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Muscle Growth in Divergent

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Mac W. Orcutt

Department of Food Science/Human Nutrition

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Ronald B. Young

Major professor

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Muscle Growth in Divergent Chick Strains

A Thesis

by

Mac W. Orcutt

Approved as to style and content by

Ronald B Young
Chairman

R. A. Merkel
Member

A. M. Dawson
Member

W. G. Bergen
Member

James F. Price
Member

L. E. Dawson
Chairman of Department

G116113

ABSTRACT

MUSCLE GROWTH IN DIVERGENT CHICK STRAINS

By

Mac Orcutt

Cultures of muscle cells derived from meat and egg type chicken strains were established for the qualitative and quantitative analysis of muscle growth. Myogenic tissue differentiated from mononucleated cells through advanced myotube maturity. Essentially no differences in morphological characteristics were seen during the examination period. There were no differences in synthesis rates of total protein synthesis/myotube nucleus, total protein/myotube nucleus, or myofibrillar protein content/myotube nucleus between the two chicken strains. However the broiler cells tended to contain more myofibrillar and total protein per myotube nucleus. The rate of myosin heavy chain synthesis found in the muscle cells of layer origin was significantly greater ($P < .05$) than that of the broiler. However, the stability of the myosin heavy chain subunit was similar in both brioler and layer muscle cultures.

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LIST OF ABREVIATIONS

acyl-tRNA	acyl-translational ribonucleic acid
BC	broiler cells
BE	broiler extract
C	centigrade
^{14}C	carbon fourteen
Ca^{++}	calcium ion
c-AMP	cyclic adenosine monophosphate
c.f.	see figure
cm	centimeter
DNA	deoxyribonucleic acid
dpm	desintegrations per minute
Fb	fibroblast
FBR	fractional breakdown rate
FdU	fluorodeoxyuridine
f-met-tRNA	N-formylmethionyl-tRNA
FSR	fractional synthesis rate
g	gravity
G_1	pre-DNA synthetic phase of mitosis
G_2	post-DNA synthetic phase of mitosis
^{3}H	tritium
hnRNA	heterogeneous nuclear ribonucleic acid
i.e.	example

IF_1	initiation factor one
IF_2	initiation factor two
IF_3	initiation factor three
LC	layer cells
LE	layer extract
M	molar
mAmp	millamperage
Mb, Mbs	myoblast, myoblasts
Mf, Mbs	myofiber, myofibers
MHC	myosin heavy chain
min	minute
ml	milliliter
MLC	myosin light chain
mM	millimolar
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
Mt, Mts	myotube, myotubes
ng	nanogram
nm	nanometer
p	probability
pg	picogram
pH	negative log of hydrogen ion concentration
pI	isoelectric point
PMb, PMbs	presumptive myoblast

PMbFb	presumptive myoblast fibroblast
poly-A	polyadenylic acid
poly-U	polyuridylic acid
RNP	ribonucleoprotein
S	period of DNA replication during mitosis
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
$t_{\frac{1}{2}}$	half-life
TCA	trichloroacctic acid
tcRNA	translational control ribonucleic acid
tRNA	transfer ribonucleic acid
v/v	volume per volume
µCi	microcurie
µl	microliter
%	percent

INTRODUCTION

Domestic animals vary drastically in body conformation. Traditionally, these differences have been incurred by planned selection practices rather than by natural selection. Presently, one of the major goals of the animal scientist is the production of maximal amounts of animal product at the lowest possible production costs. Artificial insemination and the closely associated selected sire program has been one tool which has helped achieve a portion of this idealistic goal. However, the progeny testing of breeding sires for the purposes of eventual placement within breeding herds of artificial insemination firms is a rather expensive and rigorous process.

The major goal of this research project was to investigate the growth of divergent animal strains, using broiler and layer chicks as animal models, in cell cultures of myogenic tissue in hopes that gross differences in muscle growth observed in the animals would be exhibited in cell culture. This, if found effective, could aid the initial screening of selected sires, thereby reducing the number of potential sires that need to be placed in progeny tests. Additionally, this approach could potentially evaluate the individual performance of each animal. This study also served as a pilot study for investigating muscle growth using muscle biopsies from closely related production animals by means of cell cul-

ture analysis. Ideally, differences in some of the parameters associated with growth would be found in these production animals, and growth potentials of these animals could be predicted from the cell culture experiments with some degree of accuracy. Unfortunately, reproducible differences were not observed in cell cultures. Since no differences were found in animals of divergent muscling, one could not hope to find differences in growth in animals of similar breeding.

LITERATURE REVIEW

A. The Fetal Development of Striated Muscle

1. Development of Myogenic Cells from Primordial Tissue of the Developing Embryo

Muscle tissue in general differentiates from cells of the lateral plate and paraxial (somatic) mesoderm (Allen and Pepe, 1965; reviewed by Fischman, 1972), although there are one or two general exceptions to this broad classification. Even though there is some controversy as to the origin of some vertebrate musculature, there is little doubt that limb musculature develops by the differentiation of the lateral plate (somatic) mesoderm (Rudnick, 1945; Saunders, 1948; Tschumi, 1957; reviewed by Fischman, 1972).

Presently, there are two general mechanisms by which cellular differentiation of embryonic tissue is thought to take place. Speman (1938) described a system of differentiation whereby inducer molecules bring about a cellular change which gives differentiational multipotentiality to a cell, thus allowing an undifferentiated cell to become any one of several types of terminally differentiated cell types after only one mitotic division. This theory of cellular multipotentiality was supported by Konigsberg and Hauschka (1965) and Hauschka and Konigsberg (1966), who reported that collagen served as an inducer molecule for muscle cell differentiation. Doering and Fischman (1977) also have found a macromolecule which served in this capacity. The specific role of collagen in

muscle development has been contested by several workers who have concluded that collagen merely enhances myogenic growth and differentiation and therefore has only a permissive role in muscle development (Bischoff and Holtzer, 1969; Yaffe, 1969). This enhancement of muscle growth by collagen may be necessary for normal *in vivo* muscle growth since cartilage develops prior to muscle (Abbott et al., 1974; Flickinger, 1974).

A second postulate for the mechanism of cellular differentiation argues that cells are, at most, bipotential (Abbott et al., 1974). This is accomplished by stepwise sequences of quantal mitoses, whereby a mesenchyme cell initiates the formation of a variety of other cell types (Abbott et al., 1974). Quantal mitosis is the mechanism by which cell progress from one cell compartment to another (Campbell et al., 1974; Holtzer and Bischoff, 1970) yielding daughter cells which are phenotypically distinct from that of the mother cell (Holtzer et al., 1975c). The inducers of this differentiation process may be produced endogenously rather than by exogenous means (Holtzer et al., 1973b; Konigsberg and Hauschka, 1965; O'Neil and Stockdale, 1972a).

The quantal mitosis theory of development seems more plausible since it maintains that, at any given time in the development process, each cell is fully differentiated within a cellular compartment, and a cell's progeny can enter a new cellular compartment only by means of a quantal mitosis (Abbott et al., 1974; Holtzer et al., 1975c; reviewed by Young and Allen, 1979). The former theory would seem to

permit spontaneous and perhaps uncontrolled differentiation.

Muscle, bone and connective tissue are derived from the same primordial germ layer, the mesoderm (Forrest et al., 1975). Thus, by the theory of bipotentiality, these tissue types have common ancestral cell compartments (Abbott et al., 1974). Through quantal divisions, terminally differentiated compartments are formed from more primitive compartments (Abbott et al., 1974). There are at least three cellular compartments between primitive mesenchyme cells and the terminally differentiated myoblasts (Abbott et al., 1974; Dienstman et al., 1974; Dienstman and Holtzer, 1975; Holtzer et al., 1974; 1975c), but there could be a larger number of compartments if myogenesis is similar to erythrogenesis (Weintraub et al., 1971).

During proliferation, cells enter the mitotic cycle which consists of four separate phases: 1) G_1, synthesis of machinery necessary for mechanics of cell replication (Holtzer and Bischoff, 1970; Lehninger, 1975), 2) S, in which the cell's complement of DNA is synthesized, 3) G_2, RNA and protein synthesis, and 4) M, where mitosis occur (Holtzer and Bischoff, 1970). The duration of each of these phases for replicating myogenic cells is 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, 1970).

The cellular compartment, presumptive myoblast fibroblast (PMbFb), is the myogenic stem cell that is thought to give rise to the cells of the myogenic cell line (Abbott et al.,

1974). This cell type is cytologically indistinguishable from the presumptive myoblast (PMb), the fibroblast (Fb), and other mesenchyme cell compartments from which the PMb originate (Holtzer et al., 1975d). The PMbFb is the immediate cell precursor to the PMb and Fb cell compartments; chondroblasts differentiate directly from the mesoderm rather than PMbFb (Abbott et al., 1974). PMbs comprise the penultimate compartment of the myogenic cell lineage (Abbott et al., 1974; Holtzer et al., 1974; reviewed by Young and Allen, 1979). These cells give rise to the myoblast (Mb) cell compartment by means of a terminal quantal mitosis (Abbott et al., 1974; Holtzer et al., 1974). At one time, the difference between the Mb cell compartment and the PMb compartment was thought to be quantitative rather than qualitative, based on adenylate kinase and creatine kinase levels in both the PMb and Mb (Tarikas and Schubert, 1974) and myosin heavy chain (MHC) synthesis rates in the Mb and myotube (Mt) (Emerson and Beckner, 1975; Schubert et al., 1973). However, these researchers did not examine the possibility of qualitative differences between the structural molecules of the Mb, Mt, and PMb. Myosin of the Fb and PMb is predominately the constitutive or nonmuscle type (Holtzer et al., 1975d), whereas, the vast majority of the myosin in the Mt is of the myofibrillar type (Holtzer et al., 1975d). These data indicate that a gene transition or activation has occurred between the PMb and the Mb compartments (reviewed by Young and Allen, 1979). At this time,

most investigators support the presumption that there is a qualitative difference between the PMb and Mb. This has been determined by actin to myosin ratios (Rubinstein et al., 1974), electrophoretic analysis of salt soluble protein fractions on SDS gels (Chi et al., 1975 a,b), half lives of actin and myosin molecules (Rubinstein et al., 1974) and immunological studies of the myosin molecule (Holtzer et al., 1975 c,d; Okazaki and Holtzer, 1965; Rubinstein et al., 1974). These studies also serve as direct support for the involvement of quantal mitoses in cellular differentiation. Mbs are postmitotic cells which have only the option of cellular fusion (Stockdale and Holtzer, 1961; reviewed by Fischman, 1972).

