week mice are shown in Figure 2. The concentration of viable cells in muscle decreases sharply during the period of most rapid growth (3-4 weeks of age). Although both sexes of mice were pooled for this particular experiment, two prior experiments indicated that cell yield for males of a particular age was greater than that for females of the same age (3.6×10^5 cells/g for males versus 1.8×10^5 cells/g for females at 6 weeks). This result is consistent with the fact that females approach physiological maturity at a slightly earlier age than males. As the mice approached adulthood the number of cells/g began to level off, corresponding to a flattening of the growth curve (Figure 2).

Although cell yields of 10^5 cells/g were in accordance with the results of Bischoff (1974), this value probably was an underestimate of the total number of myogenic cells/g for two reasons. First, the method of isolation did not result in the complete digestion of the tissue. Bischoff

Figure 2. Effect of growth upon the total number of cells/g of hind limb muscle determined at 5 hr in culture. Counts were made as described in Materials and Methods. Points denoting total number of cells/g are the result of one to four determinations ().

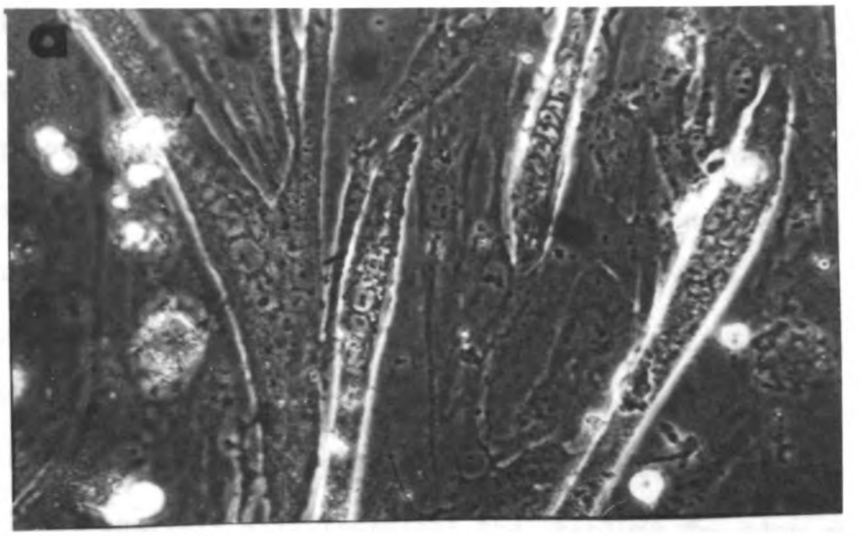

Body Weight (Grams)
Total Cells Per Gram (x 10^5)
Age (Weeks)
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
8

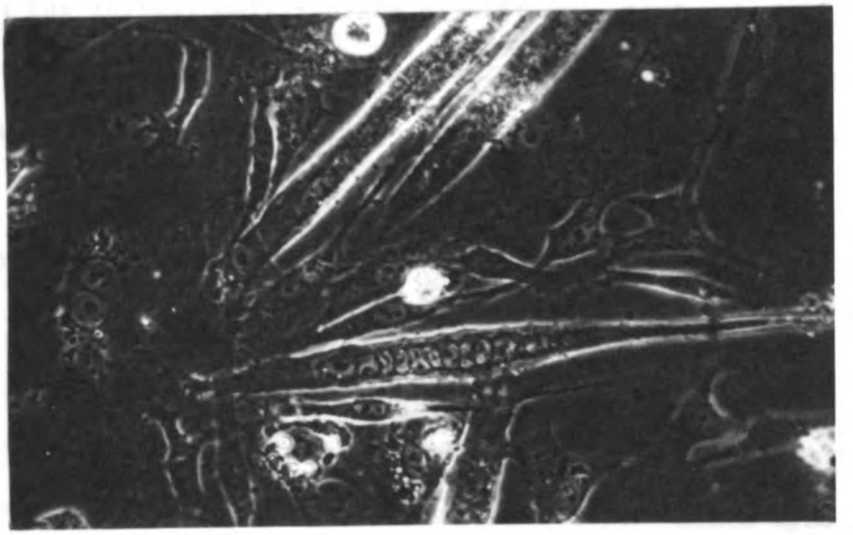
(1974) found cells by histological examination in the muscle fragments after dissociation. Secondly, not all cells remained viable after isolation or remained attached to the culture dish surface. For these reasons the actual cell yield estimated in culture was likely to be lower than that which existed *in vivo*. Figure 2 indicates a cell yield of approximately 1×10^5 viable cells/g for mice in early adulthood. Bischoff (1974), however, indicated that the number of satellite cells should be 2.5×10^5 cells/g in an adult rat based upon calculations using the results of Ontell (1973), but Bischoff (1974) in his own experiment, indicated that the number of myogenic cells/g was 1×10^5 based upon cell counts at 24 hr *in vitro*. Since preliminary experiments showed that cell attachment was completed by 5 hr *in vitro*, an overestimation of the frequency of satellite cells *in vivo* may have occurred in Bischoff's experiment if cells underwent one turn of the cell cycle during the first 24 hr in culture. In addition, when cells were isolated by the method of Bischoff (1974) and placed in culture, some of the cells never appeared bipolar or spindle shaped, and many never fused to form multinucleated myotubes. Therefore, not all of the cells isolated by Bischoff's method (1974) were myogenic. Thus, it is apparent that the satellite cell yield, based upon cell counts after 5 hr *in vitro*, is only a fraction of 1×10^5.

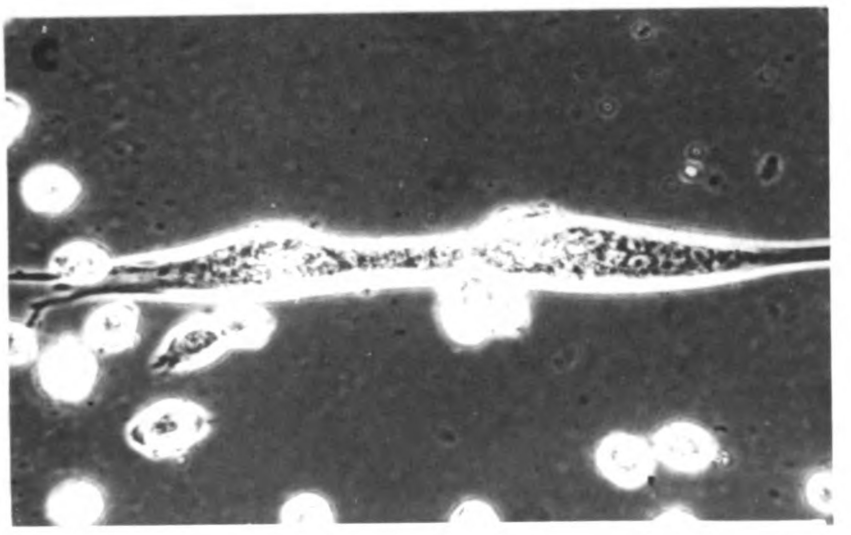
When cultures were allowed to proliferate extensively, multinucleated myotubes formed (Figure 3.a, 3.b, 3.c). Not all the cells fused, however, and single cells eventually became confluent over the entire surface of the plate between the myotubes.

In order to determine the percentage of myogenic cells isolated by enzymatic digestion, cloning experiments were performed. The cloning principle involved separating single cells by a large distance and allowing

Figure 3. Phase micrographs of multinucleated myotubes formed by the fusion of myogenic cells in vitro. Myotubes from (a) neonatal, (b) rapidly growing after 96 hr in culture, and (c) adult mice after 9 days in vitro. X 3200.


a

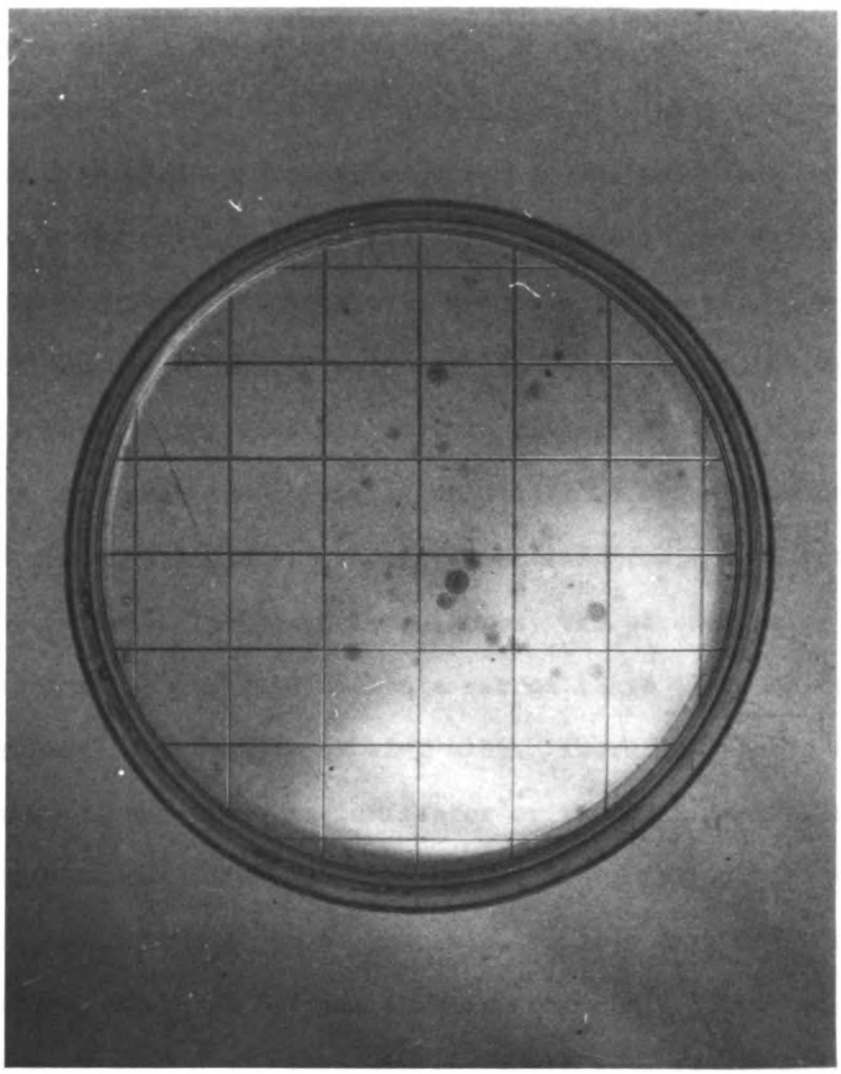



c

them to proliferate extensively. Since all cells in a clone were derived from one single cell and since only myogenic colonies exhibited fusion, the percentage of clones that contained multinucleated myotubes was indicative of the percentage of the initial cell isolate that was myogenic. In the initial cloning experiment cells were plated in 15 cm, collagen-coated polystyrene culture dishes. Three relative densities were plated in order to ensure that colony forming cells would remain separated by large distances (Figure 4). The percentage of myogenic cells determined by *in vitro* clonal analysis will represent the absolute fraction of myogenic cells in the initial cell isolate only if the plating efficiency is: (1) identical for all cell lineages; and (2) identical for all cell types in muscle of mice of various ages. If the isolated procedure was detrimental to the viability of a specific cell lineage, *in vitro* clonal analysis would indicate a higher percentage of cells in other lineages than that which existed *in vivo*. Failure of cellular attachment could result from dissociation-induced damages (enzymatic or agitative), or because of significant developmental differences (Hauschka, 1974b). The cloning principle depends on extensive proliferation of an isolated cell. Since myoblasts are post-mitotic cells, they cannot proliferate and because of low density they cannot fuse into multinucleated muscle colonies (Abbot et al., 1974). Even though this particular compartment of cells was of the myogenic lineage, cloning would cause an underestimation of the true percentage of myogenic cells. Such a population may have existed *in vivo* (Bischoff, 1974); however, the quantitiy of post-mitotic myogenic cells was not known.

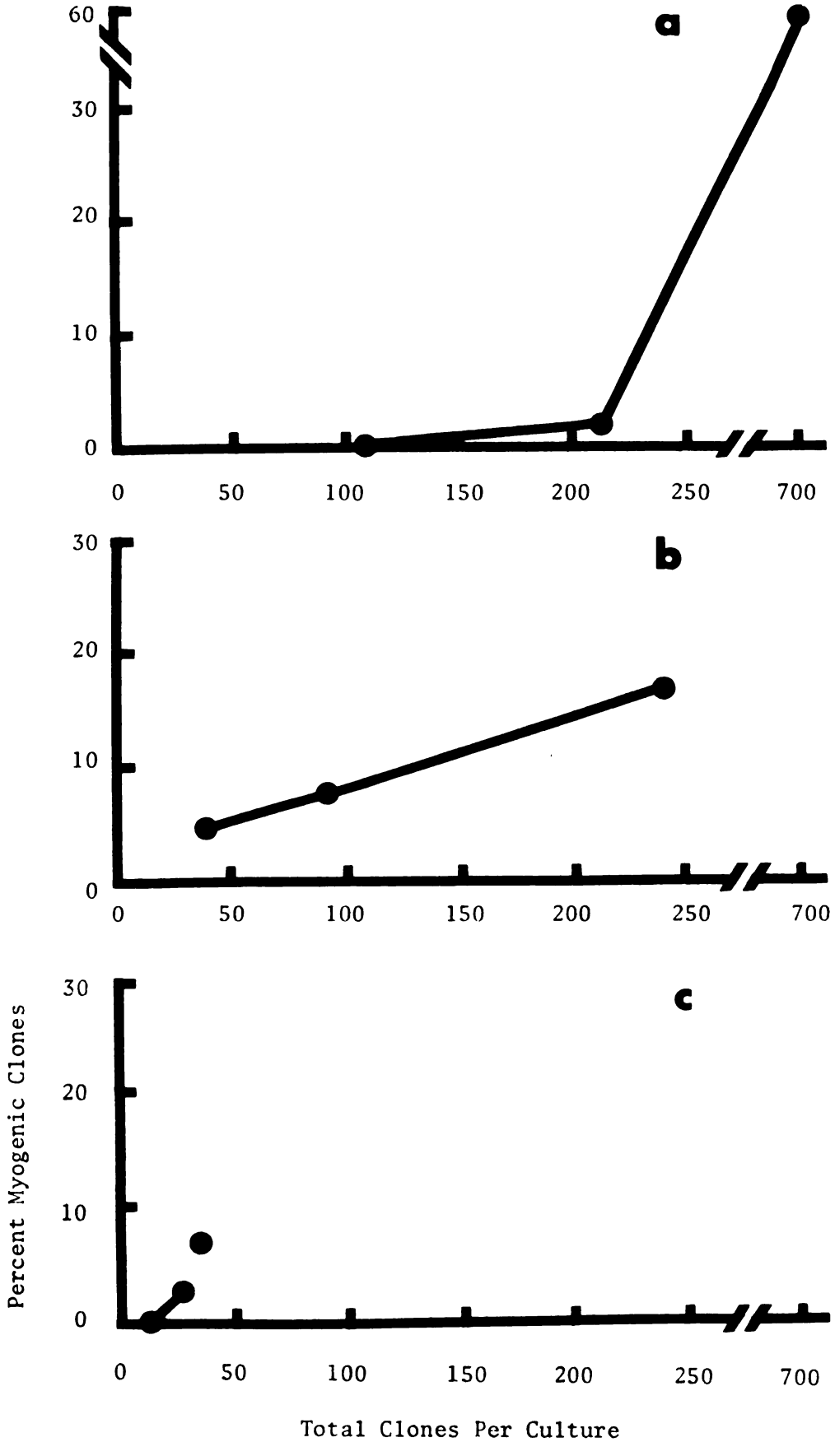
The first cloning experiment involved plating cells from three ages of mice (neonatal, rapidly growing and adult) at three densities (x, 2.5x, 5x) per age group. Plates were cultured between 11-20 days with

Figure 4. Photograph of a clonal culture which was fixed and Giemsa stained after 11 days in culture. Isolated colonies appear over the entire surface of the plate (15 cm in diameter).



medium changes after 1 and 6 days. Plates were Giemsa stained after varying periods in culture since higher density cultures displayed colonies which were confluent over the surface of the plate sooner than low density cultures. Clones were analyzed according to the procedure outlined in Materials and Methods. The data in Figure 5 indicate that the percentage of clones which displayed muscle differentiation was dependent upon the clonal density in the cultures. At all three ages the high density plates consistently had a greater percentage of muscle colonies than low density plates. The percentage of muscle colonies has been shown to be proportional to the number of colonies per culture dish (White and Hauschka, 1971) if measures are not taken to prevent it as described below (Hauschka, 1974a). This suggested that the clones somehow altered the growth medium to enhance muscle differentiation. In the adult cultures, the percentage of myogenic clones had a more rapid increase within a narrow range of clonal densities compared to neonatal and rapidly growing mice. Therefore, these myogenic cells either conditioned their medium faster or required less of a conditioning effect. Clones from neonatal cells showed very little expression of muscle differentiation at clonal densities of less than 215 clones per plate, whereas densities of less than approximately 40 clones/plate for rapidly growing and 20 clones/plate for adult were required to prevent fusion in muscle cell clones. White et al. (1975) showed that as cells were cloned from embryos of increasing age during the early stages of embryogenesis, they required less of a conditioned medium effect for fusion. The results in Figure 5 indicated that an analogous situation occurred during postnatal myogenesis. Neither the developmental significance of the changing sensitivity of cells to conditioned medium nor the mechanism of the conditioned medium effect is understood.