2. The Formation of Multinucleated Myotubes

Mt formation is the result of fusion of postmitotic Mbs (Dienstman and Holtzer, 1977; Holtzer, 1970; Holtzer and Bischoff, 1970). Structually, the onset of fusion is preceded by an end-to-end alignment of the Mbs (Stromer et al., 1974). Alignment is accompanied by a very close approach of surface membranes of adjacent cells (Stromer et al., 1974). These surface membranes gradually fuse, then disappear altogether. The cytoplasms of the fusing cells gradually become confluent, thereby, completing the fusion process. (Stromer et al., 1974). A single Mb has the option of fusing with another primed Mb or an existing Mt (Bishcoff and Holtzer,

1969; Holtzer and Bischoff, 1970; reviewed by Fischman, 1972). At one time, the increase in the number of nuclei within Mt was thought to occur by means of mitosis (Kitiyakawa and Angevini, 1963), amitosis (Godman, 1958) or endomitosis (Boyde, 1960). The evidence for the fusion process is well documented, primarily by electron microscopic studies (Przybylski and Blumberg, 1966), autoradiography of fusing Mbs and Mts, Holtzer, 1970) and clonal analysis of muscle cultures (Young et al., 1978b). Fusion appears to be the following two step process: 1) primed homotypic cells recognize each other (Okazaki and Holtzer, 1965; Yaffe, 1969) and 2) contiguous cell membranes undergo surface alterations that may be associated with the withdrawal from the cell cycle (Holtzer, 1970).

Fusion is coupled to the mitotic cycle as evidenced by fusion of Mb only after spending at least five hours in the G_1 of the cell cycle (Holtzer, 1970). Later evidence suggests that there is a repression of the cell cycle which occurs prior to, but independent of, the fusion process (Dienstman and Holtzer, 1977). The Mb probably withdraws from the cell cycle during the G_1 period of the Mb cell cycle just prior to the fusion process (Holtzer et al., 1975a; Holtzer and Bischoff, 1970). This withdrawal is likely an endogenously programed event which subsequently leads to restructuring of a cell surface which permits fusion (Holtzer et al., 1975b).

Based on research by Buckley and Konigsberg (1974; 1977) there is not sufficient evidence that cells withdraw from the

cell cycle prior to fusion, and these researchers argue that there is no endogenously programed sequence associated with fusion. Presently, there are two schools of thought as to the interrelationship between fusion and the mitotic cycle. 1) O'Neil and Stockdale, (1972b) and Buckley and Konigsberg (1974; 1977) maintain that fusion withdraws Mbs from cycle. 2) Bischoff and Holtzer (1969) and Nadal-Ginard (1978) propose that the withdrawal occurs prior to, but independently, of fusion.

The bulk synthesis of myosin and actin in the terminal G_1 is coupled to the withdrawal of the Mb from the mitotic cycle (Emerson and Beckner, 1975; Holtzer et al., 1975d Nadal-Ginard, 1975). Fusion and the bulk synthesis of the contractile tissue are thought to be related incidences, but not obligatory for one another (Bandman et al., 1978; Emerson and Beckner, 1975; Holtzer et al., 1974; 1975a,b,d; Kaufman and Parks, 1977; Reporter, 1974; Sanger et al., 1971; Sanger and Holtzer, 1972; Tarikas and Schubert, 1974; Vertel and Fischman, 1976; Young et al., 1975). It is not known at this time whether the critical event committing a Mb to fusion in the terminal quantal division is an event associated with the parental DNA synthetic period, the G_1, M, or G_2 of the Mb itself (Holtzer and Bischoff, 1970).

Mb fusion requires the presence of at least 50 μM Ca^{+2} (Holtzer, 1970) and possibly c-AMP (Zalin, 1976). Such compounds as antimycin-C (Konigsberg, 1964; Reporter and Ebert, 1965), Ca^{+2} free medium (Okazaki and Holtzer, 1965), pH

variations (Strehler, 1963), 2-4 dinitrophenol, cyanide azide, and (ethylenedintrilo)tetraacidic acid (Holtzer, 1970), are known to inhibit the fusion process by interrupting post-mitotic events occurring in the G_1 period immediately preceding fusion (Holtzer, 1970).

3. Maturation of the Myotube

Mt formation occurs in a sequential manner. Initially post-fusion, the nuclei become grouped together in the center of the sarcoplasm. At this development stage, the cytoplasm stains positive with basic dyes and there is a cytological absence of myofibrils (Carlson, 1970; Stromer et al., 1974). However, as previously discussed there is a substantial increase in myofibrillar protein synthesis at this time. Later, the nuclei become aligned in long central chains, the cytoplasm stains with eosin, and myofibrils become visible at the periphery of the developing muscle fibers. However, at this time myofibrils are not found in the more basophilic interior of the fiber (Carlson, 1970; and Stromer et al., 1974). After this stage of development, cross striations fill most of the fiber and the nuclei begin migration to the cell periphery (Carlson, 1970; Stomer et al., 1974). Finally, the nuclei migrate to locations somewhat equidistant around the periphery of the myofiber (Mf), at positions just under the sarcolemma (Muir, 1970; Stromer et al., 1974). However, more nuclei per unit area are found near the myotendonal

junction than any other location along the muscle fiber (Goldspink, 1972). The difference between a Mt and(Mf) is merely the degree of maturation. However, this demarcation has not been made clear in the literature. The decline of cellular fusion may be associated with the onset of basal lamina formation (Fischman, 1970).

4. Myofibril Assembly

The myofibrils are composed primarily of thick and thin filaments which initially form at the periphery of the Mt (Fischman, 1970; Tomanek and Colling-Saltin, 1977). The newly formed thick and thin filaments evidently self-assemble in the cytoplasm of the Mt to form heragonal myofibrillar arrays similar to those observed in mature cells (Stromer et al., 1974). It has been demonstrated that the hexagonal lattice of interdigitating thick and thin filaments forms in the absence of the Z-band (Allen and Pepe, 1965; Fischman, 1967; Warren and Porter, 1969) yet, the lattice forms prior to the synthesis of the M band (Fischman, 1967; 1972). However, microtubles and cytoplasmic streaming may play a role in the orientation of the myofilaments (Fischman, 1972). The sarcolemma may be involved in the assembly and orientation of the myofilaments. This was demonstrated by finding newly synthesized thin filaments more randomly oriented in the core of the Mt as compared to those in the periphery, which were almost perfectly aligned longitudinally with respect to the Mt (Fischman, 1967). This

can be explained by independent research carried out by Hagopian and Spiro (1970) and Kelly (1969), who suggest that the Z-band material associated with the sarcolemma may be directing orientation of the filaments. These researchers feel that the densely staining material found close to the sarcolemma is embryonic Z-band material which is derived from or associated with the sarcoplasm. Wainrach and Sotelo (1961), Hay (1963), and Heuson-Stiennon (1965) speculated that the Z-band acts as an assembly site for myofilaments. The above evidence, plus cell free assembly experiments involving purified myosin and actin (Hanson and Lowy, 1963; Huxley, 1963) serve as excellent evidence that the tertiary structures of actin and myosin molecules contain much, if not all, the necessary information required for polymerization into thick and thin filaments and subsequent aggregation of the filaments forming an ordered lattice structure. Fischman (1967) adds that it is reasonable to assume that the ionic strength and adenosine triphosphate (ATP) concentration within muscle cells allow myosin and actin monomers, once synthesized, to aggregate spontaneously into thick and thin filaments.

Myogenic cells are capable of the synthesis of a myriad of proteins, and some of these proteins form complexes that have yet to be properly characterized. The Mb and Mt assemble a 20 nm diameter filament whose function is unknown (Stromer et al., 1974). These microtubules are usually oriented parallel to the long axis of the Mt (Stromer, et

al., 1974) and are thought to function in the maintenance of the elongated shape of the Mt (Stromer et al., 1974). Stromer et al. (1974) postulate that the microtubules may form a cytoskeleton which directs assembly of myofilaments.

Another uncharacterized filament is a 10 nm diameter filament which has no peripheral organization and is found throughout the sarcoplasm of all developing muscles (Ishikawa et al., 1968; Kelly, 1969; Stromer et al., 1974; Tomanek and Colling-Stalin, 1977). At one time, it was thought these 10 nm diameter filaments were embryonic forms of the thin filaments which were later molded by means of proteolytic enzymes to the 6-8 nm diameter characteristic size of the thin filaments (Stromer et al., 1974). However, biochemical tests and structural examinations of these filaments proved these unrelated to actin or the thin filament (Ishikawa et al., 1968; Stromer et al., 1974). It is doubtful that this filament functions in myofibrillogenesis, since this molecule has been found in a variety of cell types (Ishikawa, 1968). But, it has been suggested that the myofibrils in skeletal muscle insert into the plasmalemma at myotendonal junctions, via a class of thin filaments which have been described as 5-10 nm in diameter (Auber, 1969; Ishikawa, 1965). These 10 nm diameter proteins may be related to the microtubules (Holtzer et al., 1973a).

B. Postnatal Growth of Mammalian Striated Muscle

1. Increase in Muscle Length

The available evidence suggests that Mb fusion ceases about the time of birth in domestic animals. This means that growth of skeletal muscle subsequent to birth occurs primarily through enlargement of existing cells (Stromer et al., 1974). It should be noted, that in such animals as the rat and possibly the chick, muscle growth may take place by means of increasing Mf number during the first three weeks postnatally (Chiakulas and Pauley, 1965; Goldspink, 1962; 1964; Mizuno and Hikami, 1972). Thus, increases in number of Mfs after birth may be dependent on degree of maturation of each species at the time of birth. Postnatal addition of muscle fibers should not be confused with the tremendous increase in muscle DNA during postnatal growth due to incorporation of nuclei into existing muscle fibers. The mechanism for this incorporation is probably similar to that of the activated satellite cell.

The limbs of most species of animals approximately double in length during postnatal growth (Goldspink, 1972). Some of this length is taken up by increases in the length of the tendons (Goldspink, 1972), and muscle fibers which run obliquely to the long axis of the muscle attribute some increase in length to increases in Mf girth (Goldspink, 1972). Most of the increase in muscle length, however, is due

to an increase in the number of sarcomeres positioned in series along the Mf (Goldspink, 1968; 1972; Hooper, 1976; Williams and Goldspink, 1971). Also associated with muscle growth is the decrease in the degree of overlap of thick and thin filaments resulting in an increase in the resting sarcomere length (Goldspink, 1968), thus resulting in an increase in muscle length.

The sarcomeres are postulated as adding to the myofibrils interstitially (Schmalbruch, 1968) or by serial addition of sarcomeres into the ends of existing myofibrils (Goldspink, 1972; Griffin et al., 1971; Holtzer et al., 1957; Hooper, 1976; Ishikawa, 1965; Kitiyaka and Angevini, 1963; MacKay et al., 1969; Williams and Goldspink, 1971). Presently, the later postulation is more generally accepted. The former postulated mechanism for increasing the number of sarcomeres within the myofibril would necessitate division of the myofibril transversely with the additional involvement of considerable modification of the sarcoplasmic reticulum and transverse tubule systems, which seems unlikely (Goldspink, 1972). Muir (1971) and Ishikawa (1965) suggest hypothetical mechanisms whereby peripheral clefts and fingerlike invaginations of the ends of the muscle fibers provide regions where myofilaments may be added to the ends of myofibrils without the myofibrils having to relinquish attachment of the sarcolemma. Griffin et al., (1971) and Williams and Goldspink (1971) found by means of autoradiography that newly synthesized

structural ADP of actin filaments and RNA are concentrated at the end regions of the muscle fibers and not along the periphery or in the middle of the Mfs. This gives the best evidence suggesting serial addition of sarcomeres to the ends of myofibrils. This also coincides with the observation that the terminal sarcomeres of the myofibrils are shorter than those in the middle (Goldspink, 1968; 1972). These new end terminal sarcomeres may not be fully functional (Goldspink, 1971). Williams and Goldspink (1971) determined that for normal serial addition of sarcomeres to occur, the muscle must be capable of isotonic contraction.

2. Increase in the Number of Myonuclei with Increasing Age

Moss (1968a) and Williams and Goldspink (1971) have determined that the number of nuclei within the muscle fiber increases beyond the stage at which there is no further increase in muscle fiber length. It has also been demonstrated that larger diameter fibers possess more total nuclei (Moss, 1968a; Williams and Goldspink, 1971; Swatland, 1977). Increases in muscle mass during normal growth are associated with an accompanied proportional increase in numbers of muscle nuclei (Powell and Aberle, 1973; 1975). Moss (1968b) demonstrated equal protein accretion per Mf nucleus in normal and starved chicks undergoing compensatory growth. This would indicate that protein accretion in muscle is

closely associated with increases in DNA (Allen et al., 1979).

The increase in nuclei within postnatal striated muscle is accomplished by fusion of satellite cells within the muscle fiber (Mauro, 1961; Miller, 1977; Moss and Leblond, 1970a,b; Muir, 1970; Reznik, 1970; Schultz, 1976; Shafig, 1970; Shafig et al., 1968; Young et al., 1978).

Mauro (1961) first identified the satellite cell in frog skeletal muscle. Since this time many investigators have confirmed their existence in skeletal muscles of other species (Muir, 1970). Satellite cells of striated muscle are mononucleated cells whose cytoplasm contains no myofilaments (Muir, 1970), and these cells are found under the basement membrane component of the sarcolemma but outside the plasma membrane of the Mf (Muir, 1970). The satellite cells are fusiform with the long axis of the cell aligned parallel along the muscle fibers (Muir, 1970). Satellite cells are usually closely associated with a capillary, are found within close proximity to a myonucleus (Schmalbruch and Hellhammer, 1977) and are easily identified by the abundant quantities of clumped chromatin in the nucleus with a small amount of cytoplasm containing few organelles surrounding the nucleus (Muir, 1970; Schultz, 1976). The organelles in the cytoplasm are mainly free ribsomes and mitochondria; a golgi apparatus can usually be observed in close proximity to the nucleus and isolated profiles of rough endoplasmic reticulum are located in peripheral regions of the cell

(Muir, 1970; Schultz, 1976). Differential staining has shown that satellite cells are devoid of glycogen in muscles of both the newborn and the adult (Galavazi and Szirmai, 1971; Ishikawa, 1970).

The satellite cells are regularly spaced along the Mf (Muir et al., 1965) and are found both along extrafusal and intrafusal fibers (Banker and Cooper, 1971; Maynard and Cooper, 1973). Aloisi et al., (1973) and Schmalbruch and Hellhammer (1977) determined that there are more satellite cells associated with slow phasic than with fast phasic muscle fibers. This coincides with the work of Burleigh (1977) who determined that there are more nuclei within slow phasic fibers than there are in fast phasic fibers. Church (1969) determined that satellite cells are recognizable in embryonic development as soon as the basement membrane becomes visible. Several workers (Ishikawa, 1970; Moss, 1968a,b, 1969; Moss and Leblond 1970a,b; Schmalbruch and Hellhammer, 1977; Snow, 1977; and Young et al., 1978b) found a significant decrease in the number of satellite cells per muscle as animals age. This is partially explained by the dilution of satellite cell numbers per gram of muscle due to accretion of muscle proteins during growth (Young et al., 1978b).

Most researchers class satellite cells as dormant Mbs (Schultz, 1976). However, activated satellite cells are capable of incorporating labeled thymidine into their nuclei, suggesting mitotic activity (Church, 1969; MacComachie

et al., 1964; Moss and Leblond, 1970a,b). It would seem therefore, that these cells more closely resemble a resting PMb since a Mb is incapable of replications (Holtzer, 1970).

Satellite cells play an important role in postnatal growth (Allen et al., 1979; Moss and Leblond, 1970a,b; 1971; Young et al., 1978b) and in muscle regeneration (Steen, 1970). The mechanism by which satellite cells are induced to replicate and subsequently incorporate into a Mf are presently unknown. However, satellite cells may add to the ends of the Mfs during growth (Goldspink, 1972). This would indicate that satellite cells must be capable of migration beneath the basal lamina, since the satellite cells are found spaced almost equidistantly along the length of the Mf. Electron microscopic studies by Schultz (1976) supports this postulation, based on the presence of numerous cytoplasmic processes extending from the satellite cell which could aid the migratory process. Schultz (1976) also reported that satellite cells of adult animals are dormamt. This conclusion is based on the observation of condensed chromatin material and the paucity of cytoplasmic organelles. However, injury and stress can by some unknown means activate these cells to proliferate and they or their daughters to subsequently fuse to form Mts (Schultz, 1976).

C. Mechanisms Regulating the Quantity of Proteins

1. Synthesis of Contractile Proteins

The contractile proteins are believed by some to be synthesized in close proximity to the area in which they are to be inserted into the prospective contractile unit (Etlinger et al., 1975; Larson et al., 1969; Morkin, 1970). Both synthesis and degradation of the contractile proteins seem to be coordinated in relation to one another (Devlin and Emerson, 1978; 1979; Rubinstein et al., 1976). Coordinated synthesis may be controlled by proportional numbers of polysomes corresponding to each contractile protein (Devlin and Emerson, 1979; Heywood and Rich, 1968). The rate of synthesis of each of these proteins varies with the time course of development (Devlin and Emerson, 1978). Very little, if any, myofibrillar contractile proteins (i.e. α-actin, tropomysin, and myofibrillar myosin) are synthesized in the unfused myogenic cell compartments (Devlin and Emerson, 1979; Przybyla and Strohman, 1974; Strohman et al., 1977; Young et al., 1975). However, there are changing synthesis rates of each of the contractile proteins with respect to each other as indicated by Heywood and Rich (1968); who found changing proportions of polysomal RNA of contractile proteins with respect to one another during development of embryonic chick muscle. Strohman and coworkers (1977) and Emerson and Beckner (1975) found a 30-100 fold increase in myosin heavy chain (MHC) mRNA activ-

ity during the progression from prefusion to fused myogenic cell cultures. Some of this change in mRNA activity may be attributable to the stability of the MHC mRNA which increases as differentiation proceeds from the PMb to Mb (Buckingham et al., 1974). The mechanism of this stabilization is not directly related to the entry of the messenger into polysomes, since the stable 26S MHC mRMA species appears in the cytoplasm approximately 16 hours prior to 26S polysomal MHC mRNA during Mt formation (Buckingham et al., 1974).

The 200,000 dalton MHC is synthesized on polysomes consisting of 50-70 ribosomes per mRNA (Heywood et al., 1967). Myosin light chains (MLC) on the other hand, are synthesized on smaller polysomes containing 4-9 ribosomes per mRNA (Low et al., 1971; Sarkar and Cook, 1970). This indicates not only that there are separate genes for the protein subunits but that MHC mRNA and MLC mRNA are monocistronic (Low et al., 1971; Thompson and Heywood, 1974), as are the mRNAs for actin and tropomyosin (Heywood and Rich, 1968). Generally, eucaryotic cells exhibit a direct relationship between the number of ribosomes in a polysome and the molecular weight of the protein synthesized (Heywood and Rich 1968; Low et al., 1971). The rate of MHC synthesis ranges from 24×10^3 to 37×10^3 molecules/min/Mt nucleus (Emerson and Beckner, 1975; Young et al., 1979).

2. Transcriptional Control of Protein Synthesis

Developing myogenic tissue undergoes a great deal of change during the maturation process. One of the largest changes is alteration in protein synthesizing capability. During the differentiation process this change results from either selective inactivation of existing active genes combined with an activation of previously transcribed regions of the genome, or quantitative modulation of the portion of the genome without concomitant expression of untranscribed genes (Reviewed by Young and Allen, 1979). These changes encompass the entire metabolic and functional needs of the cell compartments which undergo change during development. As cells develop they become more specialized, and this specialization process may take place by means of decreasing transcriptional diversity with each successive cell division. Ordahl and Caplan (1976) postulated, on the basis of nucleic acid hybridization data, a loss of transcriptional diversity in the myogenic cell line as proliferation of PMb ceases and the Mb form multinucleated Mts. The greatest diversity of the non-repetitive DNA sequence available for transcription into RNA occurs during the proliferative phase of myogenesis and decreases as Mt formation proceeds (Ordahl and Caplan, 1976). This is tentatively supported in earlier work by Nguyen and Cole (1972) who found the RNA synthesis per nucleus in postmitotic myogenic cell cultures to be approximately one third that of the dividing PMb. The diversity of the RNA does not seem to change at or after hatching (Ordahl and Caplan, 1976). The previous research as well as later

research by Caplan and Ordahl, (1978) supports the idea of irreversible gene suppression as a mechanism for cell specialization. All genes in the nucleus are initally transcribed, and cells become specialized by means of progressive gene amplification and/or suppression (Caplan and Ordahl, 1978). A second means whereby differentiation is thought to take place is reversible activation and inactivation of certain regions of the genome (Ptashne, et al., 1976). A similar mechansim for transcriptional regulation has recently emerged which outlines the mechanisms of attenuation and antiattenuation of transcription. Attenuation involves nonspecific initiation of transcription, resulting in early transcription termination and subsequent release of the abortive transcripts and the formation of complete but not necessarily functional transcripts (Remington, 1979). The antiattenuation scheme involves the binding of regulatory molecules to the nascent pre-mRNA results in a conformational change in the transcript near the 5' terminus, thus prohibiting the formation of the transcription termination complex (Remington, 1979). Which of these mechanisms is represents the true mechanism is presently unknown.

Transcription or the control of transcription may play the largest role in modulation of protein synthesis (Waterlow et al., 1978; Young, 1974). For example, a strict proportionality exists between the rate of ovalbumin synthesis and

the cellular content of both total and polysome-bound ovalbumin mRNA in both estrogen and progesterone treated tubular gland cells removed from the magnums from chick oviducts (Palmiter, 1972; Palmiter and Schimke, 1973). Using a similar model and a cDNA probe, similar results were found for estrogen alone (Harris et al., 1975; Means et al., 1975; Swaneck et al., 1979; Tsai et al., 1978) and estrogen and progesterone (McKnight et al., 1975). Additionally, Gurdon and coworkers (1971) found that the rate of hemoglobin synthesis increases linearly with the level of hemoglobin mRNA injected into Xenopus laevis oocytes. This evidence indicates that the cytoplasmic mRNA content is likely to be the rate limiting step in protein synthesis. Thus, transcriptional controls in general may play the most important role in controlling tissue specific protein synthesis and possible accumulation. A possible exception to the above generalization may be the myofibrillar contractile proteins during the early stages of myogenesis. The initial synthesis of contractile proteins in early myogenesis may be subject to translational controls, since mRNA for MHC seems to be present before and during fusion of Mb, with no myosin synthesis until several hours after the fusion process (Buckingham et al., 1974; Dym et al., 1979; Heywood et al., 1975; Morris et al., 1973; Paterson and Strohman, 1972; Strohman et al., 1977; Young et al., 1975). Upon completion of muscle differentiation, transcription is probably the primary regulator of protein accretion. Powell

and Aberle (1973; 1975) found increases in protein accumulation to be associated with higher levels of total RNA, and the number of polysomes per Mf may increase in maturing muscle cell cultures (Hosick and Strohman, 1971). However, it is important to realize that mRNA makes up a very small portion of the total RNA, and during muscle growth there is a decrease in rRNA accumulation after Mt fusion (Bowman and Emerson, 1977) and RNA is diluted several-fold in muscle tissue by the accumulating protein (Waterlow et al., 1978). Therefore, ribosomes between myofibrils possibly do not increase in number with age (Trayer and Perry, 1966; Young, 1970). Electron micrographs of growing muscle show that the concentration of ribosome-like granules between myofibrils decreases with age, while the populations of ribosomes at the periphery of the fibers remain constant (Galavazi and Szirmai, 1971). RNA levels may increase just prior to and shortly after birth in most tissue (including muscle), but post-natal growth and development do not involve further changes (See Waterlow et al., 1978 for a review). Waterlow et al. (1975; 1978), for example, found the RNA/DNA ratio during development in kidney, heart and muscle to remain unchanged during postnatal growth. These data imply that the amount of RNA per cell remains relatively constant from shortly after birth through maturity. It also is interesting to note in this regard that the degradation rates of rRNA and MHC mRNA are very similar in the Mb and Mt (Bowman and Emerson, 1977, Buckingham et al., 1974).