Figure 5. Effect of clonal density upon the percentage of myogenic clones in culture.
(a) neonatal
(b) rapidly growing
(c) adult mice
Clonal cultures were prepared and analyzed as described in Materials and Methods. Each point is the mean of three cultures.


a
b
c
60
30
20
10
0
0
50
100
150
200
250
700
Percent Myogenic Clones
Total Clones Per Culture

In vitro differentiation is somehow dependent upon the compartment of each MCF cell in the lineage at its time of removal from intact muscle tissue. Cells will not become capable of terminal muscle differentiation until they have entered a post-mitotic, fusion capable compartment. The properties of the medium surrounding myogenic cells changes with age of the culture. This change is the result of a rise in clonal size which increases the final concentration of the conditioning effect(s) in the culture medium. Therefore, the true percentage of MCF cells in the cell isolate from the three various ages cannot be precisely determined because of differences in the clonal density, and rates of cell proliferation within the clones. Also, the presence of a number of large colonies of mononucleated, spindle-shaped cells, similar to the single cells in clones which displayed fusion, indicated that the culture conditions did not provide for the expression of the maximal percentage of muscle colony differentiation (Figure 6).

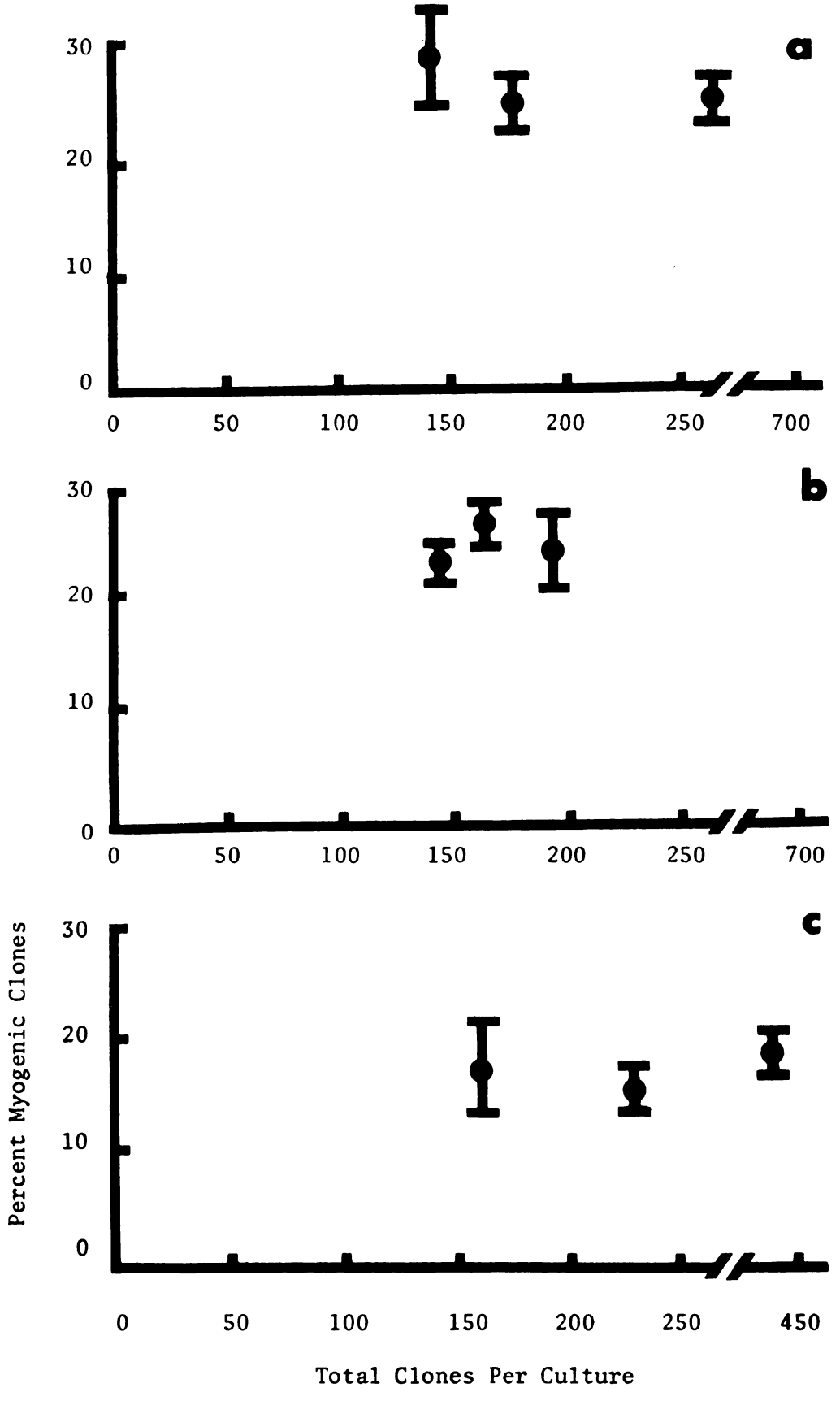
A second cloning experiment was designed with two provisions in mind to alleviate the dependence of muscle colony formation on clonal density. First, continuous replication was induced for a period of 10 days with daily medium changes to prevent conditioning effects. Changing medium daily permitted cell proliferation so that adequate numbers of cells were obtained and helped to prevent cell fusion by keeping macromolecular components added to the medium by the cells below the minimum levels that induced fusion. Secondly, cultures were provided with conditioned medium for a period of 48 hr at the end of 10 days of replication to allow for fusion. Conditioned medium prepared as described in Materials and Methods was then added to the cultures. Cultures exposed to conditioned medium for 48 hr after an extended period of proliferation displayed no significant

Figure 6. Bright field micrograph of a: (a) Giemsa stained myogenic clone, and (b) a colony of spindle-shaped, mononucleated cells similar in morphology to the mononucleated cells in the muscle forming colony. X 1250.


a


b

Figure 7. Effect of clonal density upon the percentage of myogenic clones in culture.
(a) neonatal
(b) rapidly growing
(c) adult mice
Conditioned medium was used and prepared as described in Materials and Methods. Each point is the mean of three cultures, with error bars denoting the standard deviation.


a
b
c
30
20
10
0
0
50
100
150
200
250
700
450
Percent Myogenic Clones
Total Clones Per Culture

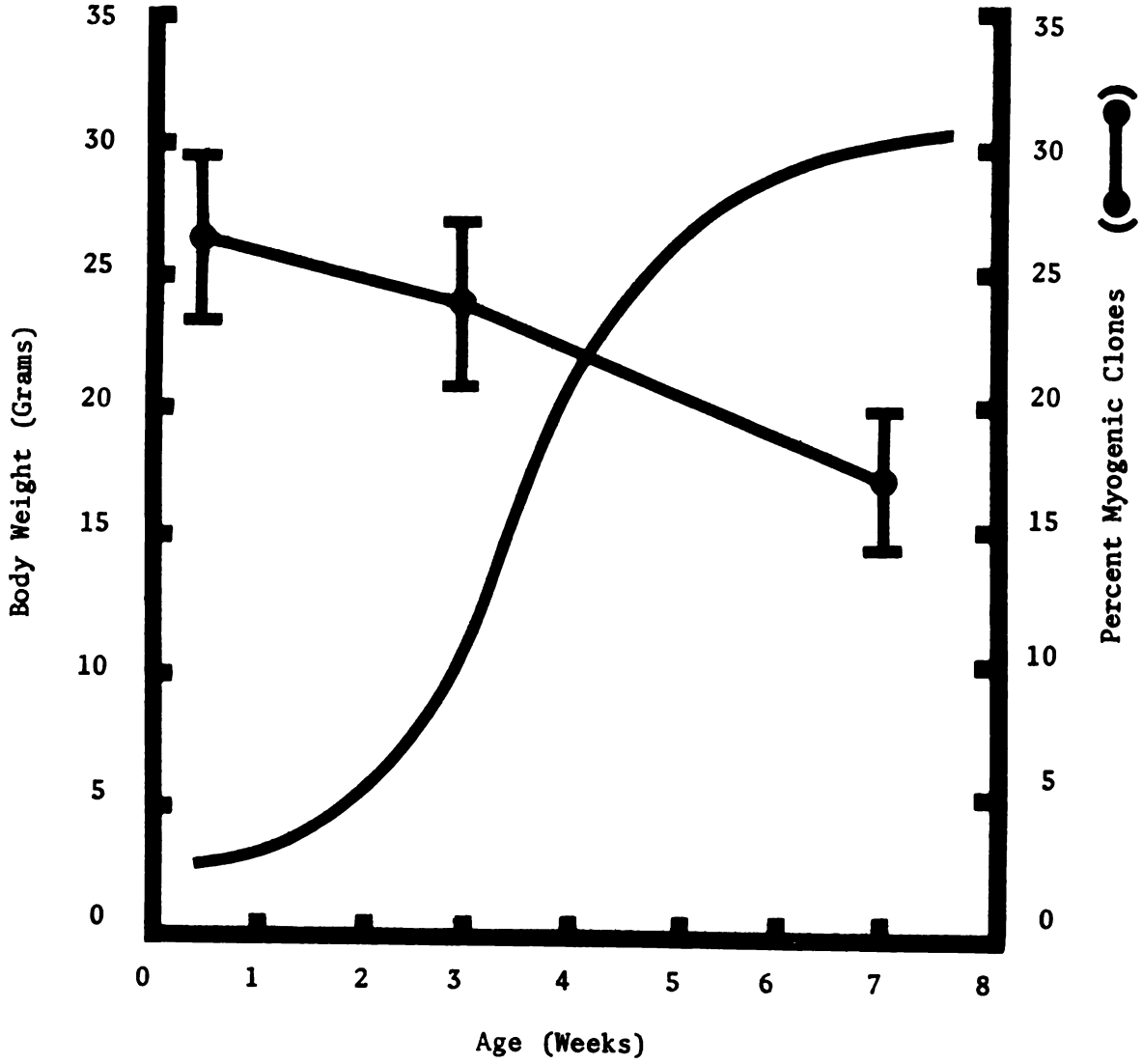
difference in the percent MCF at either of three clonal densities (Figure 7).

Although the experiments on percent MCF cells shown in Figure 7 are independent of clonal density, the percentage is substantially less than the maximum percent MCF cells observed in neonatal cultures of the highest cell density (Figure 5.a). This difference, ~60% in Figure 5.a compared to ~25% in Figure 7.a, resulted from an overestimation of the percent MCF cells in the first cloning experiment. When clones were analyzed in cultures where too many cells were initially plated (causing overlap of macroscopic clones), two clones in close proximity may have been scored as being separate colonies when they actually were derived from a single myogenic cell. It is also possible that a colony could contain both myotubes and nonmyogenic cells if two separate cells attached in very close proximity to one another. Yet, this colony would be considered myogenic (as defined by clonal analysis procedures in Materials and Methods) resulting in an underestimation of the total number of nonmyogenic clones.

With the selfconditioning effect properly controlled, the percent MCF cells isolated from hind limb muscles decreased with increasing age (Figure 8). These experiments provide an accurate appraisal of the relative change in the number of myogenic cells which existed *in vivo* as described below.

By weighing the tissue just prior to mincing and by accounting for dilutions made during plating, the total number of colony forming cells/g was determined from the total number of clones per plate. Data from both experiments were combined to yield an average number of colony forming cells/g. These data can be combined since the total number of viable cloned cells was examined without regard to the percentage that was

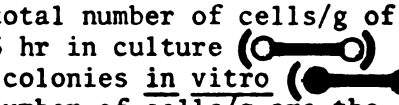
Figure 8. Percent myogenic clones in culture of cells from the hind limbs of mice at various stages of the growth curve (●—●). Error bars denote the standard deviation for nine plates counted at each age.

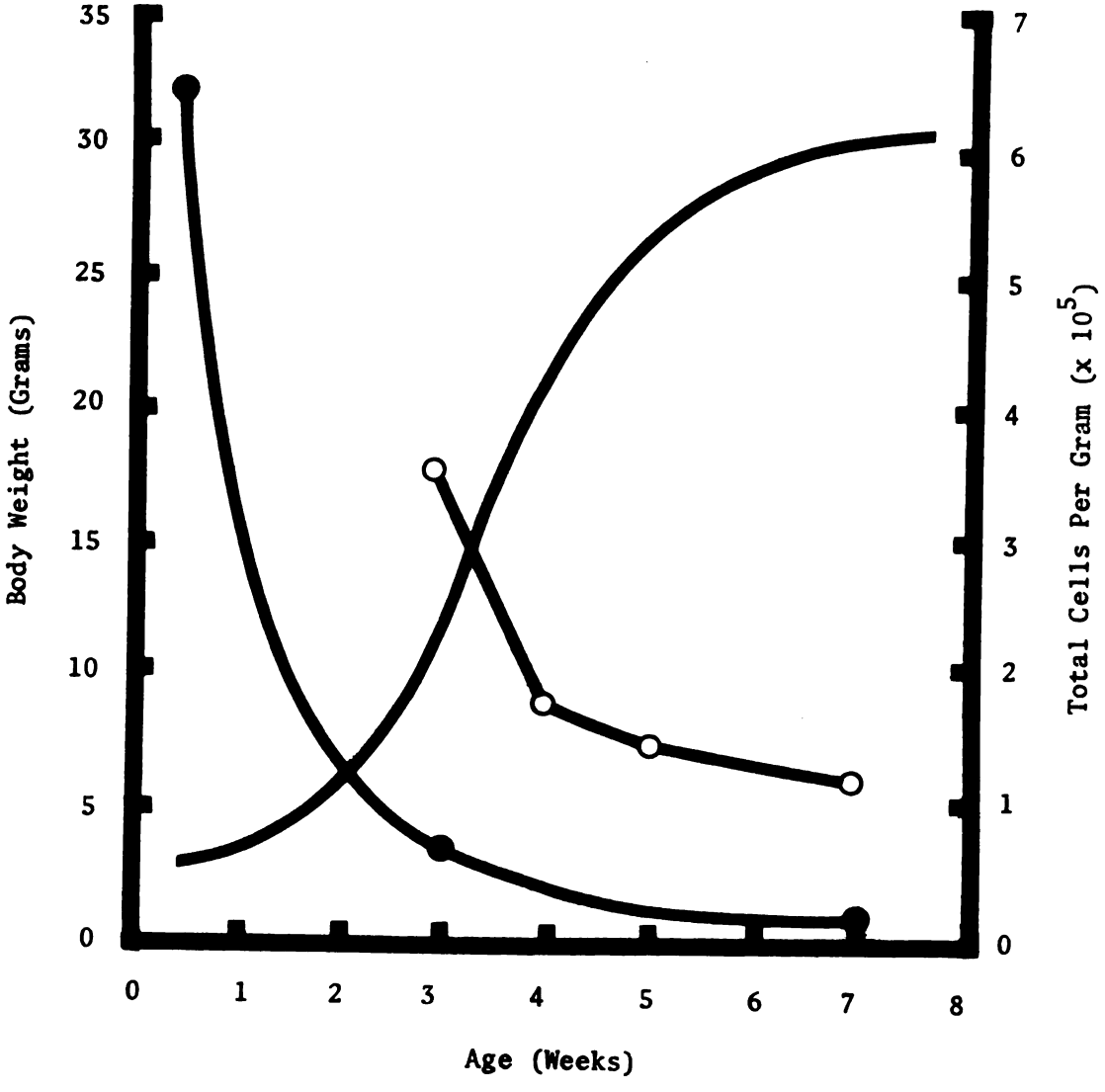

35
30
25
20
15
10
5
0
Body Weight (Grams)
0
1
2
3
4
5
6
7
8
Age (Weeks)
Percent Myogenic Clones

myogenic. The number of colony forming cells/g decreased with age of the mouse (Figure 9). A comparison of the above data to the cells/g data obtained by 5 hr counts (Figure 2) showed that the number of colony forming cells/g calculated from cloning experiments was only a small fraction of the value calculated after 5 hr in culture. This was caused by the reduced plating efficiency of these cell isolates. Clonal cultures were held for extended periods of time at extremely low cell density and both of these factors reduce cell viability *in vitro*. Clonal analysis required that cells proliferate extensively and form colonies in order to be counted, whereas cell counts at 5 hr only required cells to attach to the surface of the plate. In addition, the cell populations from the three different ages of mice were not equally affected by cloning. The number of colony forming cells/g for 3 week old mice was 21% of the 5 hr value, whereas it was 2.4% of the 5 hr value for 7 week old mice. Therefore, either the plating efficiency of the initial cell isolate or the ability to proliferate *in vitro* was reduced for mice of increasing age.

To estimate the number of myogenic cells in postnatal muscle based upon the results of cloning, the percentage of myogenic clones was multiplied by the total number of cells/g determined by 5 hr counts. Although this number was not exactly equal to the number of MCF cells/g *in vivo* since not all the cells plated attached or formed clones, the relative changes between ages were quite large. The number of MCF cells/g decreased ~330 fold between birth and early adulthood (Figure 10).

If *in vivo* proliferation of the satellite cell is followed by one of the daughter cells fusing and the other remaining beneath the basement membrane, the absolute number of satellite cells in adult muscle should remain static (Allbrook et al., 1971; Moss and LeBlond, 1971). The

Figure 9. Effect of growth upon the total number of cells/g of hind limb muscle determined at 5 hr in culture  and by the ability to form colonies in vitro. Points denoting the total number of cells/g are the result of one to four determinations.

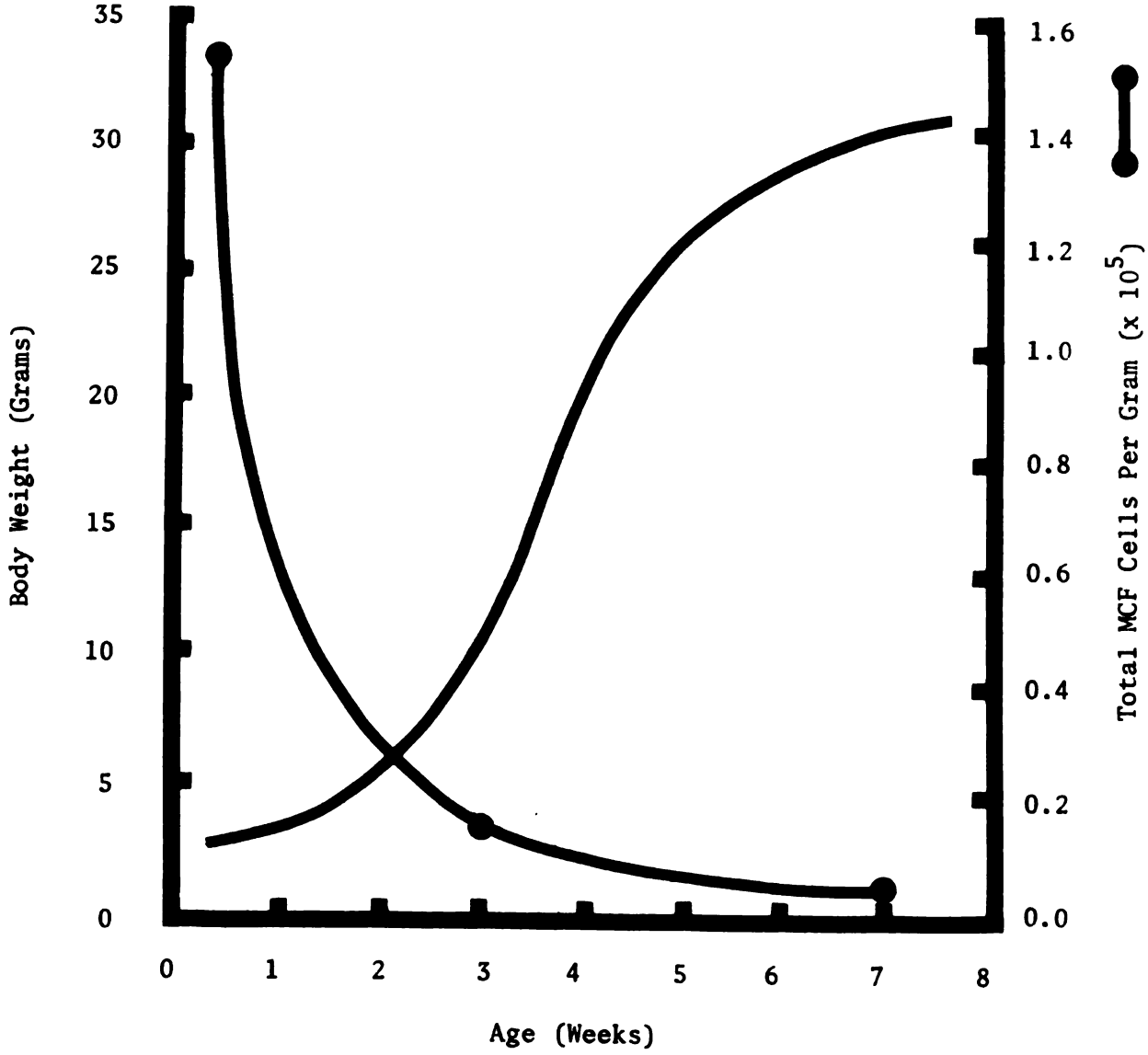

Body Weight (Grams)
Total Cells Per Gram (x 10^5)
Age (Weeks)
35
30
25
20
15
10
5
0
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8

concentration of satellite cells in muscle should still decrease, however, due to an increase in muscle mass. Results of this in vitro study showed that as mice approached early adulthood there was a much less drastic decrease in MCF cell concentration (Figure 10). This approximately 4 fold decrease in satellite cell concentration (Figure 10) between 3 and 7 weeks may have been due to the approximate 3 fold increase in body weight (Figure 1).

The frequency of replicating myogenic cells declines as myogenesis proceeds both in vitro (Bischoff and Holtzer, 1969) and in vivo (Marchok and Herrmann, 1967), yet a small number of presumptive myoblasts remain in vitro from cell suspensions of mature muscle tissue (Bischoff and Holtzer, 1969). Clonal analysis was the method by which this research attempted to determine the concentration of myogenic cells in postnatal muscle. Yet in vitro quantitation was subject to error due to the plating efficiency of the cell isolate and the compartment of each myogenic cell in the muscle lineage at the time of in vivo removal. These experiments show clearly, however, that there are significant changes with age in the composition of the colony forming cells in skeletal muscle. The concentration of myogenic cells is lower in adult skeletal muscle than in neonatal and rapidly growing muscle; however, these experiments suggest that the fusion potential is independent of age. In an attempt to extend these qualitative observations, another set of experiments was conducted to examine the relative ability of satellite cells to synthesize total protein and myosin heavy chains (MHC) and to assemble the myofibrillar proteins into apparently intact, functional myofibrils.

When cells isolated by the method of Bischoff (1974) were cultured for extended lengths of time, indications of in vitro myogenesis became

Figure 10. Total number of MCF cells/g of hind limb muscle from mice at various stages of the growth curve. Points are derived by multiplying the percentage of myogenic clones times the total number of colony forming cells/g (●—●).


35
30
25
20
15
10
5
0
Body Weight (Grams)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Total MCF Cells Per Gram (x 10^5)
0
1
2
3
4
5
6
7
8
Age (Weeks)

evident. Cells from these suspensions appeared either spindle or bipolar shaped and elongated in culture (Figure 6.b), typical of myogenic cells in vitro. These cells were capable of DNA synthesis and mitotic activity as there was an increase with time in the number of cells in each culture. Fusion requires that cell density be high enough for sufficient conditioning of the medium (White and Hauschka, 1971) and cell membrane interaction to have occurred.

Once cells were capable of fusing, they aligned in an end-to-end manner, and their membranes touched and fused (Figure 11.a, 11.b, 11.c). In immature myotubes the nuclei were randomly oriented and densely packed within the cytoplasm (Figure 12.a, 12.b, 12.c). Figure 12 also shows mononucleated, bipolar cells lined up parallel to immature myotubes. Single cells were observed to fuse with myotubes less than 3 days old (Figure 12.c). Bischoff and Holtzer (1970) have indicated that post-mitotic myoblasts will not fuse with myotubes 4 days or older in vitro. No evidence was found in this research to contradict this statement.

As early myotubes matured, the myonuclei became oriented in the center of the myotube (Figures 13 and 14.a). This morphological event is coincidental with the synthesis and assembly of the myofibrillar proteins at the periphery of the myotube (Fischman, 1970, 1972). Multinucleated syncitia were capable of fusing with one another. In vitro fusion of myotubes required no particular orientation of the myotubes to one another. They were perpendicular (Figure 13.a), oblique (Figure 13.b), or parallel to each other (Figure 13.c).

The extent of multinuclearity of myotubes in vitro varied greatly and depended upon the density of cells in the Mb compartment. Therefore, colonies formed during cloning experiments contained the highest density

Figure 11. Phase micrographs of satellite cell progeny in alignment with each other so their cytoplasms will fuse.
(a) bipolar, elongated cells from neonatal mice lining up side-by-side and end-to-end after 68 hr in culture
(b) spindle-shaped cells from rapidly growing mice after 72 hr in culture
(c) spindle-shaped cells from adult mice after 5 days in culture
X 3200.

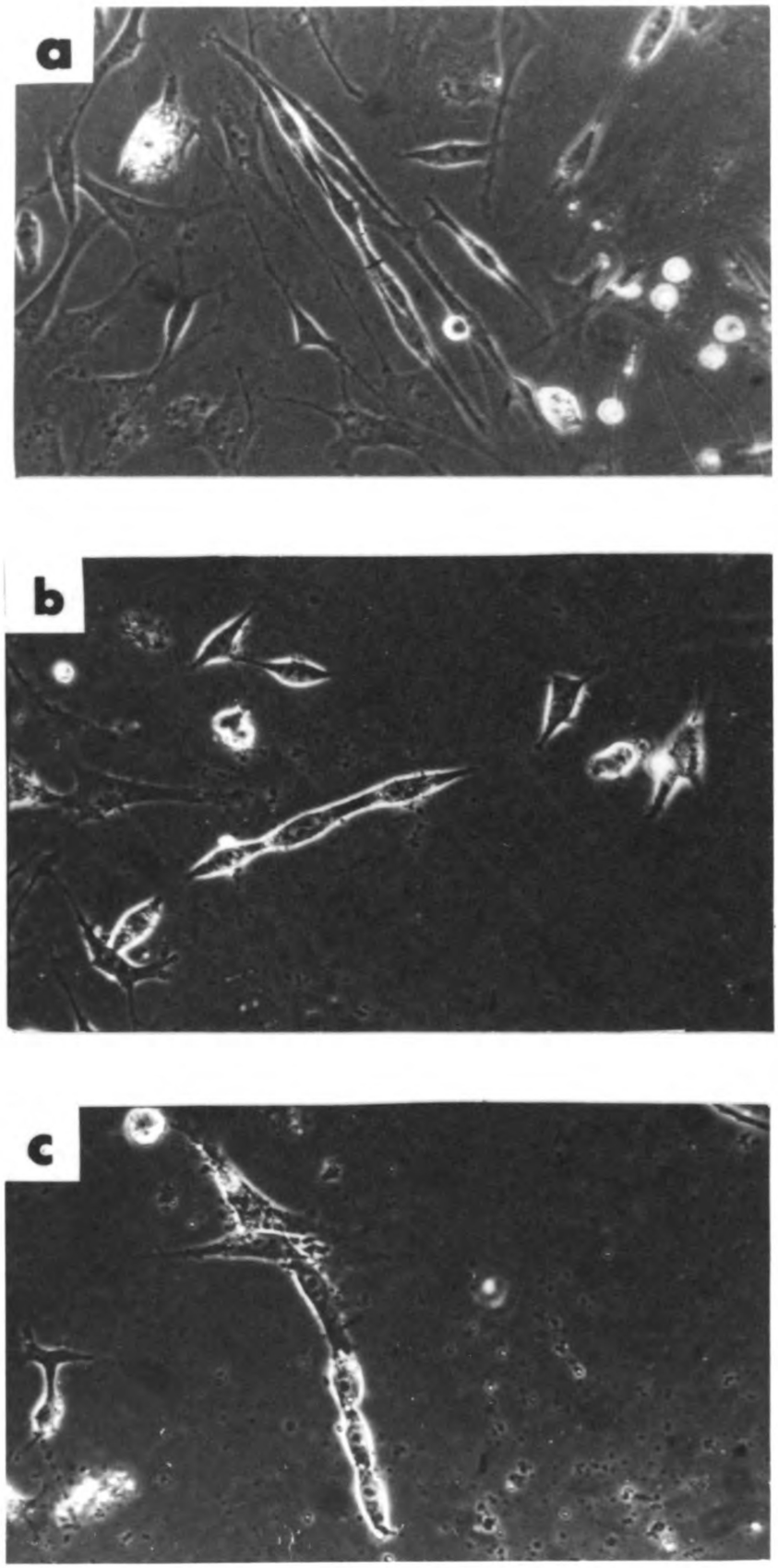
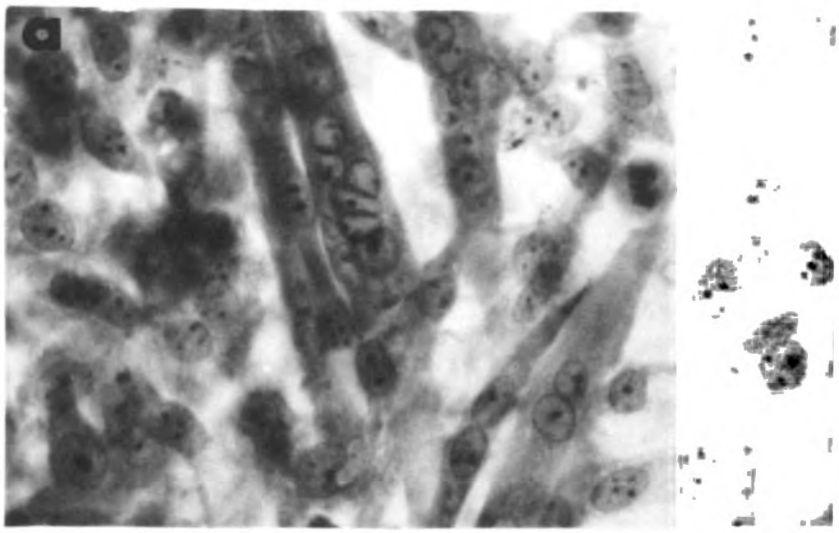
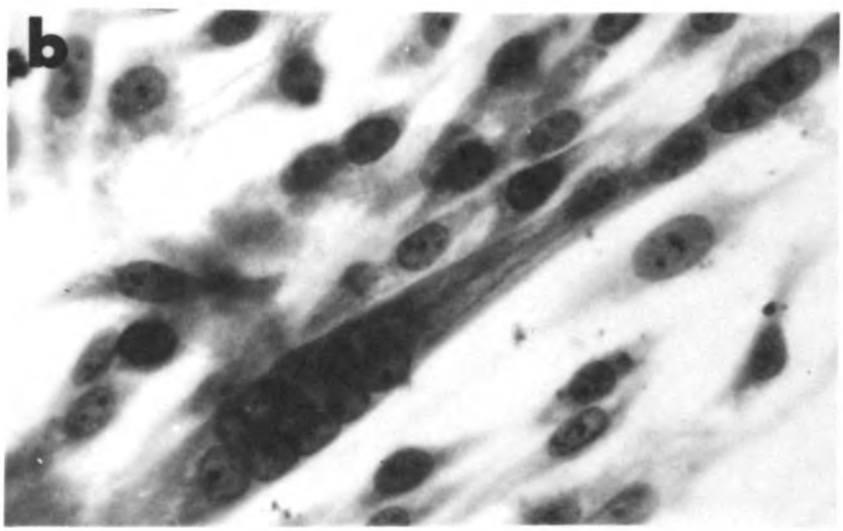

a
b
c

Figure 12. Micrographs of satellite cell progeny which have undergone fusion *in vitro* to form multinucleated myotubes.
(a) fused nuclei of neonatal satellite cell progeny after 4 days; Giemsa stained; bright field optics. X 5000.
(b) myotubes formed from the fusion of satellite cell progeny from rapidly growing mice; Giemsa stained after 5 days; bright field optics. X 5000.
(c) phase micrograph of a 48 hr old myotube formed by the fusion of nuclei derived from satellite cells of adult mice after 7 days. X 3200.