Muscle growth may diminish as a constant amount of polysomes in muscle synthesize only enough product so as to replace that which has degradated. This assumes constant transcription and translation rates and that protein degradation eventually becomes equal to synthesis rates as muscle mass reaches a steady state where no new accumulation can occur (Burleigh, 1974).

3. Post-Transcriptional Modification of mRNA and It's Role in Protein Synthesis

Post-transcriptional controls may have some importance in regulation of protein synthesis. Polyadenylation is a relatively late post-transcriptional step occurring in the latter third of the interval between transcription of the heterogeneous RNA (hnRNA) and the appearance of the mRNA in cytoplasmic polysomes and may be a rate limiting step in the processing of mRNA (Perry et al., 1974). The polyadenylation process is an integral part of the overall processing mechanism by which primary transcription products are converted to mRNAs. This may be accomplished by the poly-A tail serving as a nuclear control in selecting hnRNA which would eventually become mRNA of a polysome, or it may serve as a cytoplasmic control in determining mRNA activity. Both mechanisms seem possible since polyadenylation is known to occur in both the nucleus and cytoplasm (Diez and Brawerman, 1974; Mendecki et

al., 1972; Revel and Groner, 1978; Slater et al., 1973).

Polyadenylation may serve *in vivo* as a means of stabilization of the mRNA. Huez and coworkers (1975) demonstrated with globin mRNA injected into *Xenopus laevis* oocytes that mRNA is less susceptible to degradation when polyadenylated. The length of the poly-A tail on the 3'-OH terminus end of the mRNA decreases with the maturation time of the mRNA (Gorski et al., 1974; 1975) and may have some regulatory function in determining the life-span of mRNA. Since no RNA containing less than 20 adenylate residues could be found in the cytoplasm (Gorski et al., 1974), this population may be inactive or easily susceptible to degradation (Gorski et al., 1974). However, Greenberg (1975) demonstrated in HeLa, L, 3T3, and 3T6 cell lines that the majority of the cytoplasmic mRNA falls into only one or two classes with respect to half-life and, on this basis, he argued that the differential stability of mRNA does not have a major role in the control of translation or post-transcriptional modification of protein synthesis.

The poly-A tail is added to the hnRNA post-transcriptionally, and polyadenylated hnRNA is the immediate precusor to the mRNA which is found in the polysomes of the cytoplasm (Duncan et al., 1975). Messenger RNA seems to be selected from the hnRNA and transported to the cytoplasm where it decays much less rapidly than that of the hnRNA which is found within the nucleus (Bradhorst and Humphreys, 1972; Bradhorst and McConkey, 1974). What is interesting to

note is the high level of turnover of hnRNA found to take place within the nucleus and the drastically lesser quantity of mRNA found in the cytoplasm, which is derived from the hnRNA (Egyhazi, 1976).

Recent investigation has demonstrated that hnRNA of mouse β-globin (Tilghman et al., 1978), rabbit β-globin (Philip et al., 1978), chick ovalbumin (Dugainczyk et al., 1978), and yeast precursor tRNA (O' Farrell et al., 1978) have cleavage of an internal intervening segment and reannealing of the split ends of the mRNA and tRNA prior to biochemical activity. Philipp et al. (1978) postulates that the formation of stable hairpin loops surrounding the intervening spacer sequence plays an intergal role in ensuring that the translatable mRNA does not contain the spacer sequence. Whether any of this occurs with mRNA of the contractile proteins is yet unknown.

The mechanisms of hnRNA processing are largely unknown. Part of the mRNA is probably excised from the 3' region of the hnRNA, since hnRNA and mRNA were found to have a common 3' poly-A linked segment (Darnell et al., 1973). This also indicates that cleavage of hnRNA may take place after the addition of the poly-A segment. There is, however, contradictory evidence that the hnRNA is cleaved and processed from the 5' end after initial methylation of the guanine nucleotide, rather than processed from the 3'-OH terminus end (Perry and Kelly, 1976; Salditt-Georieff et al., 1976). The

5' terminal m^7G in the mRNA cap protects the mRNA from 5' exonucleolytic degradation (Revel and Groner, 1978); however, the main function of the cap seems to be recognition during initiation (Revel and Groner, 1978). Revel and Groner (1978) postulated that a rapid degradation of the whole mRNA molecule occurs following removal of the cap. Therefore, the cap structure may serve as an inhibitor of mRNA degradation.

Messenger ribonucleoprotein (mRNP) may be one of the post-transcription guides of gene expression of developing muscle tissue (Buckingham et al., 1974). Untranslated mRNA is located both in the nucleus and cytoplasm as mRNPs (Revel and Groner, 1978). Messenger RNP molecules are thought to be storage forms of mRNA which are found in the polysomes (Bag and Sarkar, 1976; Dym et al., 1979; Heywood et al., 1975). The proteins associated with polysomal mRNPs are quite divergent (Bag and Sarkar, 1976; Jain and Sarkar, 1979), denoting the possibility of some control mechanism for translation. It is interesting that the proteins associated with the mRNAs of free mRNPs may be common among different mRNA species (Bag and Sarkar, 1976), since the proteins associated with both actin and MHC mRNA are of similar molecular weights. This is reinforced by research of Bobel (1973) who found proteins of similar molecular weight associated with mRNP complexes associated with mRNA from rat liver hepatic cells, L cells, and globin mRNP.

Post-transcriptional alteration of the mRNA also in-

volves methylation of the adenosine units. The functional significance of methylation is to date unknown, but internal methylation may increase the affinity of mRNA for ribosomes and thereby serve to prevent breakdown of the mRNA (Revel and Groner, 1978).

4. Translational Controls of Protein Synthesis

Much of the short term regulation of protein synthesis may take place at the level of translation and many factors could alter the rate of mRNA translation (Lodish, 1976). Bowman and Emerson (1977) and Hirsch et al. (1973) demonstrated that the pool of native ribosomal subunits does not change in size to any great extent when the rate of protein synthesis varies; therefore, the rate of translational initiation during protein synthesis may not change to any great extent in growing tissue. However, Srivastava (1969) postulated that there are fewer ribosomes associated with the polysome during progressive growth. This could be the result of mRNA accretion without concomitant rRNA accumulation or a decrease in both mRNA and rRNA.

Under most physiological conditions, initiation is the rate limiting step for translation (Waterlow et al., 1978). One explanation for the reduction in the rate of initiation would be lack of translatable mRNA. This is probably not the case in most incidences, since reduction in the rate of protein synthesis by nutritional or hormonal deficiencies can be

immediately reversed by replacement of the missing substances (Waterlow et al., 1978). This indicates that sufficient mRNA is present, but some factor which is highly influenced by the nutritional status of the cell is needed for protein translation. RNPs may have some role in controlling protein synthesis under these circumstances, since RNPs are postulated as the cytoplasmic storage form of mRNA (Dym et al., 1979).

Regulation of translation may be mediated in some situations through reduced availability of one or more amino acyl-tRNA species to the ribosome. This could be due to shortages of essential amino acids, defects in the tRNA, or reduced activity of amino acyl-tRNA synthetases (Waterlow et al., 1978). The first factor can probably be disregarded in most cases since Allen and coworkers (1969) found the tRNA populations in rat liver to be nearly 100 percent amino acylated in fasting and normal rats. Measurable decreases in amino acylation were observed only when amino acid inbalances in diets were induced (Allen et al., 1969; Fleck et al., 1965).

Translational control RNAs (tcRNA) specific for myosin have been isolated from the intiation factor three (IF_3) preparations from muscle (Bester et al., 1975; Heywood et al., 1974). Specifically, two classes of tcRNAs have been isolated. One specifically inhibits the translation of heterologous mRNAs and myosin mRNPs (Bester et al., 1975; Heywood et al., 1974; 1975) and the other enhances polysomal mRNA translation with a great deal of specificity (Bester et al., 1975; Heywood

et al., 1975). However, the mechanism for binding differs between the tcRNA for inhibition and the one for enhancement of translation (Heywood et al., 1975). Translational control RNA tcRNA isolated from mRNP molecules was demonstrated to inhibit both myosin heavy chain and globin synthesis in heterologous reticulocyte cell free systems involving the specific mRNP-mRNA as templates (Bester et al., 1975; Heywood et al., 1974). The tcRNA which inhibits myosin synthesis is thought to be a mRNA protein tcRNA containing an oligo uridine region which binds to myosin RNPs and non-polysomal mRNA at the poly-A tail (Heywood et al., 1975). The poly-U tail on the mRNA·protein tcRNA may be cleaved during processing, forming a tcRNA which stimulates mRNA translation (Bester et al., 1975; Heywood et al., 1974; 1975). Both classes of tcRNA are very mRNA species specific (Heywood et al., 1975). This is thought to be due to a region on the tcRNA which can specifically recognize base sequences at or near the 5'OH end of the mRNA (Heywood et al., 1975). The protein component of the mRNA·protein tcRNA complex is involved in the tcRNA-mRNA interaction (Kennedy et al., 1978). It is thought that the protein component is absent in the tcRNA complex which stimulates translation of myosin (Heywood et al., 1975).

The IF_3 promotes the recycling of ribosomal subunits into polysomes (Benne and Hershey, 1978; Kafatos and Gelinas, 1973; Whalen and Gros, 1977). Primarily, initiation factors are responsible for influencing the binding of mRNA and f-met-

tRNA during initiation (Jackson, 1975). The process is further aided by ribosomal proteins such as S-1 and S-12 with which the initiation factors produce changes in mRNA or rRNA configuration and subsequently affect the stability of the interaction between the two (Adams et al., 1976).

The initiation factor, IF_3, from muscle ribosomes is the only tissue-specific initiation factor known at the present time which may be required for MHC mRNA translation, as IF_1 and IF_2 from other sources are equally effective in initiating MHC synthesis (Rourke and Heywood, 1972). Whalen and Gros (1977) do not feel there is sufficient evidence to suggest species specific initiation factors for each mRNA. These researchers found no real specificity between initiation factors and the message being translated.

Ribosome translation time for the synthesis of one MHC molecule in chick embryos is 5 to 10 minutes (Kabat and Rich, 1969). And 50 to 75 ribosomes simultaneously translate each active myosin mRNA (Heywood et al., 1967). Using these figures each myosin mRNA could produce 5 to 15 MHC subunits per minute (Young, 1974). A Mb or Mt nucleus which is fully activated and synthesizing MHC at 30,000 molecules/nucleus/min would need to contain at least 2000 to 6000 MHC mRNA molecules per Mf nucleus (Young, 1974). The measured quantities of MHC mRNA per Mt nucleus varies a great deal between laboratories. John et al., (1977), Robbins and Heywood (1978), and Dym et al., (1979) report values for the

number of MHC mRNA per Mt nucleus which are of the same order of magnitude, these values being 1406, 1200-2200 and 650-4800 MHC mRNA molecules per Mt nucleus respectively. These values compare reasonably well with the earlier predicted values made by Young (1974). Benoff and Nadal-Ginard (1979a) measured 47,500 MHC mRNA molecules per Mt nucleus. However, they measured poly-A minus mRNA whereas Robbins and Heywood (1978) and Dym et al., (1979) measured poly-A MHC mRNA and John et al. (1977) obtained their values from hybridization analyses of total RNA muscle preparations. Benoff and Nadal-Ginard (1979a,b) may have technical problems inherent in their systems which would influence their result.

5. Protein Degradation

Generally, proteins turn over at a rate proportional to their subunit molecular weight (LaGrange and Low, 1976). This holds true under both *in vitro* and *in vivo* conditions, and proteins which degrade more rapidly *in vivo* behave similarly *in vitro* (Dice et al., 1973; Goldberg and Dice, 1974). Characteristics such as large target sizes, higher incidences of transcriptional and translational errors manifested within the primary structure and more inherent instability of large proteins may serve as partial explanations for the larger proteins degrading more rapidly (Goldberg and Dice, 1974; Goldberg and St. John, 1976; Waterlow et al., 1978). Dice et al., (1979) reports that proteins with a low isoelectric point (pI) are

more susceptible to degradation. This research stemmed from earlier observations of faster turnover of acidic proteins (Dice and Goldberg, 1979). Susceptability to endproteases can be ruled out as the mechanism whereby acidic proteins degrade more repidly, because Dice et al., (1970) demonstrated that acidic proteins were no more susceptible to various endoproteases than were neutral or basic proteins. Proteins within specific pI ranges still exhibit a relationship between subunit size and half-life (Dice et al., 1979).

The 200,000 dalton molecular weight MHC subunit of myosin seems to be an exception to the general idea of direct proportionality between protein size and degradation rate. LaGrange and Low (1976) determined that the MHC subunit turns over at a rate only slightly faster than the 20,000 dalton MLC subunit of myosin. McManus and Mueller (1966) found MHC to turnover at a rate similar to that of MLc in vivo and Low and Goldberg (1973) discovered MHC degraded at a rate just slower than actin, tropomyosin, and MLC. Based on the majority of the evidence it is reasonable to assume that MHC is more stable than one would expect for a molecule of it's molecular weight. A partial explanation for the stability of myosin may result from its highly ordered structural arrangement within the interior of the myofibril.

Muscle contratile proteins turnover slower than enzymes, ans slower than the contractile proteins of the heart and

smooth muscle (Kimata and Morkin, 1971; Waterlow et al., 1978). Evidence indicates that the rate limiting step in proteolysis is probably prior denaturation (Reviewed by Waterlow et al., 1978).

Kimata and Morkin (1971) determined that myosin turnover follows first order kinetics, indicating that myosin breakdown is a random process. Swick and Song (1974) also favor random degradation as the means by which myosin is degraded, but they concede that some proteins involve cellular life spans. Earlier research such as that carried out by Dreyfus (1960) postulated that myosin is not in a state of dynamic equilibrium, but rather governed by lifetime kinetics.

Factors affecting the turnover of proteins may be such things as stabilization by ligand binding (documented in enzymes only), primary structure, conformation, workload or amount of contraction (in cases of contractile proteins), the tissue location of the protein, hormone treatment and enzymatic activity of the protein (Dice and Goldberg, 1974; Goldberg and St. John, 1976; Kimata and Morkin, 1971; Waterlow et al., 1978).

6. Protein Accumulation within Muscle

The intensity of myosin synthesis is higher in red or oxidative muscle fibers than in white or glycolytic fibers (Kimata and Morkin, 1971; Waterlow et al., 1978). However, there is no noticeable difference in myosin degradation between the fiber

types (Kimata and Morkin, 1971). This seems odd since white fibers are larger, yet according to these data proteins should accumulate more in red muscle fibers. There may be some experimental error in one or both determinations.

Present evidence suggests that the relative rates of synthesis of particular proteins are altered in such a way during growth, so that proteins with the slowest breakdown rate show the greatest developmental fall in fractional synthesis rate (FSR) (Waterlow et al., 1978). As the rate of protein synthesis (FSR) declines a concomitant decreases in the activity of the RNA is seen (Millward et al., 1975).

Muscle hypertrophy occurs during growth, work, passive stretch and denervation (diaphragm only) (Waterlow et al., 1978). In all cases of muscle hypertrophy (except possibly growth), the accumulation of muscle protein is attributable to an increase in the FSR without an increase in the fractional breakdown rate (FBR) (Waterlow et al., 1978). The increase in synthesis rate is due to an induced increase in the level of mRNA accumulation and an increase in DNA (Hubbard et al., 1975). This indicates incorporation of satellite cell progeny into muscle fibers during hypertrophy. In summary, although the mechanisms responsible for changes in protein content in skeletal muscle are not known, it is clear that protein accretion occurs when net synthesis rate of protein exceeds the net degradation rate (Millward et al., 1975).

D. Postnatal Muscle Growth in Avian and Mammalian Animal Models

The use of divergent strains or breeds of animals as models for growth studies, may the potential of being of great value in evaluating changes and differences in muscle growth from early embryonic stages through senility.

Mizuno and Hikami (1972) found day old chicks of the White Cornish breed (meat type) to be somewhat heavier than those of the White Leghorn (egg type); however, no significant differences between muscle weights were observed. The mature weight of White Leghorns is about two thirds that of the White Cornish. There is no increase in muscle fiber number after hatching in chickens (Mizuno and Hikami, 1972; Moss et al., 1964; Smith, 1963), and muscles of the White Cornish chicks had significantly more muscle fibers than those of the White Leghorns. DNA per muscle was equal in the two types at hatching, indicating equal numbers of myofiber nuclei per muscle (Mizuno and Hikami, 1972). Nuclear division of myogenic cells took place more rapidly in the White Cornish breed than in the White Leghorn after hatching (Mizuno and Hikami, 1972). This indicates that there are more nuclei being incorporated into the Mfs of the White Cornish chicks. Accompaning the addition of nuclei are greater serial additions of sarcomeres into the myofibrils of the White Cornish breed. This assumption is based on research by Hooper (1976) who found more

sarcomeres in heavy body weight mice compared to the light body weight counterpart and research by Hegarty et al., (1973) who determined that sarcomere length is equal for all individuals within the same species.

For the most part there was no difference in the N/DNA ratio between White Cornish and White Leghorn chicks from the time of hatching through 8 weeks of age (Mizuno and Hikami, 1972). Thus, the amount of cytoplasm controlled by each Mf nucleus is approximately the same size. However, a difference was found between the two breeds at 8 weeks in the M. satorius and M. gastrocnemius muscles, where there was a higher N/DNA ratio in the White Leghorn chicks. As indicated earlier, the DNA content per muscle in all muscles studied was essentially equal for the two breeds at hatching (Mizuno and Hikami, 1972); however, two and eight week old chicks exhibited significantly more DNA/muscle in all muscles of the White Cornish breed.

The study by Mizuno and Hikami (1972) showed that part of the difference in muscle weight is due to Mf number and another portion is due to the ability of muscles to activate satellite cell populations to replicate and subsequently fuse to existing Mfs.

Other researchers agree that the majority of the differences observed in the size of muscles in mature animals is due primarily to muscle fiber numbers (Ezekwe and Martin,

1975; Hanrahan et al., 1973; Luff and Goldspink, 1970). This is genetically programed and to a limited extent, is an inherent property of breeds, strains or herds depending on selection practices. Fiber diameter also had an important role in controlling the overall differences in muscle size (Ezekwe and Martin, 1975; Lepore et al., 1965). However, Simmonds and coworkers (1964) fell that accumulation of extracellular material such as collagen may be the main constituent which accounts for differences in ultimate muscle size.

Since muscle fiber number is a genetic trait, it could be used as selection criteria for meat animals. The degree of emphasis placed on this trait would depend on heritability of the trait and the degree to which this trait is affected by environmental conditions. Inadvertently, meat animal selection over the years has involved selection for animals of higher fiber number. Increases in Mfs per muscle have been shown to occur in mice when selection pressure is applied toward large mature size (Aberle and Doolittle, 1975; Ezekwe and Martin 1975; Luff and Goldspink, 1967) and postulated to occur in swine (Powell and Aberle, 1976; Ezekwe and Martin, 1975). The remaining challenge seems to be maximization of Mf numbers per muscle by selection, at the same time altering environmental conditions so as to allow maximal expression of genetic potential and possibly manipulate physiological conditions so as to maximize protein accretion. The

goal would be to achieve this condition at the least cost and in the shortest time possible.

E. Conclusion

Some animals are more heavily muscled than others of the same breed or species, primarily because the more heavily muscled individuals have more and larger muscle fibers. Although the number of muscle fibers is determined at fertilization, the extent to which an animal can express its genetic potential is not known until the animal is nearly grown. Selection of breeding animals for the meat industry could be made easier and less expensive if a method could be devised to accurately assess an animal's own performance. This would be most adventageous if the sample could be obtained at a very early age (i.e. at birth). The use of cell cultures of myogenic tissue of production animals could eventually be a means of screening production animals. Thus, the goal of the present experiments was to measure growth parameters of muscle cells in culture from grossly divergent animal strains in order to determine whether these parameters could reflect the gross differences found in the animals.

MATERIAL AND METHODS

All preparations were at temperatures of 0-4°C unless otherwise stated.

A. Experimental Design

1. Culture System

The study involved growth comparisons between meat and egg type chickens. This process was complicated because embryo extract was required for optimum growth in cell cultures. Therefore, four treatments were required so the exogenous and endogenous growth differences between cell types could be distinguished; broiler cells nourished with media containing broiler embryo extract (BC x BE), and broiler cells nourished with media containing layer embryo extract (BC x LE) comprised the broiler cell treatments. The same extract combinations were established for layer cells (LC), forming treatments LC x BE and LC x LE.

Almost the entire pattern of muscle differentiation can be reproduced in muscle culture over a period of approximately 8 days. Collection of muscle tissue for monitoring growth was made on days 1, 2, 3, 4, 6, 8 and 10. Cultures can be maintained for approximately 14 days with very few problems. However, cultures frequently undergo deterioration after 8 or 10 days.