a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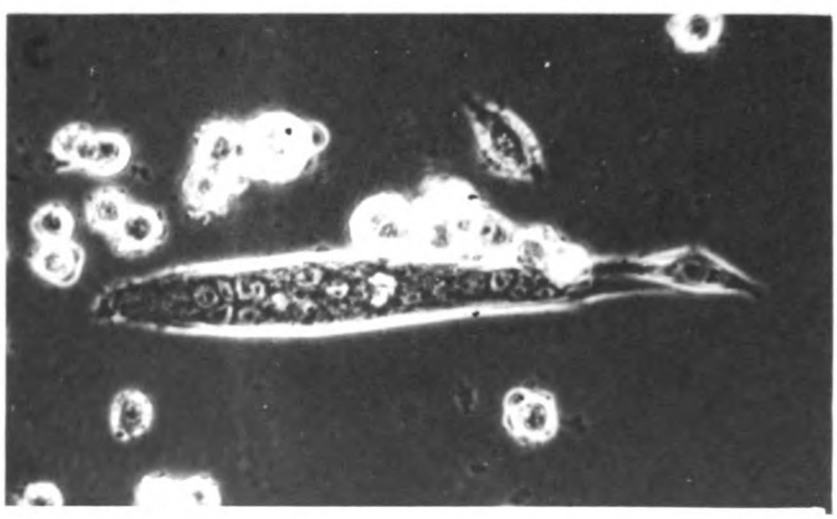
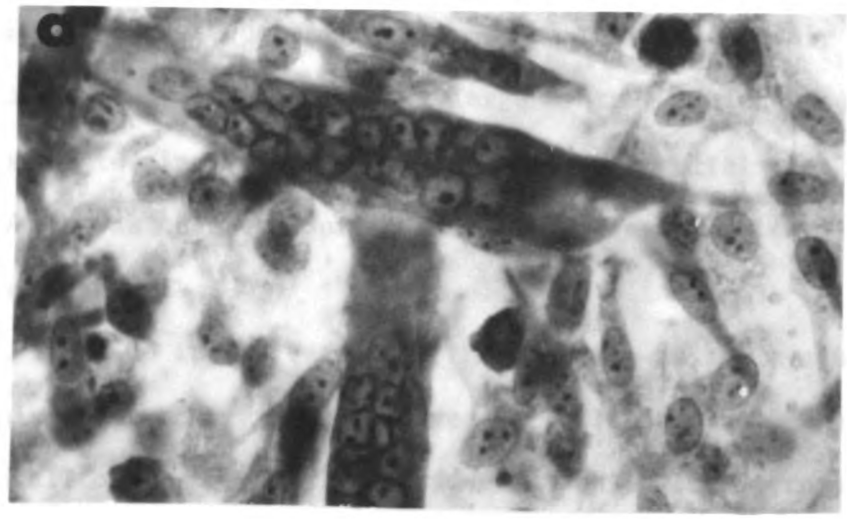
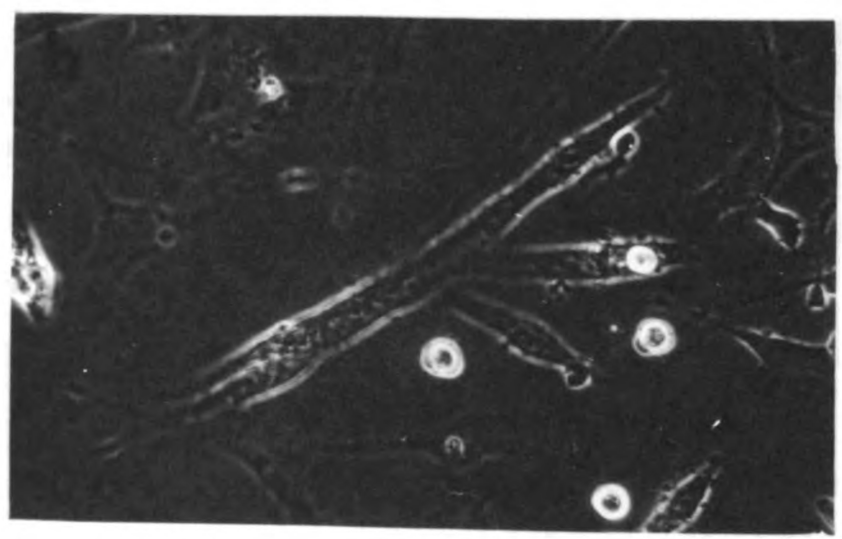
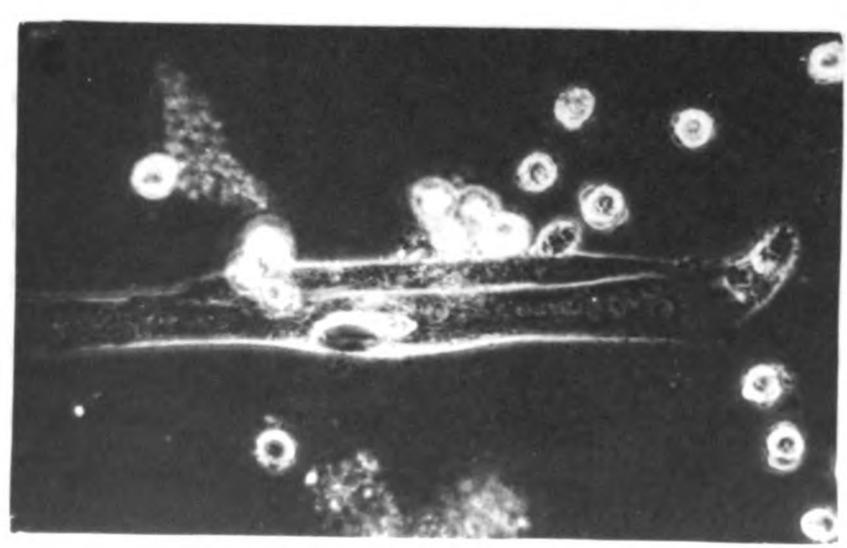

c

Figure 13. Micrographs depicting the *in vitro* fusion of myotubes with one another.
(a) two myotubes from neonatal cells after 6 days in culture; Giemsa stained; bright field optics. X 5000.
(b) fusion of myotubes formed from the progeny of satellite cells from rapidly growing mice after 72 hr in culture; phase contrast optics. X 3200.
(c) two myotubes from satellite cell progeny of adult mice after 8 days *in vitro*; phase contrast optics. X 3200.







of myogenic cells and consequently displayed the most extensively multinucleated myotubes. Figure 14.b shows a large myotube, which resulted from the fusion of two individual myotubes (the two parallel ones in Figure 13.c) which was observed during in vitro differentiation with the use of phase microscopy.

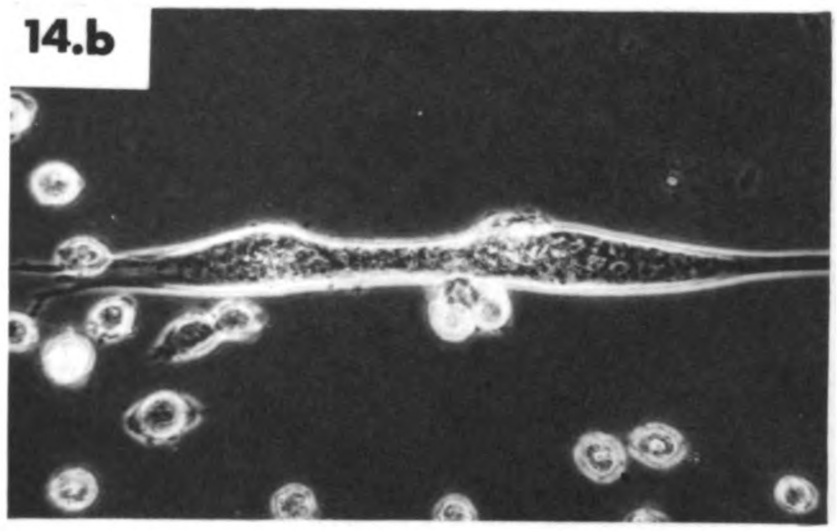
Myonuclei lined up in the center of the myotubes as the myotubes elongated. This event occurred in cultures of cells from all three ages of mice as did all other previously-mentioned morphological events. However, elongation did not occur in some myotubes in adult cultures with fused nuclei remaining as large "myosacs" (Figure 15). These "myosacs" stained very intensely due to the dense packing of the myonuclei and the specificity of the Giemsa stain for nucleic acids which are highly concentrated within the myosac. It is possible that satellite cell progeny from adult muscle required more of the "elongation factor" reported to be in the embryo extract component of culture medium (de la Haba and Amundsen, 1974).

Myotubes sometimes displayed a high degree of branching with increasing age of the culture (Figure 16.a, 16.b). In order to evaluate this morphological occurrence and also the maximum potential for myogenic differentiation, cultures were maintained for up to 21 days in vitro. The fact that nonmyogenic cells in the plate would completely overgrow the cultures and obscure the myotubes was a limiting factor in maintaining cultures for extended periods. Therefore, inhibitors of cell replication were used to prevent proliferation of the nonmyogenic cell population after approximately 10% of the myogenic cells had fused. The presence of Ara-C- or FdUrd-containing medium prevented the confluence of nonmyogenic cells which often lead to the loss of material off the collagen-coated plate surface. Figure 16.b was an eleven day old culture from cells of rapidly growing

Figure 14. Micrographs of large multinucleated myotubes *in vitro*.
(a) a large myotube in culture from rapidly growing mice after 9 days; Giemsa stained; bright field optics. X 1250.
(b) phase contrast micrograph of a myotube from adult mice 9 days *in vitro* (24 hr later than Figure 13.c). X 3200.

Figure 15. Bright field micrograph of elongated myotubes and myosacs (arrows) derived from the progeny of satellite cells of adult mice; Giemsa stained. X 1250.


14.a


14.b

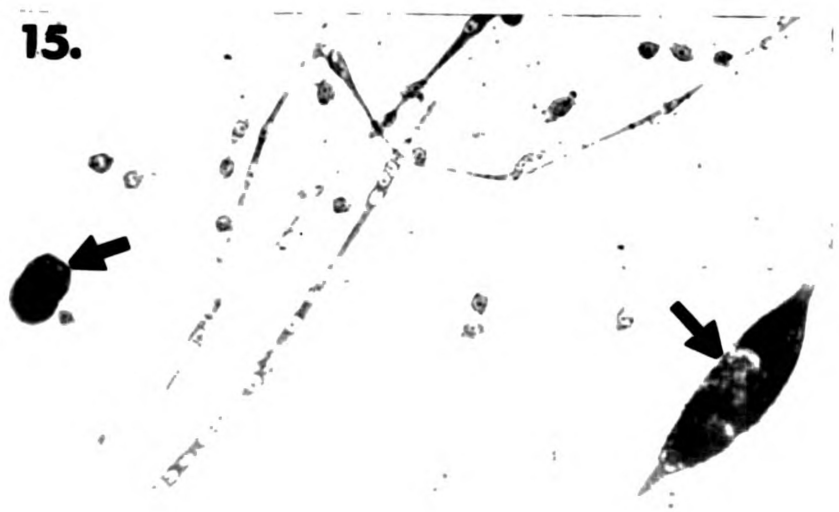
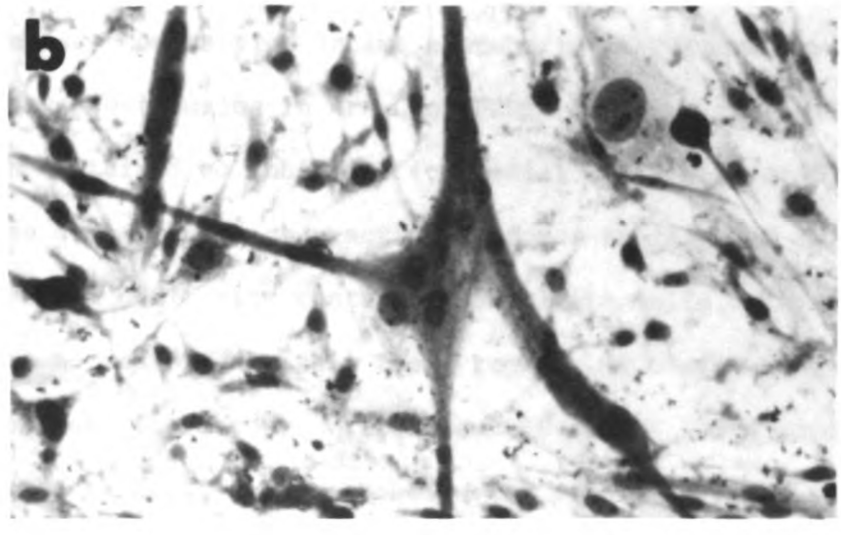

15.

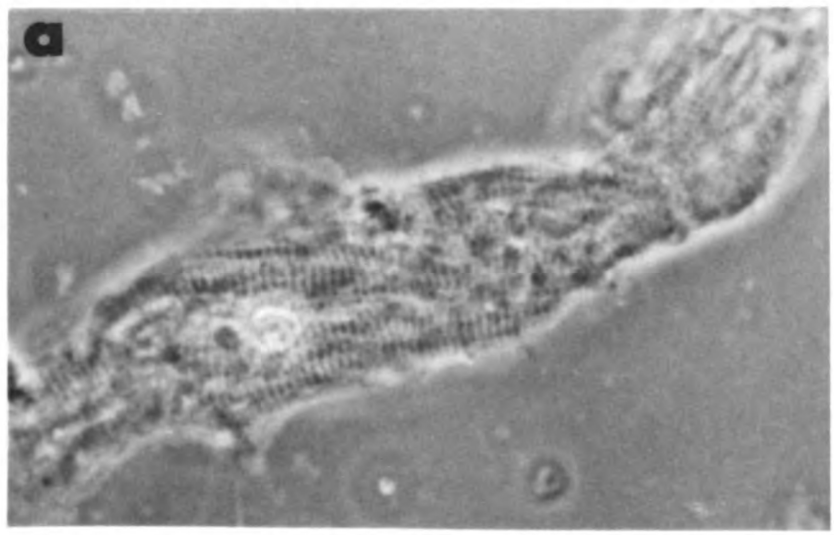
Figure 16. Bright field micrographs of branched myotubes derived from satellite cell progeny of rapidly growing mice. Eleven days in culture; Giemsa stained. X 2000.
(a) Ara-C culture
(b) control culture


b

mice. Ara-C (which inhibits DNA polymerase activity) was placed on this culture on day 7. By day 11 there was a larger percentage of nuclei within myotubes than in control cultures (see Figure 16.a, 16.b). Preliminary results showed that FdUrd prevented cell replication but did not increase the percent fusion as much as Ara-C because FdUrd was not as toxic. The use of inhibitors of cell proliferation permitted cultures containing satellite cells to be maintained *in vitro* for extended periods of time, allowing for more extensive *in vitro* muscle differentiation and less interference by nonmyogenic cells.

A number of observations were made to determine if differentiating myotubes, formed by the fusion of satellite cell progeny from neonatal, rapidly growing, and adult mice are capable of synthesizing and assembling the myofibrillar proteins. First, spontaneous contraction of myotubes in cultures from each age of mice was observed with an inverted phase microscope. This observation was good evidence for the presence of contractile proteins. Contraction became a property of cultured myotubes as early as two days post-fusion in Ara-C or FdUrd cultures, as well as in cultures which contained no inhibitor (controls). The work of Bischoff (1974) had also demonstrated contracting myotubes in cultures derived from cell suspensions of adult mice containing satellite cells. Cross-striated myofibrils were not observed within the cytoplasm of contracting myotubes using phase contrast microscopy. Yet, Bischoff (1974) found evidence of cross-striated myofibrils in myotubes using polarized light microscopy. Although some degree of myofibril organization must have been present in order for contraction to occur, banding in myotubes *in vitro* was not visible when using phase contrast optics. When myofibrils were prepared from myotube cultures (as outlined in Materials and Methods), however, phase

Figure 17. Phase micrographs of myofibrils from neonatal myotubes grown 11 days in culture.
(a) fragmented myofibrils with striations. X 12,500.
(b) fragmented portion of a myotube depicting striations and myonuclei. X 20,000.


a

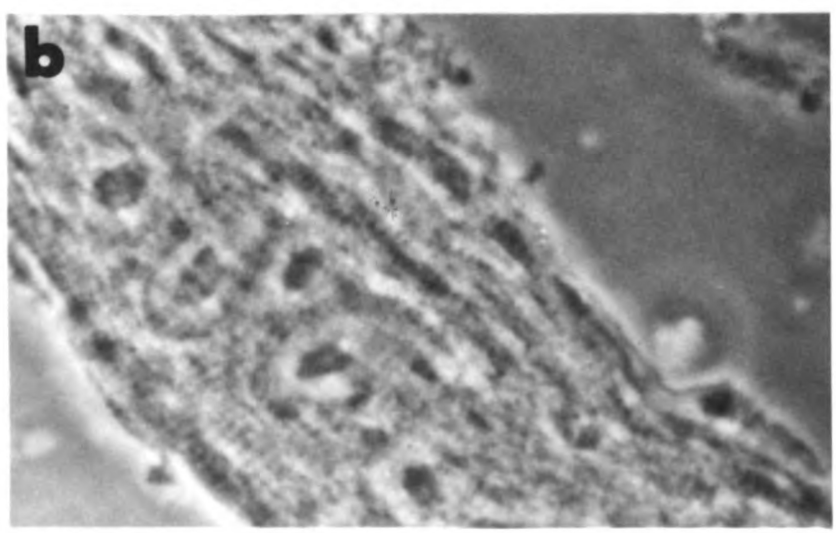
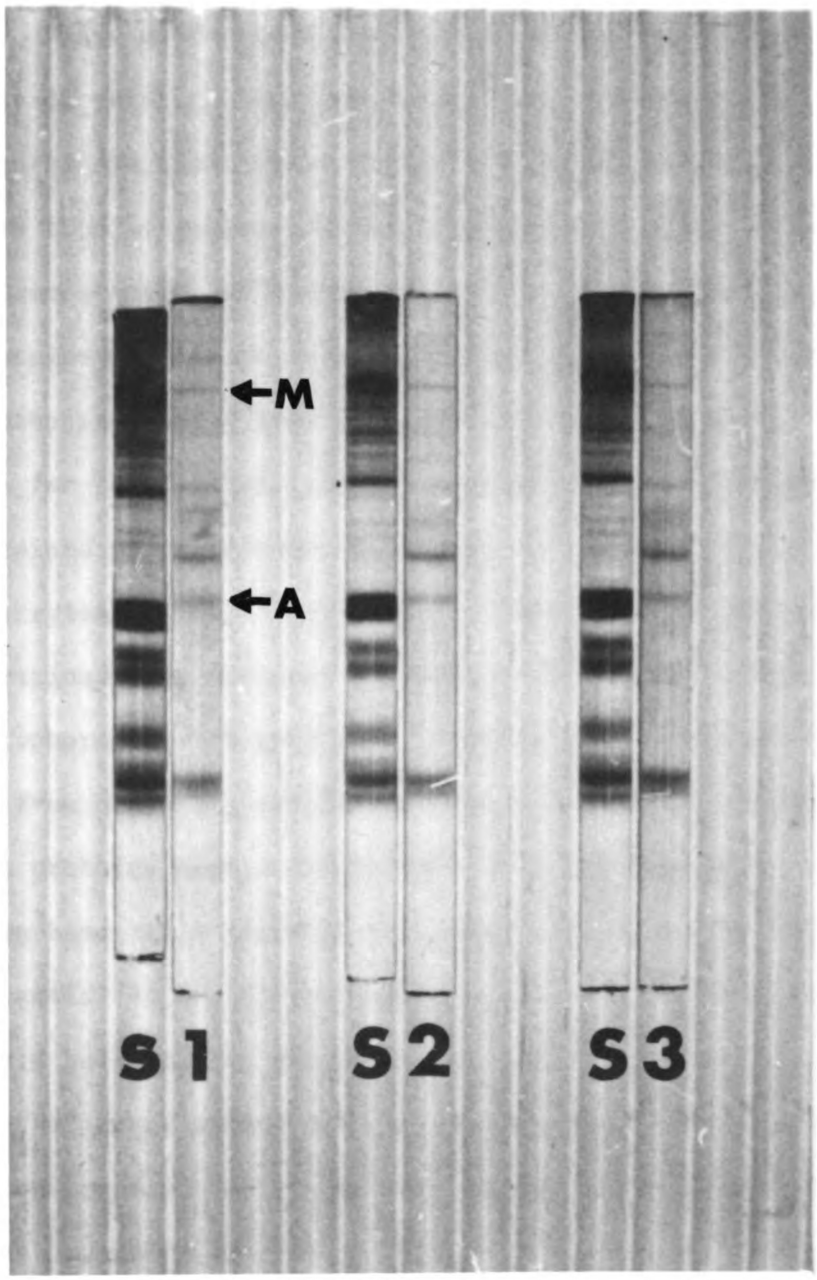

b

Figure 18. SDS-polyacrylamide gel electrophoresis of myofibril preparations from neonatal, rapidly growing, and adult Ara-C cultures. Standards (S) consist of 125 ug of rabbit myofibrils loaded onto 10% gels. Gels of myofibril preparations from: (1) neonatal, (2) rapidly growing, and (3) adult cultures were coelectrophoresed with standards.


M
A
S1
S2
S3

microscopy revealed fragments of cross-striated myofibrils (Figure 17.a, 17.b). Striations were evident on cylindrically shaped fragments of myofibrils in myofibril preparations from cultures of cells from all three ages of mice. Phase micrographs demonstrated alternate light (I-band) and dark (A-band) zones. Therefore, it is evident that myofibrillar myosin (thick filaments) and actin (thin filaments) were synthesized. These filaments were then assembled into the structural unit of muscle, the sarcomere. Demonstration of contraction also indicated that these myofibrillar proteins which were involved in the contractile mechanism must have been synthesized and assembled by satellite cell progeny.

Evidence for the synthesis of the myofibrillar proteins myosin and actin was obtained by electrophoresing aliquots of myofibril preparations on 10% SDS-polyacrylamide gels as outlined in Materials and Methods. Myofibril preparations from cultures containing fused satellite cell nuclei were coelectrophoresed with myofibril preparations from fresh rabbit muscle. Preparations from neonatal, rapidly growing, and adult Ara-C cultures contained the proteins myosin and actin. This was evidenced by the presence of two protein bands that migrated the same distance as the MHC (200,000 daltons) and actin (44,000 daltons) polypeptides of the rabbit myofibrils on the standard gels (Figure 18).

The observation of contracting myotubes in cultures from which the myofibrils were prepared in conjunction with the above results supports the conclusion that myotubes derived from satellite cells are capable of synthesizing and assembling the myofibrillar proteins into intact, functional myofibrils in vitro.

The results discussed up to this point have qualitatively dealt with the relative ability of satellite cells to fuse and to assemble the

myofibrillar proteins into contractile units. Another goal of this study was to determine if the rates of myofibrillar protein synthesis, total protein synthesis and degree of differentiation were influenced by the age of mice from which the satellite cells were isolated. Rates were determined at different stages of differentiation in both control and Ara-C treated cultures. Stages of differentiation were defined by the extent of myotube formation and the results were expressed as the percentage of cell fusion.

Ara-C prevents mitosis by inhibiting DNA polymerase activity (Chuang and Chuang, 1976). While most single cells were prevented from replicating by Ara-C, post-mitotic Mb were still capable of fusing since Ara-C does not interfere with the fusion process. Thus, Ara-C prevented proliferation of the nonmyogenic cell population and its eventual confluence over the culture surface. The number of nuclei per culture dish decreased after Ara-C administration, indicating that Ara-C not only inhibited replicating single cells but was also cytotoxic to some of them. Therefore, addition of Ara-C resulted in cultures with a higher percent fusion than control cultures. The use of Ara-C provided a culture model in which myofibrillar protein synthetic ability of myogenic nuclei could be more accurately assessed.

Ara-C was added to cultures after approximately 10% of the nuclei were in multinucleated myotubes. The amount of time between plating of the cell suspension and the initiation of fusion varied and depended upon the initial plating density (Konigsberg, 1971). Cells were plated at 2.25×10^4 cells/cm^2 which allowed for at least 10% fusion before nonmyogenic cells became confluent.

The extent of muscle differentiation was determined just prior to the addition of Ara-C and every 48 hr thereafter for a period of 10 days.

Cultures were Giemsa stained and the percent fusion calculated as outlined in Materials and Methods. The first time point in the controls was also used as the first time point for the Ara-C treatment as both cultures were plated from the same cell suspension and were not under the influence of Ara-C. The percent fusion for the remaining time points in the controls was measured every 48 hr for a period of 6 days. Each point used for the fusion curve was the average of two determinations in a single experiment (Figure 19.a, 19.b, 19.c). The main burst of fusion in neonatal Ara-C cultures occurred between 40-72 hr (Figure 19.a). Control cultures reached a peak percent fusion of approximately 40% after 4 days in vitro. The continued increase in percent fusion after 48 hr for the Ara-C cultures resulted from a decrease in the number of nonmyogenic cells, as well as the continued fusion of myogenic cells. As the percent fusion for Ara-C cultures reached its maximum, the loss of nonmyogenic cells was essentially reduced to zero. The rapid decline in percent fusion for the control cultures was the result of proliferation of nonmyogenic cells (Figure 19.a).

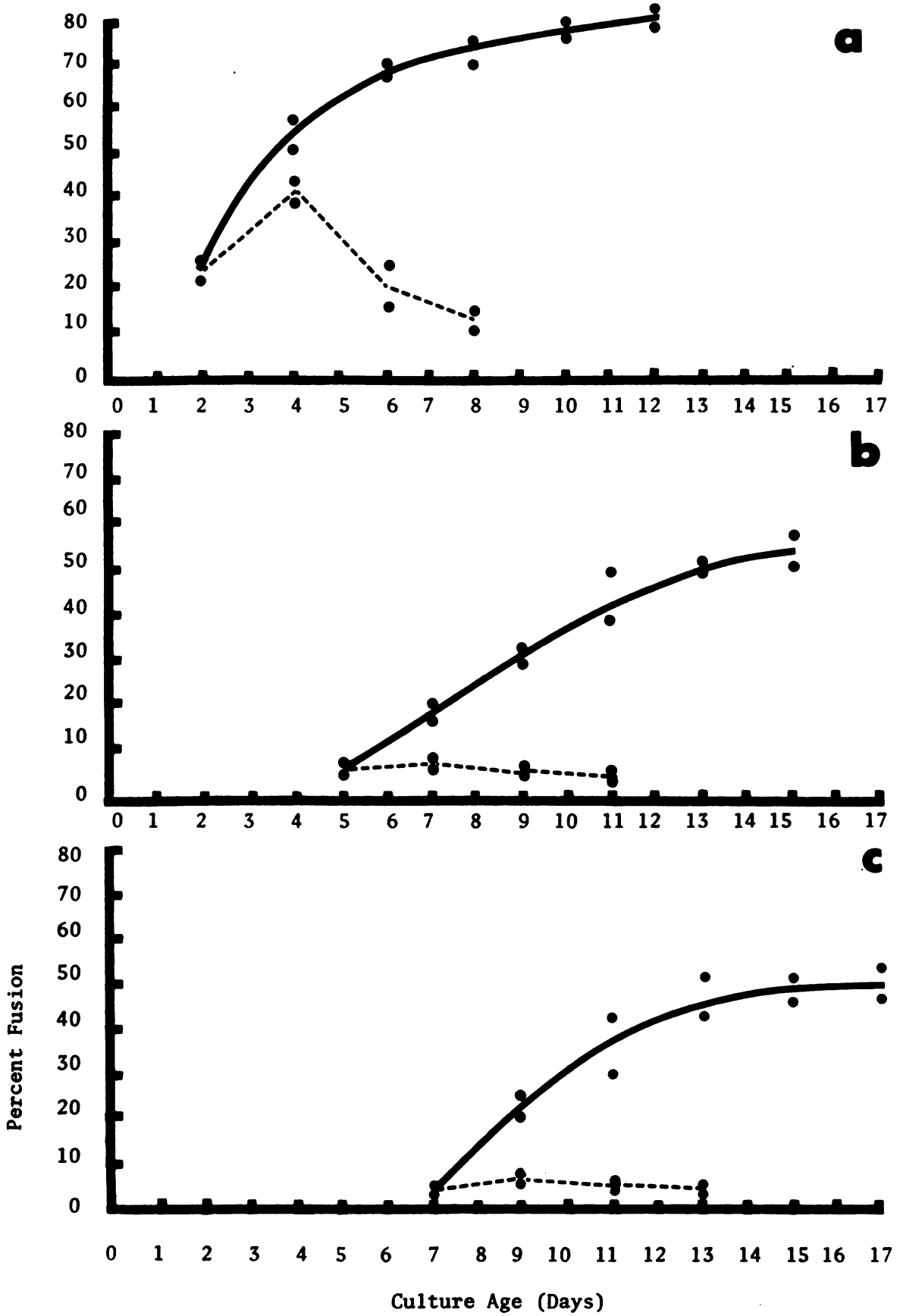
The fusion curves for rapidly growing and adult myogenic cell cultures displayed the same general kinetics of fusion as did neonatal cultures (Figure 19.b, 19.c). Controls displayed a maximum percent fusion 2 days after fusion was first observed and then declined. The percent fusion in Ara-C cultures increased over the next several days and reached a maximum 8-10 days after fusion was first noted.

Differences were evident in both the onset and maximum percentage fusion in cell cultures from different age mice. The initiation of fusion was delayed in cultures from mice of increasing age. Yet, fusion is cell density dependent (O'Neill and Stockdale, 1972; Konigsberg, 1971) and

Figure 19. Kinetics for fusion in myogenic cell cultures which were derived from the satellite cell population of: (a) neonatal, (b) rapidly growing, and (c) adult mouse hind limb muscle. Percent fusion, cell isolation, and culture methods were performed or determined as described in Materials and Methods. The points denoting each day in culture are the means of separate experiments.

Control cultures (-------).

Ara-C treated cultures (———).


a
b
c
Percent Fusion
Culture Age (Days)
0 10 20 30 40 50 60 70 80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

earlier results from this research indicated a decreased percentage of myogenic cells in the cell isolate from mice of increasing age. Therefore, this delay may not have resulted from a declining myogenic potential of satellite cells but from unequal satellite cell densities being plated. The maximum percent fusion was approximately 77% in Ara-C, neonatal cultures and was in compliance with results for cell cultures from embryonic muscle (Yaffe, 1971); however, the maximum percent fusion for rapidly growing and adult mice was 51 and 44%, respectively (Table 1).

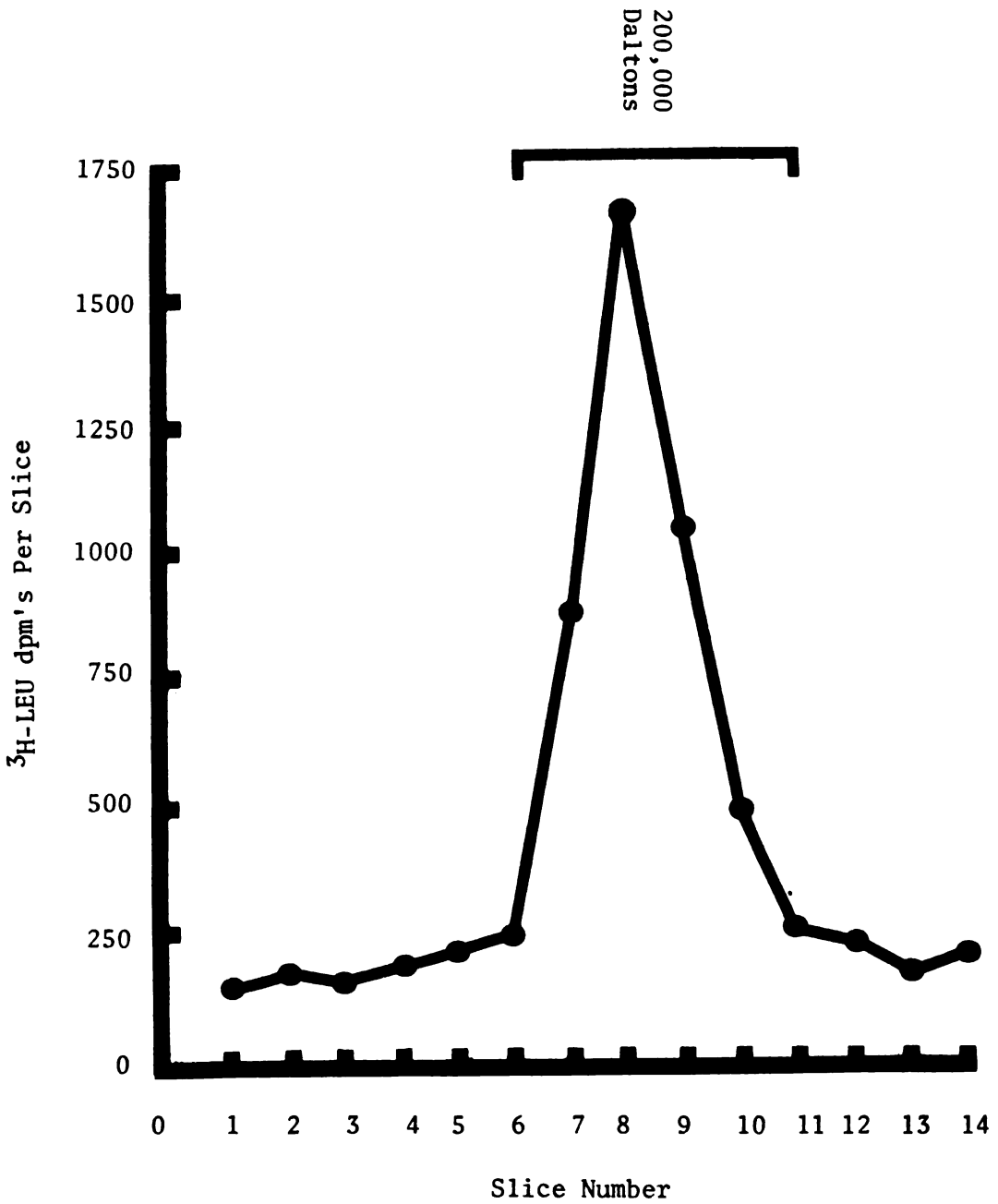
Table 1. Percentage Fusion at Various Days in Culture for Both Control and Ara-C Cultures

	$\bar{X}$ % Fusion					
	Neonatal		Fast Growing		Adult	
Culture Age (Days)	Control	Ara-C	Control	Ara-C	Control	Ara-C
1	---	---	---	---	---	---
2	23.2	23.2	---	---	---	---
3			---	---	---	---
4	40.3	56.2	---	---	---	---
5			5.4	5.4	---	---
6	19.4	68.4			---	---
7			7.8	18.4	2.8	2.8
8	12.8	71.9				
9			6.2	30.2	7.6	22.3
10		75.3				
11			5.3	42.2	3.8	35.0
12		77.0				
13				50.0	2.7	43.0
14						
15				51.2		43.3
16						
17						44.0

Rates of total protein synthesis and MHC synthesis were determined by incubating cultures in medium containing ^{3}H-leucine. Data were expressed as either the rate of total protein or MHC synthesis per nucleus. This allowed for comparison of results from cultures containing an unequal number of cells. Since the differentiation process varied between ages of mice (i.e., onset of fusion, maximum percent fusion, rate of increase in percent fusion), a particular day common to all cultures could not be directly selected for comparing the data. Therefore, the time point closest to one-half the maximum percent fusion was selected as the best comparison time. One-half the maximum percent fusion was a function of the degree of muscle differentiation rather than the number of days in culture.