2. Number of Experiments, Replicates, and Statistical Analysis

Five experiments were established within each treatment, and triplicate samples were taken at each time point within experiments. Statistical analysis was analysis of variance of the mean growth response using the F test for significance determination.

3. Logistics of Culturing All Experiments per Treatment at One Time

Culture systems can be extremely variable. Therefore, to obtain consistancy within treatments, the five experiments within each treatment required culturing at one time.

B. Preparation Methods

1. Procurement of the Animal Model

Broiler strain fertilized eggs (Hubbard X Hubbard) were obtained from Lamkin's Hatchery, Inc., Skowhegan, Maine. The fertilized eggs of the layer strain (DeKalb Leghorns) were purchased from Reichard's Hatchery, St. Louis, Michigan. These fertilized eggs were incubated for 12 days at 37.5°C, at which time the hind limbs were excised and myogenic tissues prepared for tissue culture.

2. Preparation of Cells for Muscle Culture

Embryos were removed and placed on their backs in the top half of a 10 cm culture dish (Corning). Skin from the hind limbs was removed with forceps. The legs were then sep-

arated from the embryo by pinching at the point where the thigh joins the body, and the legs were placed in the bottom half of a culture dish containing buffered saline solution (0.137 M NaCl, 0.0027 M KCl, 0.001 M MgCl, 0.00015 M NaH_2PO_4, 0.000136 M $NaHPO_4$, 0.006 M $NaHCO_3$, 0.0055 M glucose, 37°C, pH 7.4). The remainder of the skin was removed and the lower leg was separated and subsequently discarded, leaving only the thigh in the BSS. All bone was removed from the thigh muscle and discarded. The muscle was then placed in a 50 ml disposable conical test tube (Corning) containing 10 ml of complete medium per dozen embryos. The previously outlined procedure was repeated until sufficient tissue had been obtained. For reference, each twelve day old embryo yielded approximately 4.5×10^7 myogenic cells.

When an adequate number of eggs had been processed, the tissues in the 50 ml test tube were vortexed on a Votrex Genie (Fisher Scientific) at maximum speed for 20 seconds. The vortexed suspension was poured into a 10 cm culture dish and was immediately pulled into a 50 ml disposable syringe. A Swinney filter was attached to the syringe, and the syringe inverted for at least 30 seconds to allow the larger particles in the suspension to settle toward the plunger in the barrel of the syringe. The suspension was then forced through the filter into an empty 50 ml conical test tube. The nylon filter, the material remaining in the Swinney filter and the material in

the 10 cm culture dish were then placed in the original 50 ml conical test tube, and 10 ml of fresh complete medium was added. This was then vortexed and filtered in the same manner as just described. The two cell suspensions were combined and centrifuged at 700 x g for 3.5 minutes. The supernatant was discarded and the cells (pellet) were resuspended in an appropriate volume of complete medium by careful aspiration with a pipet; a small aliquot was removed for cell quantitation with a hemocytometer. Cells were plated onto 10 cm Corning culture dishes which had previously been coated with approximately 3.6 g/cm^2 of sterile collagen and then air dried. Cultures were incubated in 4 ml of complete medium at 37°C in the presence of 5% CO_2 and 95% humidity. Medium changes were made every 24 hours with 4 ml of fresh medium prewarmed to 37°C.

Complete medium was made up of 85% Eagles Minimum Essential Medium, 10% horse serum, 5% embryo extract, fungizone 2 mg/L, penicillin-streptomycin 100,000 Units/L, and gentamycin sulfate 90 mg/L.

3. Chicken Embryo Extract

Tweleve-day chicken embryos were decapitated and forced through a 50 ml disposable syringe. An equal volume of BSS was added, the mixture was placed at room temperature for 0.5 hr and the suspension was centrifuged at 1500 x g min. The supernatant from this centrifugation was saved and stored

at -20 °C for up to 6 months. After the extract had been thawed for use in complete medium, it was again centrifuged at 1500 x g for 10 minutes to remove insoluble material.

4. Preparation of Collagen

Collagen (calf skin) was dissolved in 0.1% acetic acid to a final concentration of 0.2 mg/ml, steam autoclaved and stored at -5°C.

5. Preparation of Myosin

Connective tissue and fat were trimmed from 300 g of mature chicken breast and leg muscle. The muscle was immediately chilled on ice until mincing. The excised muscle was placed in 3.3 volumes (w/v) of extraction buffer (0.3 M KCl, 0.15 M K_2HPO_4, 0.02% sodium azide, pH 6.5, 2°C) and homogenized for 30 seconds in a Virtis 45 homogenizer. This suspension was allowed to set at 2-3 °C for 30 minutes. Cold, soluble distilled H_2O (13.3 volumes) was added slowly while stirring and the suspension was strained through glass wool. An additional 20 volumes of cold water were added slowly, and the suspension was allowed to set at 2-3°C for two hours. At the end of the two hours the supernatant was decanted and the precipitate collected by centrifugation at 2000 x g for 45 minutes at 2°C. The precipitate was dissolved in 245 ml of 0.3 M KCl and 0.5 ml of 1.0 M Tris, pH 7.5. The volume was made to 320 ml with

cold double distilled water, and the solution was centrifuged at 55,000 x g for 30 minutes; this was followed by filtration through glass wool and dilution to 0.03 M KCl by the addition of cold distilled water. A crude myosin precipitate was collected by centrifugation at 2000 x g for 45 minutes. This pellet was redissolved in 20 ml of 3 M KCl and 7.5 ml of 1.0 M Tris-Acetate, pH 7, and the volume was brought to 120 ml. The myosin solution was clarified by centrifugation at 65,000 x g for 45 minutes, filtered through glass wool, and diluted with cold double distilled water to 0.23 M KCl. Dilution was made by adding the cold water in four small increments preventing KCl concentration at the interface between the protein solution and the added H_2O from falling low enough to precipitate the myosin. Clarification of the solution was made by centrifugation at 58,000 x g for 30 minutes. The supernatant was then filtered through glass wool and diluted to 0.03 M KCl. Myosin was then collected by centrifugation at 2000 x g for 45 minutes. The pellet was redissolved, clarified and collected one more time using the previously outlined procedure. The pellet obtained from the last precipitation was washed three times with cold distilled water. After each wash the myosin was collected by centrifugation at 200 x g for 30 minutes. The myosin pellet after the last wash was dissolved in 0.5 M NaCl, 0.1 M $KHCO_3$, pH 7.0. Cold glycerol was added to the myosin solution until the final solution contained 40 %

glycerol. At that time the final concentration of the NaCl was 0.16M. Protein concentration was determined by the method of Lowry et al., (1951), and the myosin stored at -20 °C until use.

C. Analytical Procedures

1. Determinations of Percentage Fusion, and the Number of Myotube Segments

Muscle cell cultures were prepared for cell quantiation by removal of the culture medium followed by rinsing twice with cold BSS. Cells were then fixed in absolute methanol for 10 min and stained with Giemsa stain for 20 min at room temperature. Nuclei were counted in 10 randomly selected fields, and the percentage of nuclei within Mts was calculated by dividing the total number of fused nuclei by the total number of nuclei and multiplying by 100. The area of the individual culture dishes and the diameter of the field of vision of the inverted phase microscope (Opton by Zeiss) were known. Therefore, the number of total nuclei, Mt nuclei, Mt nuclei per Mt segment and Mt segments per culture plate could be calculated. Total nuclei was the estimated number of both fused and unfused nuclei; obtained by multiplying the average number of total nuclei determined in 10 fields by the number of fields found in a 10 cm culture dish. Total Mt nuclei were determined in a manner similar to

that described above, however, only nuclei which were contained within Mts were quantitated. Mt segments and Mt nuclei per Mt per plate were calculated in a similar manner Mt segments refers to only those protions of Mts that were visible in one field of vision, rather than entire Mts. For this reason the value is only indirectly related to the total number of muscle fibers within a muscle.

2. Pulse Labeling of Cultures for Measurement of Myosin Heavy Chain Synthesis Rate

Muscle cell cultures were evaluated for rate of myosin heavy chain (MHC) synthesis at various culture ages by pulse-labeling with [^{3}H]-L-leucine. Cultures in 10 cm plates were labeled at 37°C for 4.0 hr with 1.5 ml of complete medium containing 10 µCi of [^{3}H]-L-leucine/ml (New England Nuclear). At the end of the labeling period, dishes were rinsed twice with cold BSS followed by a double rinse with cold 0.25 M KCl, 0.01 M $MgCL_2$, 0.01 M Tris-Hcl, pH 7.4, and then 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. The culture tissue was homogenized with 20 strokes of a 7 ml Dounce-Type homogenizer (Wheaton Scientific, A pestle) and the homogenate was placed for 15 min at 2°C. The homogenate volume was measured using a 1.0 ml disposable pipet. A 20 µl aliquot of the crude homogenate was removed for protein quantitation. The homogenate was then centrifuged at 1600 x g for 20 minutes at 2°C. The

KCl concentration of the supernatant was lowered to 0.025 M by adding 4.5 ml of cold water, tubes were left at 2°C for at least 8 hr, and the myosin-containing material was pelleted at 1600 x g for 50 minutes. The supernatant was combined with the original pellet (which contained nuclei, mitochondria, and cellular debris) for measurements of total protein synthesis rate and specific activity of the intracellular amino acid pools. The myosin pellet was dissolved and the precipitated protein was analysed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described later under SDS-Polyacrylamide Gel Electrophoresis of Proteins.

3. Pulse-Labeling of Cultures for MHC Turnover Rates

Muscle cell cultures were evaluated for rate of MHC degradation by pulse-labeling cultures at three days of age with 1 μCi [^{3}H]-L-leucine per 10 cm culture dish for 24 hours. After the 24 hr labeling period, all dishes were rinsed twice with BSS at 37°C. Following rinsing, plates were collected for cell quantitation and for MHC extraction. The remaining plates were refed with medium containing 5 mM unlabeled amino acids. Subsequent, samples were collected at 12, 24, 48 and 72 hours following the initial collection.

Culture dishes to be harvested were washed twice with cold BSS followed by a double rinse 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 dish surface into 0.5 ml of cold 0.25 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4. The muscle tissue was homogenized with 20 strokes in a 7 ml Wheaton homogenizer (A pestle) and the homogenate was placed at 2°C for 15 minutes. 2 µl of unlabeled myosin (concentration 13.1 mg/ml) was added to the crude homogenate. This homogenate was then centrifuged at 1600 x g for 20 minutes at 2°C, the supernatant was transferred to a second 15 ml conical test tube and the KCl concentration lowered to 0.025 M KCl by the addition of 4.5 ml cold distilled water. The pellet from the previous centrifugation was discarded. Myosin precipitation required at least 8 hr at 2°C, then the myosin-containing material was pelleted at 1600 x g for 50 minutes. This pellet was dissolved and analyzed by SDS-polyacrylamide gel electrophoresis as described below.