Since myosin is the major myofibrillar protein, relative rates of MHC synthesis were determined. Duplicate cultures were used for both fusion curve data and ^{3}H-leucine incorporation into MHC. Samples were prepared by low ionic strength (0.025 M KCl) precipitation of myosin as outlined in Materials and Methods, followed by SDS-polyacrylamide gel electrophoresis. Gels were stained, sliced, and the slices counted for radioactivity. Radioactivity in gel slices was most prevalent at a point corresponding to a molecular weight of 200,000 daltons (Figure 20). The total dpm's in slices which made up the peak were summed and used as a measure of the rate of ^{3}H-leucine incorporated into the MHC. Total protein synthesis and MHC synthesis were determined from the pooled material of two cultures of the same time point in culture. Results were expressed on the basis of total nuclei in culture or on the basis of fused nuclei in culture to eliminate effects of unequal cell densities.

Figure 20. The distribution of radioactivity in 0.5 mm gel slices throughout the portion of gel corresponding to the distance of MHC migration.


200,000
Daltons
1750
1500
1250
1000
750
500
250
0
^{3}H-LEU dpm's Per Slice
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Slice Number

The rate of MHC synthesis/nucleus appeared to decline with cells from mice of increasing age (Figure 21). The rate of MHC synthesis was consistently lower in control cultures (no Ara-C), indicating that myotubes synthesized myosin at a faster rate than nonmyogenic cells. The rate of MHC synthesis/fused nucleus in Ara-C cultures also decreased in cells from mice of increasing age (Figure 22). The rate of MHC synthesis per fused nucleus in control cultures was not expressed in Figure 22 because the contribution of ^{3}H-leucine incorporation directed by nonmyogenic nuclei would yield an overestimation of the protein synthetic abilities of the comparatively small number of fused nuclei.

To determine if the observed effect on MHC synthesis was specific for MHC, the rate of total protein synthesis was also measured. Total protein synthesis per nucleus was observed to follow a pattern similar to that observed for MHC synthesis (Figure 23). Once again the Ara-C cultures, with a larger percentage of their nuclei within myotubes, displayed a greater total protein synthesis rate than controls. Total protein synthesis per fused nucleus also declined in cultures from mice of increasing age (Figure 24). As before, total protein synthesis per fused nucleus in control cultures was not expressed, because the contributions by nonmyogenic cells would result in a gross overestimation of the potential of fused nuclei.

To determine more precisely whether myosin synthesis and total protein synthesis were affected to the same extent in cultures of different aged mice, MHC synthesis was expressed as a percentage of the total protein incorporation in Ara-C cultures. These data revealed that the percentage of MHC in cells of neonatal and rapidly growing mice were nearly identical and slightly higher than the adult (Figure 25).

Figure 21. Effect of mouse age on the rate of MHC synthesis in myogenic cell cultures.

Ara-C (●—●).

Control (○—○).

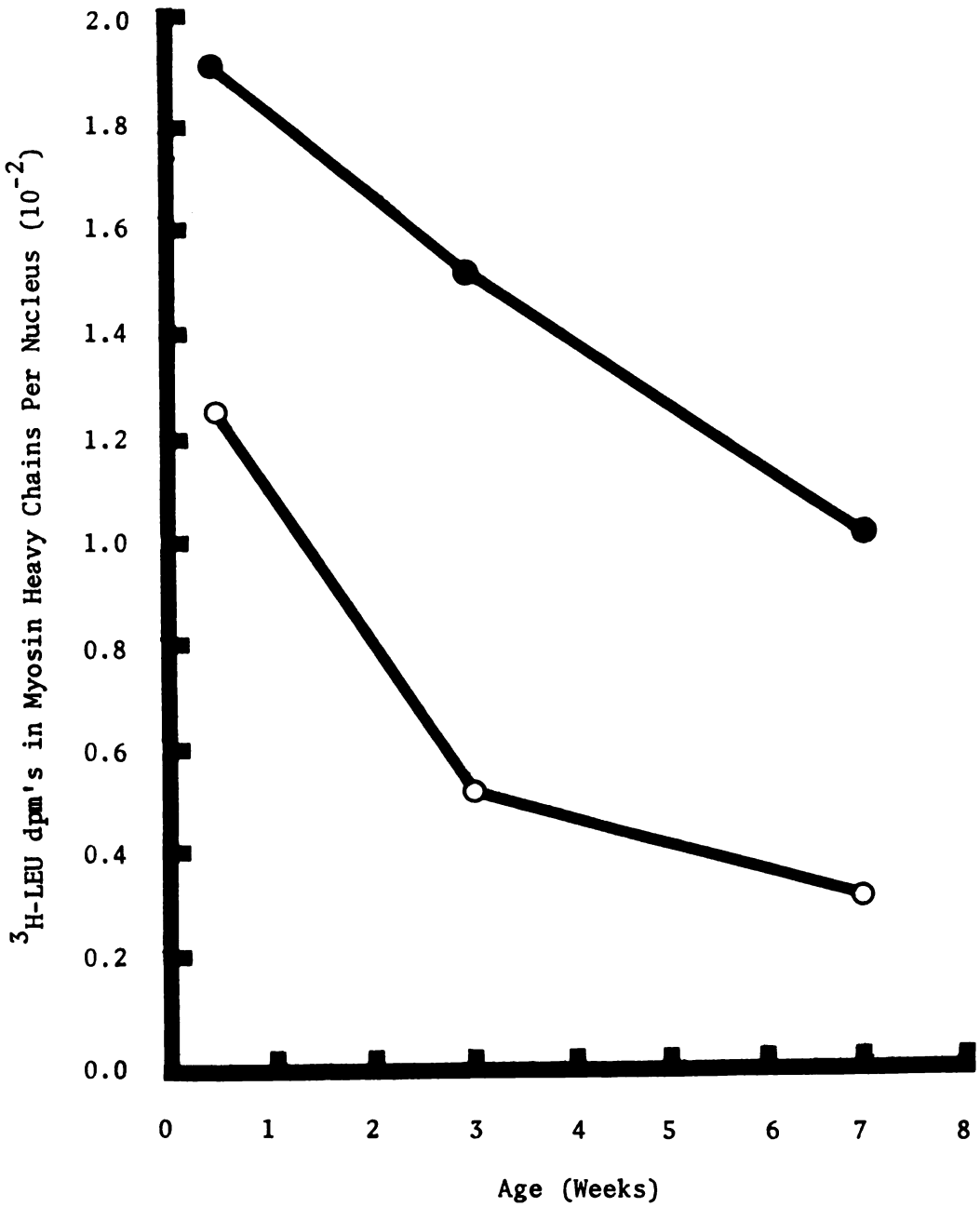

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
^{3}H-LEU dpm's in Myosin Heavy Chains Per Nucleus (10^{-2})
0
1
2
3
4
5
6
7
8
Age (Weeks)

Figure 22. Effect of mouse age on the rate of MHC synthesis per fused nucleus in myogenic cell cultures under the influence of Ara-C.

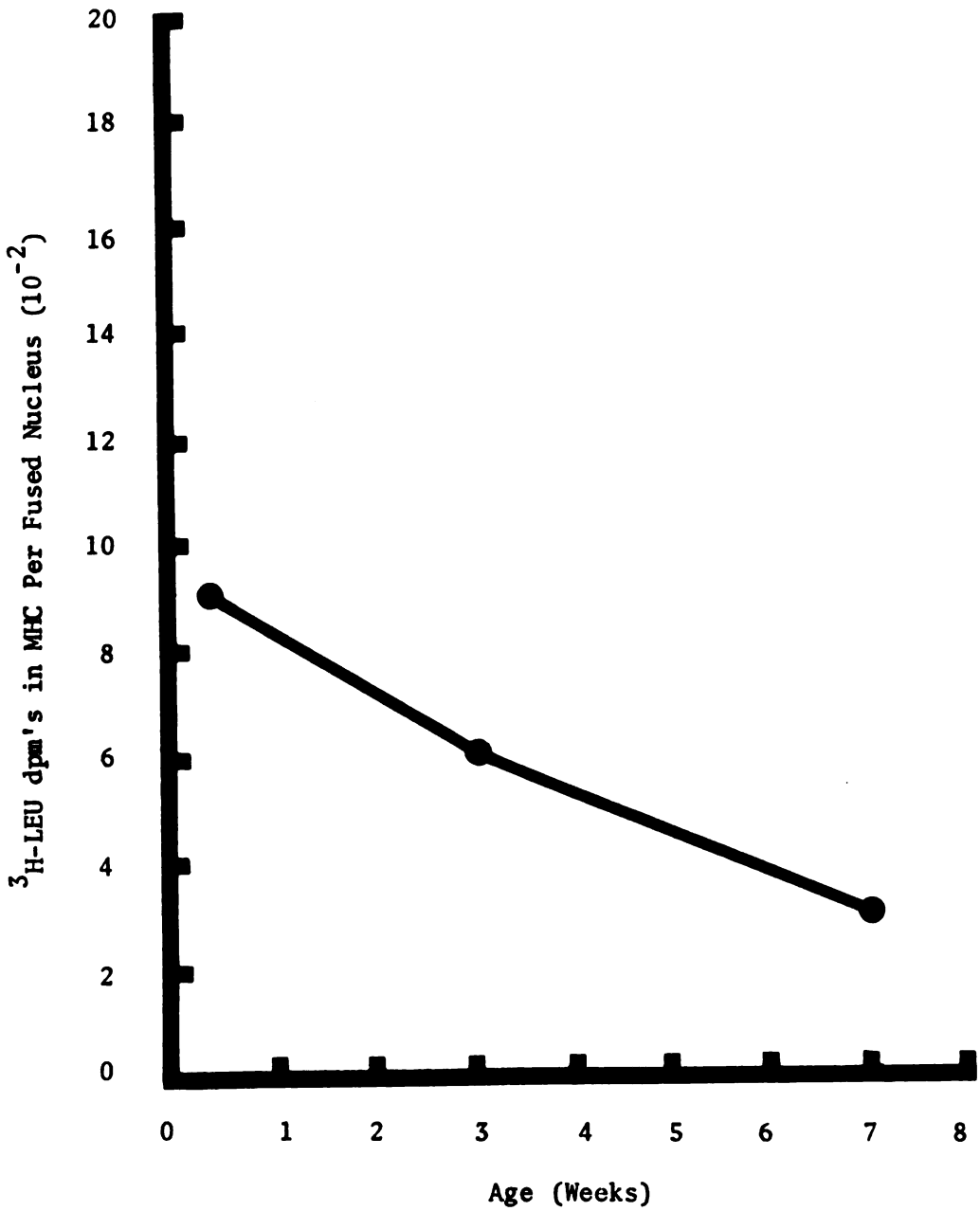

20
18
16
14
12
10
8
6
4
2
0
^{3}H-LEU dpm's in MHC Per Fused Nucleus (10^{-2})
0
1
2
3
4
5
6
7
8
Age (Weeks)

Figure 23. Effect of mouse age on the rate of total protein synthesis per nucleus in myogenic cell cultures.

Ara-C (●—●).

Control (○ ○).

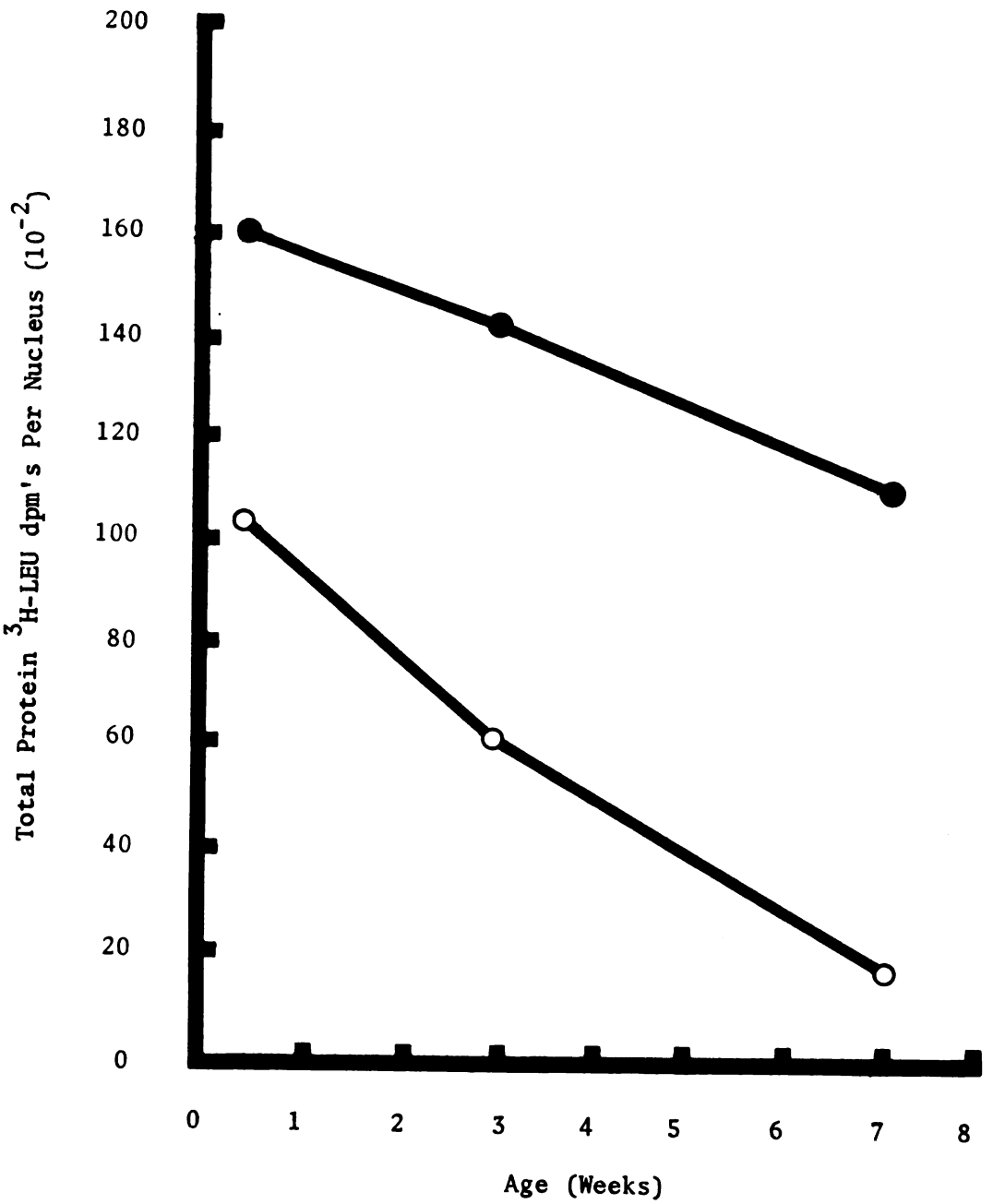

Total Protein ^{3}H-LEU dpm's Per Nucleus (10^{-2})
200
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Age (Weeks)

Figure 24. Effect of mouse age on rate of total protein synthesis per fused nucleus in myogenic cell cultures.