4. SDS-Polyacrylamide Gel Electrophoresis of Proteins

Myosin-containing pellets were dissolved by heating at 80°C for 20 minutes in 75-100 µl of a solution containing 1.0% SDS, 0.05 M Tris-HCl, 20% glycerol, 0.5% mercaptoethanol, 0.01% Pyronin Y, pH 7.2. The dissolved samples were layered quantitatively onto 8 cm x 0.5 cm, 10.0% polyacrylamide gels. The gels were electrophoresed at 0.8 mAmps per gel tube in a buffer containing 0.1% SDS, 0.20 M Tris-Glycine, pH 8.8. Gels were stained in a 0.033% Coomassie Brilliant Blue 250 R solution for 24 hours. The gels were destained electrophoretically by two, 15 min bursts in a model 1801 Quick Gel

Destainer (Ames Company) in a destaining solution containing H_2O:glacial acetic acid:methanol (87.5:7.5:5v/v). Further destaining was achieved by diffusion in 15 ml disposable conical test tubes for 3 days. When degradation rates were measured, the gels were stained for 4 hr followed by immediate electrophoretic destaining and no diffusion destaining was required.

5. Measurement of Radioactivity in the 200,000 Dalton Subunit of Myosin in SDS-Polyacrylamide Gels

Destained gels from MHC half-life experiments were frozen in dry ice, and 10 consecutive 1.00 mm slices were taken through the 200,000 dalton subunit band. Slices were dissolved in 0.2 ml of 30% hydrogen peroxide by heating at 50°C for 8-12 hours in glass scintillation vials (type 3 glass, 4 ml capacity, Rochester Scientific Company). Aquasol-2 (New England Nuclear) was added to each vial and the vials were vortexed. Vials were equilibrated in the dark for 15 minutes prior to counting in a Beckman 3131P liquid scintillation spectrometer. Counts per minute (cpm) were converted to disintegrations per minute (dpm) using the external standard channels ratio method utilizing chloroform as the quenching agent.

6. Measurement of Coomassie Brilliant Blue Dye Binding to the 200,000 Dalton Band and Measurement of Radioactivity within the MHC Band

Destained gels were frozen in dry ice, and 10 consecutive

1.00 mm slices were taken through the 200,000 dalton subunit band. Each slice was placed in a 20 ml disposable glass scintillation vial (Scientific Products). Slices were dissolved at 25°C for 2 hr in 0.6 ml 2.0% periodic acid, 4.0% lactic acid, pH 3.0. The digested sample was transferred to a 1.5 ml cuvette, and the absorbance was measured spectrophotometerically at 560 nm in a Beckman model 35K spectrophometer. Ten ml of Aquasol-2 were added to each vial and mixed. Vials were equilibrated in the dark for 15 min and analyzed as described in Section 5.

Spectrophotometric quantitation of MHC was optimized for pH, temperature, staining time, migration distance and sample volume.

7. Total Radioactivity Incorporated into TCA Precipitable Protein

The pellet from the first centrifugation of the pulse-labeled homogenate and the supernatent from above the myosin-containing pellet were combined. This material was mixed thoroughly, and trichloroacetic acid (TCA) was added to a final concentration of 10.0%. The suspension was placed at 2°C for 2 hr and centrifuged at 700 x g for 15 min. The supernatant was retained for measurement of intracellular amino acid specific activity. The pellet was dissolved by the addition of 4-5 ml H_2O_2, followed by heating at 100°C for 3-4 hours. This solution was then placed in a 10.0 ml volumetric flask, brought to volume with H_2O_2, mixed by inversion,

and 0.100 ml aliquots placed in 20 ml disposable scintillation vials. Aquasol-2 was added and samples were analyzed as detailed in section 5.

8. Radioative Complete Medium

Radioactive complete medium was prepared similar to regular complete medium with the exception that labeled L-(4,5^{3}H) leucine (Amersham or New England Nuclear) was added to the complete medium to a concentration of 10 µCi/ml.

9. Preparation of Medium and Intracellular Amino Acid Samples for Reaction with Dansyl Chloride

Culture medium and the cell homogenates (minus the salt soluble fraction) were prepared in similar manner for amino acid extraction. The nuclear-mitochondrial precipitate and the supernatant remaining after centrifugation of the myosin-containing material were combined and stored in 15 ml conical test tubes at -20°C until analysis. Upon thawing, the material was mixed and TCA was added to a final concentration of 10%. This suspension was placed a 2°C for 2 hr, after which it was centrifuged at 700 x g for 15 min. The supernatant was removed and extracted three times with 2 volumes of diethyl ether. The sample was lyophilized (Virtus Model 10-117) and dried amino acids were dissolved in a small volume of $NaCO_3$, pH 8.5 buffer.

10. Dansylation of Amino Acids

The dansyl chloride solutions (50 µCi in benzene) were

taken to dryness under a nitrogen stream and resuspended in a 1.0 ml acetone. A 10 µl aliquot of sample from cell cultures was mixed with 10 µl ^{14}C dansyl chloride reagent (50 µCi/ml acetone) followed by incubation at room temperature in the dark for 1 hr. Following incubation, the reaction mixture was again taken to dryness under nitrogen. The reaction products were resuspended in 10 µl of acetone and then spotted on a 7.5 x 7.5 cm polyamide thin-layer sheet. The chromatogram was developed by two dimensional chromatography. Two percent formic acid comprised the first solvent system and benzene and acetic acid (90:10, v/v) was utilized in the second, after drying the spots were visualized under short ultraviolet light. The leucine spot was identified, cut from the chromatogram and subsequently digested with 0.5 ml NCS tissue solubilizer (Amersham) at room temperature for 1 hr. Following digestion the solution was neutralized with 17 µl glacial acetic acid. After the addition of 15 ml of toluene·PPO (6g PPO/1X toluene) liquid scintillation cocktail, the samples were counted for (^{14}C) and (^{3}H). Spillage of (^{14}C) into the (^{3}H) channel was corrected, and the dpms of both the (^{14}C) and (^{3}H) were calculated using external standards ratio method.

11. Measurement of Protein Concentration

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

D. Microscopy

Light microscopy was preformed on Giemsa stained cultures with the use of a Zeiss Opton phase and bright-field inverted microscope. Bright-field micrographs were made to provide evidence for various degrees of muscle differentiation which developed in the in vitro model. A Zeiss Photomicroscope III was used for photographing cell cultures.

RESULTS AND DISCUSSION

Muscle growth has been shown in the literature to be a complex sequence of ordered events encompassing cell proliferation, differentiation and myofibrillar protein accretion. Muscle cell cultures were employed in this study as models for the examination of several parameters associated with muscle growth. These parameters were monitored over a 10 day culture period, which permitted examination of cells throughout proliferative and stationary growth phases. Percentages of myogenic and nonmyogenic cells in cultures at each day were quantitated in Giemsa stained cultures as described in Materials and Methods. Figures 1 and 2 are photomicrographs representative of culture fields of each of the four treatments after eight days in culture.

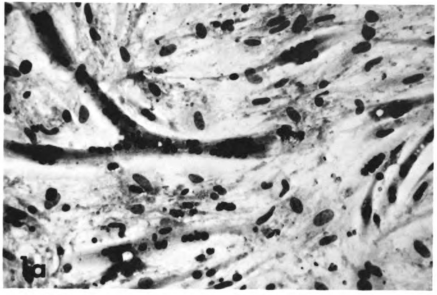
The original purpose of this project was to identify and measure differences in parameters of muscle growth between embryos of broiler and layer chicks. One complicating factor in carrying out a study of this nature was that the specific control exerted by chick embryo extract on chick muscle cells in culture was unknown. Microconstituents present in the embryo extract are required for muscle growth in cell cultures, but it was not known if these constituents were present in the same concentration in both the layer and broiler embryos at the same embryonic age. Therefore, embryo extract from 12 day old embryos became a second experimental

consideration. Embryo extract derived from embryos of both chick sources was used for medium supplementation at a final concentration of 5% (v/v). Thus, the effects of broiler extract in both broiler and layer cells and the reciprocal combinations were studied.

Cultures at 8 days of age were maximally differentiated in this cell culture system. At this time many of the Mt nuclei had migrated to somewhat equidistant positions just underneath the sarcolemma, and cross striations were visible in the central portions of the Mt. Photomicrographs were taken at day 8 to visibly demonstrate the lace of variation in Mt size and number between cultures of different treatments (Figures 1 and 2). This lack of variation was observed throughout all experiments. After day 8 the cultures deteriorated gradually, and comparisons after this time were of little interest.

The general developmental pattern of the muscle cultures was the same in all treatments. Immediately after plating, only mononucleated cells were observed. This group of cells consisted primarily of Fbs, Mbs, and myosinic precursor cells. The cells, once in the artificial environment, underwent normal differentiation. Rapid proliferation by the contaminating Fb population resulted in an increasing proportion of Fbs, but overgrowth of the muscle culture by Fbs was controlled by the addition of 10^{-7} M fluorodeoxyuridine (FDU) to

Figure 1. Micrographs of Giemsa stained Mt formed by the fusion of myogenic cells in culture. Myotubes from (a) broiler cells nourished with broiler origin embryo extract and (b) broiler cells nourished with layer origin extract after 8 days in culture. x500.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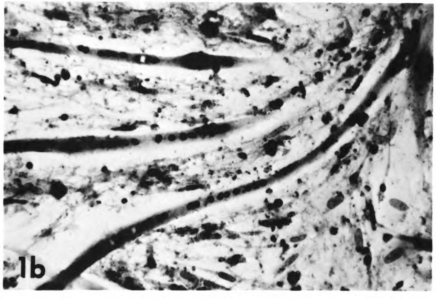
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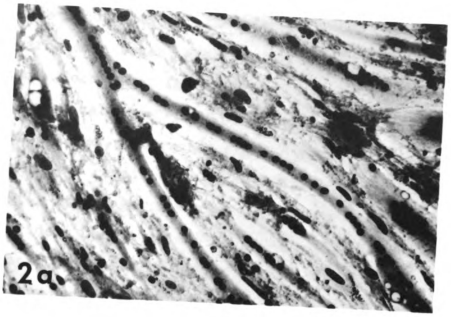
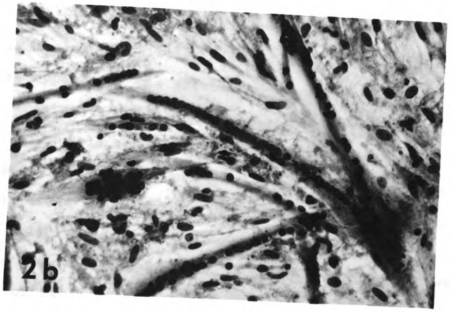
Figure 2. Micrographs of Giemsa stained Mt formed by the fusion of myogenic cells in culture. Myotubes from (a) layer cells nourished with layer origin embryo extract and (b) layer cells nourished with broiler origin extract after 8 days in culture. x500.


2a


2b

after two days.

Morphological differentiation was seen in cultures when two or more mononucleated cells fused. With time, dissolution of the sarcolemma became apparent and Mt formation was completed. Soon after fusion, the nuclei of each of the once mononucleated cells were found clumped together near the center of the Mt. Later, the nuclei began movement away from each other, yet remained aligned within the central core of the Mt. It was at this time that cross striations became evident near the cell periphery. The final step of differentiation observed in the muscle cultures was the migration of the nuclei to locations just under the sarcolemma. Concomitantly, the concentration of myofibrillar proteins increased in the center of the Mt. During the time after fusion and through senescence, mononucleated cells were continually fusing to existing Mts and continual Mt elongation was apparent. The growth patterns were very similar in all treatments and did not deviate from what is considered normal for muscle cell cultures; however, advanced development was not evident in all cultures. The last developmental stage where nuclei migrate to positions equidistantly spaced under the sarcolemma was not evident until day 10 in culture. Many of these 10 day old cultures however, were in a phase of rapid deterioration.