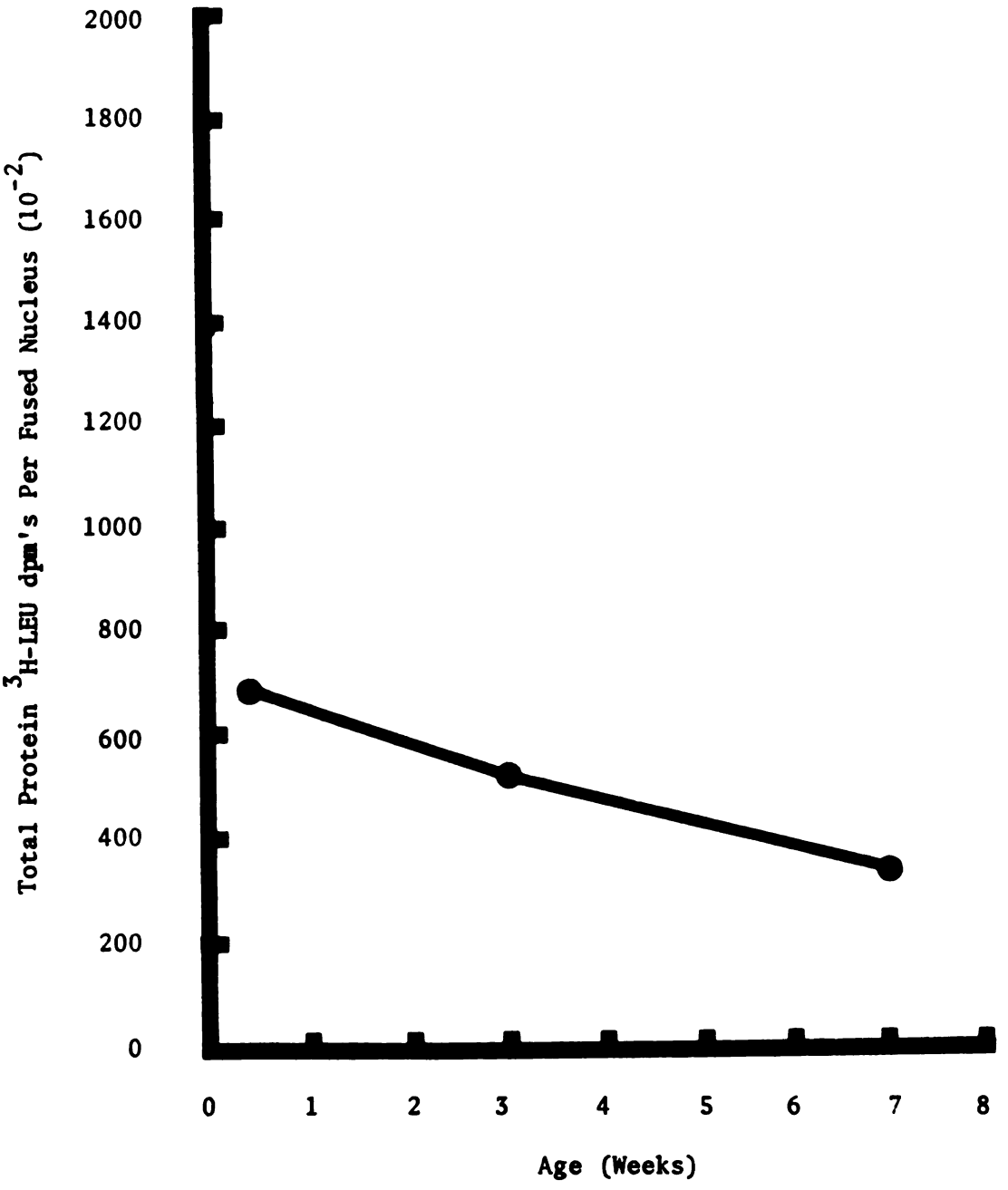
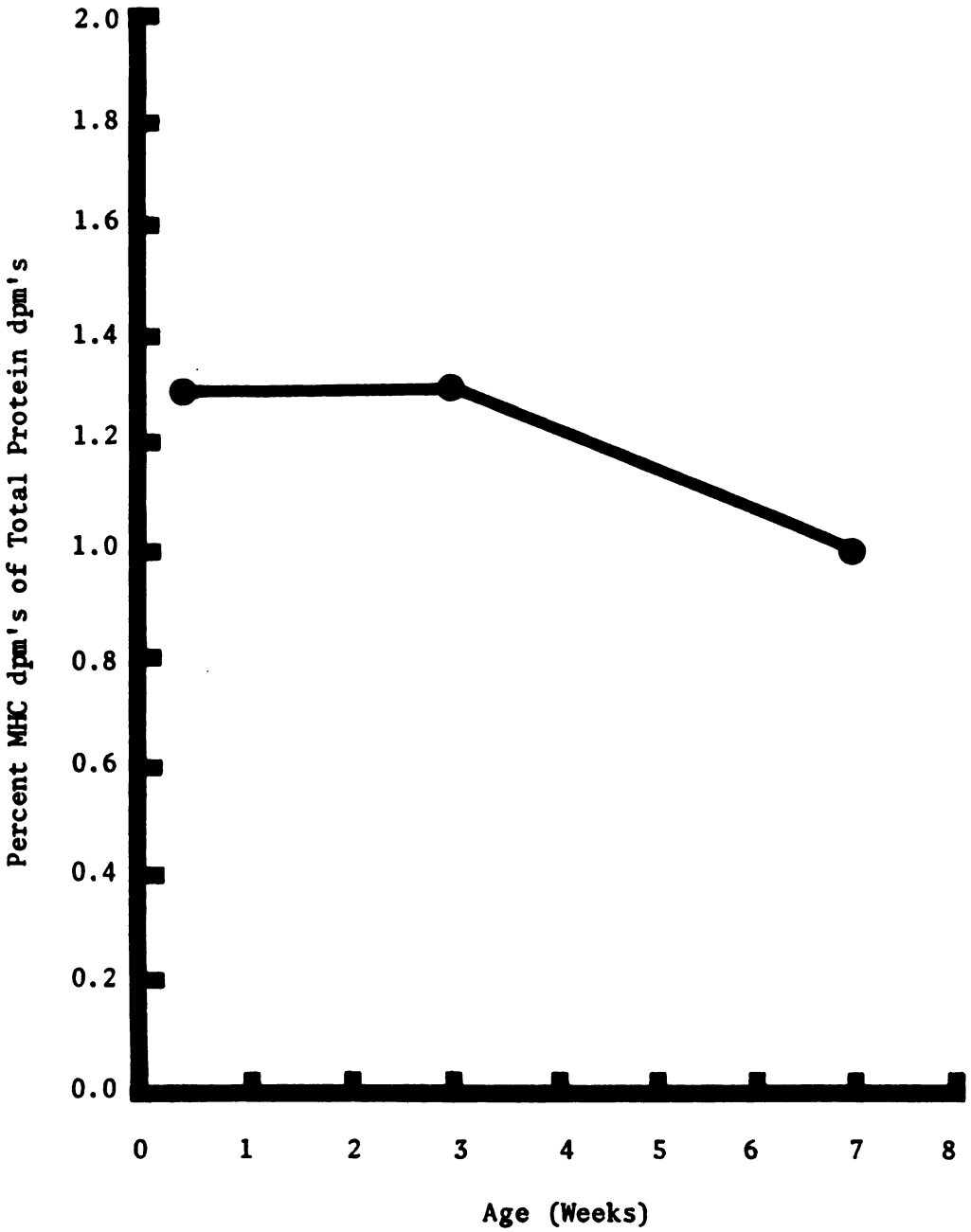

Total Protein ^{3}H-LEU dpm's Per Fused Nucleus (10^{-2})
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
Age (Weeks)

Figure 25. Effect of mouse age on MHC synthesis as a percentage of total protein synthesis in myogenic cell cultures under the influence of Ara-C.


Percent MHC dpm's of Total Protein dpm's
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
7
8
Age (Weeks)

Since one of the goals of this project was to compare the capacity for myofibrillar protein synthesis among satellite cell progeny from mice of various ages, data expressed on a fused nucleus basis from Ara-C "myotube" cultures more accurately assessed myogenic cell potential. In these cultures there was less influence by nonmyogenic cells. Therefore, using MHC synthesis as a single indicator of myofibrillar protein synthesis, the ability of satellite cell progeny to synthesize the myofibrillar proteins, along with the capacity for total protein synthesis (Figures 24 and 23), appeared to decline with increasing age. The decline in the protein synthetic abilities of satellite cells from mice of increasing age reported here is consistent with the results of morphological examination of satellite cells (Schultz, 1976). With increasing age a larger percentage of the satellite cells found, displayed a qualitative and quantitative decrease in the number of polysomes, and a decrease in the size of the rough endoplasmic reticulum and Golgi apparatus within their cytoplasm. Although the study by Schultz (1976) was conducted on in vivo satellite cells and the present study examined differentiating cultures of activated satellite cells, it is possible that extended periods of dormancy or quiescence cause a loss in the ability of satellite cells to activate their genes for synthesizing the myofibrillar proteins.

The conclusion that satellite cell progeny from mice of increasing age lose some of their protein synthetic ability can be drawn only with certain qualifications. The maximum percent fusion in both control and Ara-C cultures tended to be lower in mice of increasing age. Because it was evident that fused nuclei possessed greater protein synthetic ability than nonmyogenic nuclei (see Figures 21 and 23 where

controls display a negligible percent fusion), cultures containing a greater percentage of fused nuclei had the potential to synthesize more protein. Expressing the results on a fused nucleus and total nucleus basis accounted for differences in total cell numbers between cultures, but the extent of muscle differentiation was not entirely equalized by comparing results at one-half the maximum percent fusion.

The degree of error incurred when counting cells in culture was large enough to result in the small differences in the in vitro protein synthetic ability of satellite cell progeny. Yet it seems unlikely that errors in cell counting or slight differences in the extent of muscle differentiation could mask large differences in protein synthetic ability. It seems unlikely, therefore, that satellite cells are drastically different in their ability to synthesize and assemble the muscle proteins.

The production of mRNA may be the limiting factor in myofibrillar protein synthesis in growing muscle. The percentage of ribosomes associated with polysomes may reflect a reduction in mRNA synthesis during growth (Goldspink, 1972). Because satellite cells are the mode of increase in myonuclei during postnatal growth, and because the RNA producing capacity of an individual muscle fiber may be determined by the number of nuclei within the fiber, the DNA of satellite cell-derived myonuclei may lose some of its transcriptional ability. Alternatively, activation of the myofibrillar protein genes may require an increased mode of activation with increasing time in vivo.

A note concerning the data presented in this research is the report that Ara-C inhibits RNA polymerase II activity, resulting in decreased mRNA synthesis in chicken leukemic cells (Chuang and Chuang, 1976).

In Ara-C treated neonatal myotube cultures, MHC synthesis was approximately 1.3% of total protein synthesis at 2 days post-fusion. At this time culture myotubes displayed one of the most convincing pieces of evidence for muscle differentiation--contraction. Therefore, it appears that synthesis and assembly of the myosin molecules into thick filaments is not totally inhibited by Ara-C. While only 1.3% of the total protein synthesis in Ara-C-treated, neonatal myotube cultures was myosin (Figure 25), values of 6-15% have been reported for embryonic chicken myotubes (Chi et al., 1975). Even though this is a large difference it cannot solely be attributed to the adverse effects of Ara-C because the above experiment of Chi et al. (1975) was also under the influence of Ara-C.

V. SUMMARY

Satellite cell populations were isolated from the hind limb muscles of neonatal, rapidly growing, and adult mice. Cells were placed in an *in vitro* system, allowed to proliferate and permitted to fuse into the multinucleated myotubes. By determining the extent to which this myogenic population exists in muscle, it may be possible to evaluate the potential for postnatal muscle growth. The prevalence of this muscle forming cell population *in vivo* was approximated by *in vitro* clonal analysis. Myotube formation within a colony was used to determine whether or not the cell from which the colony was derived was myogenic. Preliminary results showed that muscle colonies somehow altered the growth medium to enhance muscle differentiation and that this effect was dependent upon clonal density. Yet, one interesting note was that adult satellite cell progeny either conditioned their medium faster or required less of a conditioning effect. In a subsequent experiment cells were kept proliferating with daily medium changes and then conditioned medium was added. Results showed that there was no significant difference in the percent MCF cells at either of three clonal densities. This same experiment revealed that during postnatal muscle accretion the concentration of MCF cells decreased and then leveled off corresponding to a flattening of the growth curve. Therefore, the potential for postnatal muscle growth declines with increasing age. During postnatal growth the decrease in concentration of satellite cells in hind limb muscles is accompanied by a slight decline in the protein

synthetic capacity of satellite cell progeny. The rate of total protein synthesis and MHC synthesis were determined by incubating cultures in medium containing ^{3}H-leucine. Ara-C provided for a culture model in which the myofibrillar protein synthesizing ability of myogenic nuclei could be more accurately assessed. MHC synthesis/fused nucleus decreased with increasing age. Total protein synthesis was also measured to determine if the observed effect for MHC synthesis was specific for MHC. Total protein synthesis/fused nucleus also decreased with increasing age. Coincident with the possible decreased protein synthetic ability of the satellite cell in maturing muscle were two other indicators of decreased myogenic activity. First, myotubes derived from cultured adult satellite cells often failed to display elongation. Secondly, clonal analysis revealed a decrease in the viability of cells isolated from adult muscle.

Observation of contracting myotubes in satellite cell cultures of all three ages and the presence of striations in myofibril preparations revealed that satellite cell progeny are not only capable of contractile protein synthesis but also capable of organization of these proteins into myofibrillar arrays.

The results of this study show that there is a population of dormant presumptive myoblasts in postnatal muscle capable of muscle regeneration and responsible for postnatal muscle accretion. Yet, it is unlikely that satellite cells are drastically different in their ability to synthesize and assemble the muscle proteins and therefore a decline in muscle accretion with increasing age is also likely due to a decline in satellite cell concentration in muscle.

BIBLIOGRAPHY

VI. BIBLIOGRAPHY

Abbot, J., J. Schiltz, S. Dienstman and H. Holtzer. 1974. The phenotypic complexity of myogenic clones. Proc. Nat. Acad. Sci. 74: 1506-1510.

Adams, S., B. Zani, G. Siracusa and M. Molinaro. 1976. Expression of differentiative traits in the absence of cell fusion during myogenesis in culture. Cell Differentiation 5:53-67.

Adelstein, R. S. and M. A. Conti. 1972. The characterization of contractile proteins from platelets and fibroblasts. Cold Spring Harbor Symp. Quant. Biol. 37:599-605.

Allbrook, D. B., M. F. Han and A. E. Helmuth. 1971. Population of muscle satellite cells in relation to age and mitotic activity. Pathology 3:233-243.

Aloisi, M. 1970. Patterns of muscle regeneration. Pages 180-193 in A. Mauro, S. A. Shafiq, A. T. Milhorat, eds. Regeneration of Striated Muscle and Myogenesis. Excerpta Medica Foundation, Amsterdam.

Bischoff, R. 1974. Enzymatic liberation of myogenic cells from adult rat muscle. Anat. Rec. 180:645-662.

Bischoff, R. 1975. Regeneration of single skeletal muscle fibers in vitro. Anat. Rec. 182:215-236.

Bischoff, R. and H. Holtzer. 1968. The effect of mitotic inhibitors on myogenesis in vitro. J. Cell Biol. 36:111-127.

Bischoff, R. and H. Holtzer. 1969. Mitosis and the process of differentiation of myogenic cells in vitro. J. Cell Biol. 41:188-200.

Bischoff, R. and H. Holtzer. 1970. Inhibition of myoblast fusion after one round of DNA synthesis in 5-bromodeoxyuridine. J. Cell. Biol. 44:134.

Bonner, P. H. and S. D. Hauschka. 1974. Clonal analysis of vertebrate myogenesis. I. Early developmental events in the chick limb. Develop. Biol. 37:317-328.

Buckingham, M. E., D. Caput, A. Cohen, R. G. Whalen and F. Gros. 1974. The synthesis and stability of cytoplasmic messenger RNA during myoblast differentiation in culture. Proc. Nat. Acad. Sci. USA 71: 1466-1470.

Burleigh, I. G. 1974. On the cellular regulation of growth and development in skeletal muscle. Biol. Rev. 49:267-320.

Carlson, B. M. 1973. The regeneration of skeletal muscle -- a review. Amer. J. Anat. 137 : 119-150.

Chi, J. C. H., S. A. Fellini and H. Holtzer. 1975a. Differences among the myosins synthesized in non-muscle cells, presumptive myoblasts, and myoblasts. Proc. Nat. Acad. Sci. 72:4999-5003.

Chi, J. C. H., N. Rubenstein, K. Strahs and H. Holtzer. 1975b. Synthesis of myosin heavy and light chains in muscle cultures. J. Cell Biol. 67:523-537.

Chuang, R. Y. and L. F. Chuang. 1976. Inhibition of RNA polymerase as a possible anti-leukaemic action of cytosine arabinoside. Nature 260:549-550.

Church, J. C. T. 1969. Satellite cells and myogenesis -- a study in the fruit-bat web. J. Anat. 105:419-438.

Church, J. C. T. 1970a. A model for myogenesis using the concept of the satellite cell segment. Pages 118-121 in A. Mauro, S. A. Shafiq, A. T. Milhorat, eds. Regeneration of Striated Muscle and Myogenesis. Excerpta Medica Foundation, Amsterdam.

Church, J. C. T. 1970b. Cell populations in skeletal muscle after regeneration. J. Embryol. Exptl. Morph. 23:531-537.

Coleman, J. R. and A. W. Coleman. 1968. Muscle differentiation and macromolecular synthesis. J. Cell Physiol. 72:supplement, 19-34.

de la Haba, Gabriel and R. Amundsen. 1972. The contribution of embryo extract to myogenesis of avian striated muscle in vitro. Proc. Nat. Acad. Sci. USA 69:1131-1135.

Devriotes, P. N. and D. M. Fambrough. 1976. Synthesis of acetylcholine receptors by cultured chick myotubes and denervated mouse extensor digitorum longus muscles. Proc. Nat. Acad. Sci. USA 73:161-164.

Dienstman, S. R., J. Biehl, S. Holtzer and H. Holtzer. 1974. Myogenic and chondrogenic lineages in developing limb buds grown in vitro. Develop. Biol. 39:83-95.

Dienstman, S. R. and H. Holtzer. 1975. Myogenesis: A cell lineage interpretation. Pages 1-25 in J. Reinert and H. Holtzer, eds. Cell Cycle and Cell Differentiation. Springer-Verlag, Berlin.

Doering, J. L. and D. A. Fischman. 1974. The in vitro cell fusion of embryonic chicken muscle without DNA synthesis. Develop. Biol. 36:225-235.

Elzinga, M., B. J. Maron and R. S. Adelstein. 1976. Human heart and platelet actins are products of different genes. Sci. 191:94-95.

Emerson, C. P., Jr. and S. K. Beckner. 1975. Activation of myosin synthesis in fusing and mononucleated myoblasts. J. Mol. Biol. 93: 431-447.

Enesco, M. and D. Puddy. 1964. Increase in the number of nuclei and weight in skeletal muscle of rats of various ages. Amer. J. Anat. 114:235-244.

Fambrough, D. and J. E. Rash. 1971. Development of acetylcholine sensitivity during myogenesis. Develop. Biol. 26:55-68.

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

Fischman, D. A. 1970. The synthesis and assembly of myofibrils in embryonic muscle. Curr. Top. Develop. Biol. 5:235-280.

Fischman, D. A. 1972. Development of striated muscle. Pages 75-148 in G. H. Bourne, ed. The Structure and Function of Muscle. Volume I. Academic Press, Inc., New York.

Fluck, R. A. and R. C. Strohman. 1973. Acetylcholinesterase activity in developing skeletal muscle cells in vitro. Develop. Biol. 33: 417-428.

Gearhart, J. D. and B. Mintz. 1975. Creatine kinase, myokinase, and acetylcholinesterase activities in muscle-forming primary cultures of mouse teratocarcinoma cells. Cell 6:61-66.

Goldberg, A. L. 1969. Protein turnover in skeletal muscle. I. Protein catabolesin during work-induced hypertrophy and growth induced with growth hormone. J. Biol. Chem. 244:3217-3222.

Goldspink, G. 1970. The proliferation of myofibrils during muscle fiber growth. J. Cell Sci. 6:593-603.

Goldspink, G. 1972. Postembryonic growth and differentiation of striated muscle. Pages 179-236 in G. H. Bourne, ed. The Structure and Function of Muscle. Volume I. Academic Press, Inc., New York.

Hanzlikova, V., E. Mackova and P. Hnik. 1975. Satellite cells of the rat soleus muscle in the process of compensatory hypertrophy combined with denervation. Cell Tiss. Res. 160:411-421.

Hauschka, S. D. 1974a. Clonal analysis of vertebrate myogenesis. II. Environmental influences upon human muscle differentiation. Develop. Biol. 37:329-344.

Hauschka, S. D. 1974b. Clonal analysis of vertebrate myogenesis. III. Developmental changes in the muscle--colony-forming cells of the human fetal limb. Develop. Biol. 37:345-368.

Hartzell, H. C. and D. M. Fambrough. 1973. Acetylcholine receptor production and incorporation into membranes of developing muscle fibers. Develop. Biol. 30:153-165.

Heywood, S. M., D. S. Kennedy and A. J. Bester. 1975a. Stored myosin messenger in embryonic chick muscle. FEBS Letters 53:69-72.

Heywood, S. M., D. S. Kennedy and A. J. Bester. 1975b. Studies concerning the mechanism by which translational-control RNA regulates protein synthesis in embryonic muscle. Eur. J. Biochem. 58:587-593.

Hilfer, S. R., R. L. Searls and V. G. Fonte. 1973. An ultrastructural study of early myogenesis in the chick wing bud. Develop. Biol. 30:374-391.

Holtzer, H. 1961. Aspects of chondrogenesis and myogenesis. Page 35 in D. Rudnick ed. Synthesis of Molecular and Cellular Structure. Ronald Press, New York.

Holtzer, H. 1970. Myogenesis. Pages 476-503 in O. Schjeide and J. Vellis eds. Cell Differentiation. Von Nostrand Reinhold, New York.

Holtzer, H. and R. Bischoff. 1970. Mitosis and myogenesis. Pages 29-51 in E. J. Briskey, R. G. Cassens, and B. B. Marsh eds. The Physiology and Biochemistry of Muscle as a Food. Volume 2. The University of Wisconsin Press, Madison, Wisconsin.

Holtzer, H., J. Marshall, and H. Finck. 1957. An analysis of myogenesis by the use of fluorescent antimyosin. J. Cell Biol. 3:705-723.

Holtzer, H., N. Rubenstein, S. Fellini, G. Yeah, J. Chi, J. Birnbaum and M. Okayama. 1975a. Lineages, quantal cell cycles and the generation of cell diversity. Quarterly Reviews of Biophysics 4:523-557.

Holtzer, H., K. Strahs, J. Biehl, A. P. Somlyo and H. Ishikawa. 1975b. Thick and thin filaments in postmitotic, mononucleated myoblasts. Science 188:943-945.

Holtzer, A., H. Weintraub, R. Mayne and B. Mochani. 1973. The cell cycle, cell lineages and cell differentiation. Curr. Top. in Develop. Biol. 7:229-256.

Ishikawa, H. 1970. Satellite cells in developing muscle and tissue cultures. Pages 167-179 in A. Mauro, S. A. Shafiq, A. T. Milhorat, eds. Regeneration of Striated Muscle and Myogenesis. Excerpta Medica Foundation, Amsterdam.

Ishikawa, H., R. Bischoff and H. Holtzer. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. J. Cell Biol. 38: 538-555.

Kahn, E. B. and S. B. Simpson, Jr. 1974. Satellite cells in mature, uninjured skeletal muscle of the lizard tail. Develop. Biol. 37: 219-233.

Ketley, J. N., R. W. Orkin and G. R. Martin. 1976. Collagen in developing chick muscle in vivo and in vitro. Exptl. Cell. Res. 99: 261-268.

Konigsberg, I. R. 1971. Diffusion-mediated control of myoblast fusion. Develop. Biol. 26:133-152.

Konigsberg, Ursula R., B. H. Lipton and I. R. Konigsberg. 1975. The regenerative response of single mature muscle fibers isolated in vitro. Develop. Biol. 45:260-275.

Lough, J. and R. Bischoff. 1976. Differential sensitivity to 5-bromodeoxyuridine during the S-phase of synchronized myogenic cells. Develop. Biol. 50:457-475.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biophys. Biochem. Cytol. 3:705-723.

MacConnachie, H. F., M. Enesco and C. P. LeBlond. 1964. The mode of increase in the number of skeletal muscle nuclei in the postnatal rat. Amer. J. Anat. 114:245-253.

Marchok, A. C. and H. Herrmann. 1967. Studies on muscle development. I. Changes in cell proliferation. Devel. Biol. 15:129-155.

Mauro, A. 1961. Satellite cell of skeletal muscle fibers. J. Cell Biol. 9:493-495.

Mauro, A., S. A. Shafiq and A. T. Milhorat. 1970. "Regeneration of Striated Muscle and Myogenesis". Excerpta Medica, Amsterdam.

Mayne, R., J. Abbot and J. Schiltz. 1972. Studies concerning the divergence of myoblast and fibroblast precursor cells during myogenesis in vitro. J. Cell Biol. 55:168a (abstract).

Millward, D. J., P. J. Garlick, W. P. T. James, D. O. Nnanyelugo and J. S. Ryatt. 1973. Relationship between protein synthesis and RNA content in skeletal muscle. Nature 241:204-205.

Millward, D. J., P. J. Garlick, R. J. C. Stewart, D. O. Nnanyelugo and J. C. Waterlow. 1975. Skeletal-muscle growth and protein turnover. Biochem. J. 150:235-243.

Morris, G. E., E. A. Buzash, A. N. Rourke, K. Tepperman, W. C. Thompson and S. M. Heywood. 1972. Myosin messenger RNA: Studies on its purification, properties and translation during myogenesis in culture. Cold Spring Harbor Symp. Quant. Biol. 37:535-541.

Morris, E. G., M. Piper and R. Cole. 1976. Differential effects of calcium ion concentration on cell fusion, cell division and creatine kinase activity in muscle cell cultures. Exptl. Cell Res. 99:106-114.

Moss, F. P. 1968. The relationship between the dimensions of the fibers and the number of nuclei during normal growth of skeletal muscle in the domestic fowl. Amer. J. Anat. 122:555-564.

Moss, F. P. and C. P. LeBlond. 1970. Nature of dividing nuclei in skeletal muscle of growing rats. J. Cell Biol. 44:459-462.

Moss, F. P. and C. P. LeBlond. 1971. Satellite cells as the source of nuclei in muscles of growing rats. Anat. Rec. 170:421-436.

Moss, P. S. and R. C. Strohman. 1976. Myosin synthesis by fusion-arrested chick embryo myoblasts in cell culture. Develop. Biol. 48:431-437.

Muir, A. R., A. H. M. Kanji and D. Allbrook. 1965. The structure of the satellite cells in skeletal muscle. J. Anat. 99:435-444.

Munro, H. N. 1976. Eukaryote protein synthesis and its control. Pages 3-18 in D. J. A. Cole et al., eds. Protein Metabolism and Nutrition. Butterworths, London.

Nameroff, M. and H. Holtzer. 1969. Interference with myogenesis. Develop. Biol. 19:380-396.

Okazaki, K. and H. Holtzer. 1965. An analysis of myogenesis using fluorescein-labeled anitmyosin. J. Histochem. Cytochem. 13:726-739.

Okazaki, K. and H. Holtzer. 1966. Myogenesis, fusion, myosin synthesis and the mitotic cycle. Proc. Nat. Acad. Sci. USA 59:1484-1490.

O'Neill, M. C. and F. E. Stockdale. 1972. Differentiation without cell division in cultured skeletal muscle. Develop. Biol. 29:410-418.

O'Neill, M. C. and F. E. Stockdale. 1974. 5-Bromodeoxyuridine inhibition of differentiation kinetics of inhibition and reversal in myoblasts. Develop. Biol. 37:117-132.

Ontell, M. 1973. Muscle satellite cells: A validated technique for light microscopic identification and a quantitative study of changes in their population following denervation. Anat. Rec. 178:211-228.

Ontell, M. 1975. Evidence for myoblastic potential of satellite cells in denervated muscle. Cell Tiss. Res. 160:345-353.

Paterson, B. and R. C. Strohman. 1972. Myosin synthesis in cultures of differentiating chicken embryonic skeletal muscle. Develop. Biol. 29:113-138.

Prives, J. M. and B. M. Paterson. 1974. Differentiation of cell membranes in cultures of embryonic chick breast muscle. Proc. Nat. Acad. Sci. USA 71:3208-3211.

Przybyla, A. and R. C. Strohman. 1974. Myosin heavy chain messenger RNA from myogenic cell cultures. Proc. Nat. Acad. Sci. USA 71:662-666.

Reporter, M. 1974. Divergent synthesis of actin and myosin in fused and unfused muscle cells. Biochem. and Biophys. Res. Comm. 56:1086-1092.

Reznik, M. 1970. Satellite cells, myoblasts and skeletal muscle regeneration. Pages 133-156 in A. Mauro, S. A. Shafiq, A. T. Milhorat, eds. Regeneration of Striated Muscle and Myogenesis. Excerpta Medica Foundation, Amsterdam.

Rogers, J., S. K. C. Ng, M. B. Coulter and B. D. Sanwal. 1975. Inhibition of myogenesis in a rat myoblast line by 5-bromodeoxyuridine. Nature 256:438-440.

Rubenstein, N., J. Chi and H. Holtzer. 1976. Coordinated synthesis and degradation of actin and myosin in a variety of myogenic and non-myogenic cells. Expt. Cell Res. 97:387-393.

Schmalbruch, H. and V. Hellhammer. 1976. The number of satellite cells in normal human muscle. Anat. Rec. 185:279-288.

Schubert, D., H. Tarikas, S. Humphreys, S. Heinemann and J. Patrick. 1973. Protein synthesis and secretion in a myogenic cell line. Develop. Biol. 33:18-37.

Schultz, E. 1976. Fine structure of satellite cells in growing skeletal muscle. Amer. J. Anat. 147:49-70.

Shizuta, Y., P. J. A. Davies, K. Olden and I. Pastan. 1976. Diminished content of plasma membrane-associated myosin in transformed fibroblasts. Nature 261:414-415.

Slater, C. R. 1975. Control of myogenesis in vitro by chick embryo extract. Develop. Biol. 50:264-284.

Stockdale, F. E. and H. Holtzer. 1961. DNA synthesis and myogenesis. Expt. Cell Res. 24:508-520.

Stockdale, F. E. and M. C. O'Neill. 1972. Deoxyribonucleic acid synthesis, mitosis, and skeletal muscle differentiation. In Vitro 8:212-225.

Storti, Robert V. and A. Rich. 1976. Chick cytoplasmic actin and muscle actin have different structural genes. Proc. Nat. Acad. Sci. USA 73:2346-2350.

Stromer, M. H., Goll, D. E., Young, R. B., Robson, R. M. and Parrish, F. C., Jr. 1974. Ultrastructural features of skeletal muscle differentiation and development. J. Anim. Sci. 38:1111-1141.

Tarikas, H. and D. Schubert. 1974. Regulation of adenylate kinase and creatine kinase activities in myogenic cells. Proc. Nat. Acad. Sci. USA 71:2377-2381.

Teng, N. N. H. and M. Y. Fiszman. 1976. Appearance of acetylcholine receptors in cultured myoblasts prior to fusion. J. Supramol. Structure 4:381-387.

Tennyson, V. M., M. Brzin and L. T. Kremzner. 1973. Acetylcholinesterase activity in the myotube and muscle satellite cell of the fetal rabbit. An electron microscopic-cytochemical and biochemical study. J. Histochem. Cytochem. 21:634-652.

Trotter, J. A. and M. Nameroff. 1976. Myoblast differentiation in vitro: Morphological differentiation of mononucleated myoblasts. Develop. Biol. 49:548-555.

Trupin, G. L. 1976. The satellite cells of normal anuran skeletal muscle. Develop. Biol. 50:517-524.

Turner, D. C., R. Gmur, M. Siegrist, E. Burckhardt and H. M. Eppenberger. 1976. Differentiation in cultures derived from embryonic chicken muscle. Develop. Biol. 48:258-283.

Vertel, B. M. and D. A. Fischman. 1976. Myosin accumulation in mononucleated cells of chick muscle cultures. Develop. Biol. 48:438-446.

Venable, J. H. 1969. Turnover of myofibrils in stable and hypertrophing perineal muscles of mature mice. Anat. Rec. 163:279-280.

Wahrmann, J. P., G. Drugeon, E. Delain and D. Delain. 1976. Gene expression during the differentiation of myogenic cells of the L_6 line. Biochimie 58:551-562.

White, N. K., P. H. Bonner, D. R. Nelson and S. D. Hauschka. 1975. Clonal analysis of vertebrate myogenesis. IV. Medium-dependent classification of colony-forming cells. Develop. Biol. 44:346-361.

White, N. K. and S. D. Hauschka. 1971. Muscle development in vitro: A new conditioned medium effect on colony differentiation. Expt. Cell Res. 67:479-482.

Winand, R. and D. Luzzati. 1975. Cell surface changes during myoblast differentiation: Preparation and carbohydrate composition of plasma membranes. Biochimie 57:765-771.

Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Nat. Acad. Sci. 61:477-483.

Yaffe, D. 1971. Developmental changes preceding cell fusion during muscle differentiation *in vitro*. Expt. Cell Res. 66:33-48.

Yaffe, D. and H. Dym. 1972. Gene expression during differentiation of contractile muscle fibers. Cold Spring Harbor Symp. Quant. Biol. 37:543-547.

Young, R. B., D. E. Goll and M. H. Stromer. 1975. Isolation of myosin-synthesizing polysomes from cultures of embryonic chicken myoblasts before fusion. Develop. Biol. 47:123-135.

Young, V. R., S. C. Stothers and G. Vilaire. 1971. Synthesis and degradation of mixed proteins and composition changes in skeletal muscle of malnourished and refed rats. J. Nutr. 101:1379-1390.