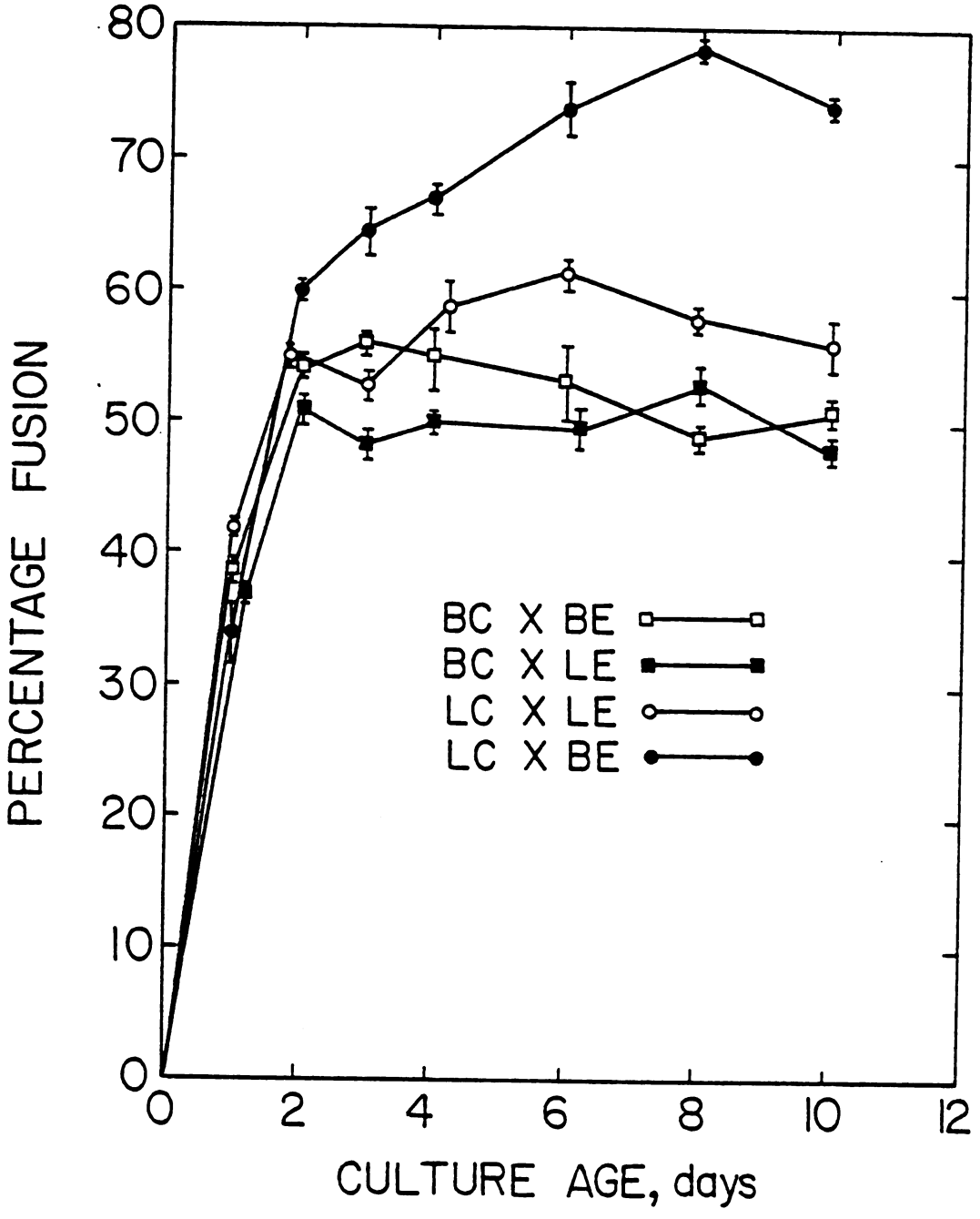
Cultures were obviously composed of mixed populations

cultures of muscle percursors because, had the cells been of the Mb cell compartment only, the percentage fusion would have progressed from zero to maximal fusion within the first 24 hours of culture, with no further increase in number of nuclei or percentage fusion.

Percentage fusion was indicative of the degree of myogenic maturity and/or muscle cell contamination. Fusion kinetics were examined to demonstrate changes in myogenic cultures as progressive differentiation resulted in Mt formation. FdU was added to the cultures at day 2 in order to block DNA synthesis and thereby ultimately inhibit cell replication. FdU is thought to have little effect on cellular metabolism unrelated to DNA synthesis (Vogel et al., 1978).

Fusion kinetics were similar in all treatments except LC x BE (Figure 3 and Table 1). The varied response seen in this treatment may have been the result of interactions between broiler extract and layer cells which ultimately changed the differentiation pattern of the myogenic cells, or from the fortuitous use of a different lot of FdU which had to be purchased to complete the experiment. Three explanations for the LC x BE response were therefore possible: 1) the LC populations nourished with medium containing broiler extract may have initially contained a larger proportion of myogenic cells; 2) the greater percentage fusion may have been the direct result of an extract-cell interaction; and 3) the FdU was of a different concentration than that used for the other three treatments.

Figure 3. Fusion kinetics in Experimental Series 1 of myogenic cells obtained from 12 day embryonic chick skeletal muscle. Percentage fusion was measured as described in Materials and Methods. Muscle cell cultures were grown over a ten day period. Each line represents the experimental results of one of four different treatment groups. Each value represents the mean of five experiments in which all measurements were obtained in duplicate. Error bars represent $\pm$ 1 SEM.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PERCENTAGE FUSION
CULTURE AGE, days
BC X BE
BC X LE
LC X LE
LC X BE

Table 1. Mean responses in Experimental Series 1 of percentage fusion, myotube nuclei per culture and total nuclei per culture[a]

Treatment[e]	Percentage Fusion	Myotube Nuclei per Culture $x10^{-6}$	Total Nuclei per Culture $x10^{-6}$
BC x BE	53.23^{cd}	2.66^{c}	5.07^{c}
BC x LE	48.33^{d}	2.88^{c}	5.97^{b}
LC x LE	55.24^{c}	3.34^{b}	6.10^{b}
LC x BE	64.51^{b}	1.99^{d}	3.04^{d}

[a]Cell cultures were examined on days 1, 2, 3, 4, 6, 8 and 10. Values represent the mean response over the entire experimental range.

[b,c,d]Means in the same column with unlike superscripts differ significantly ($P < .05$).

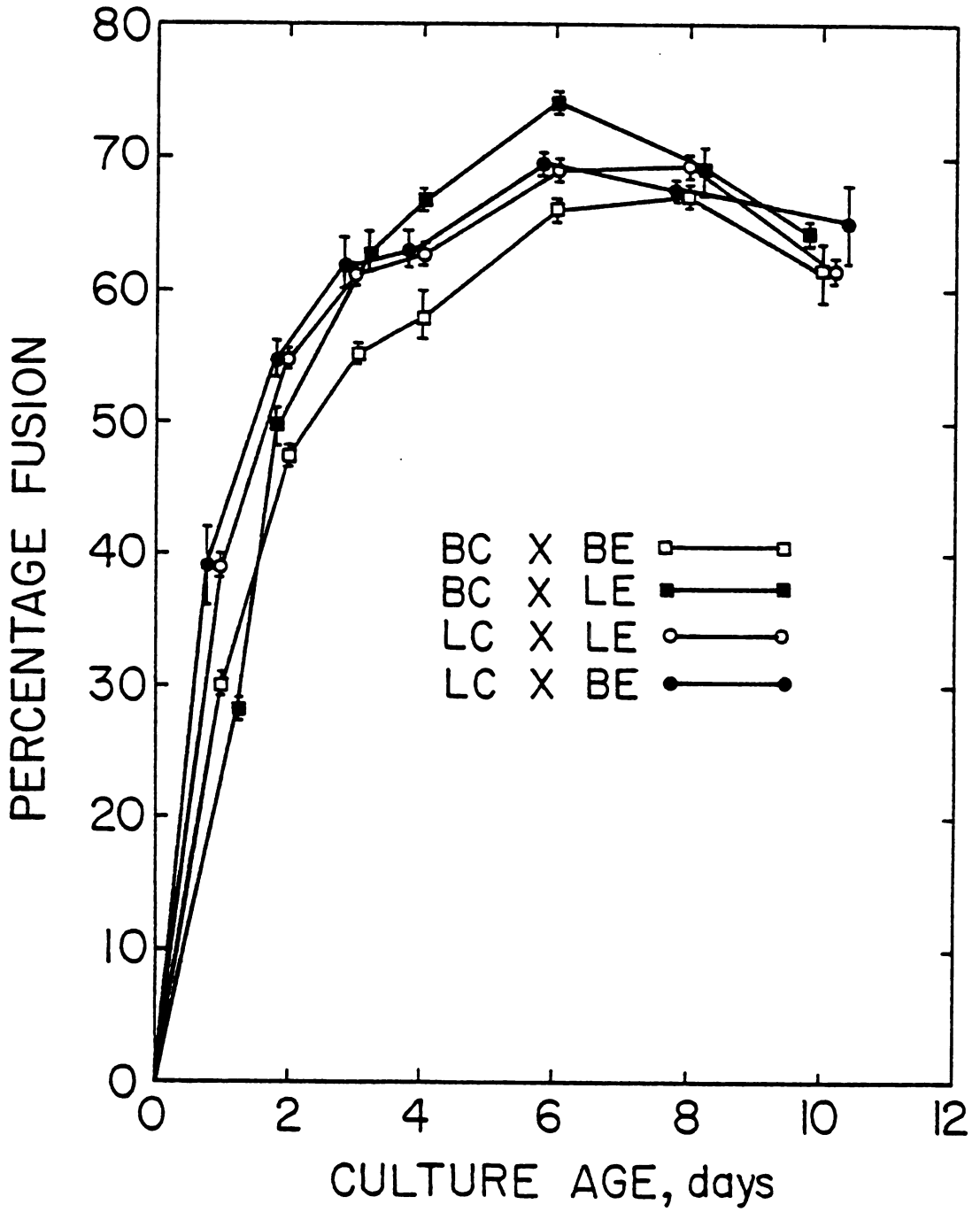
[e]BC = Broiler Cells, LC = Layer Cells; BE = Broiler Extract; LE = Layer Extract

The magnitude of each treatment dictated that the entire project could not be duplicated. However, since it was known that a separate batch of FdU was utilized for the LC x BE treatment, fusion kinetics were reexamined in a separate trial. Experimental conditions were identical to the original experimental series, except that a single vial of FdU was used for the entire experiment. The results obtained from this experiment demonstrated that the response in LC x BE treatments was due to the FdU, rather than to inherent properties of the LC x BE treatment (Figure 4 and Table 2). Thus the first two possibilities were eliminated.

Because of the differences observed in Figure 3 and Table 1 in the LC x BE treatment, and because it is not known whether these differences in cell density would affect other parameters such as turnover and synthesis that were also measured in these same cell cultures, a note of caution is urged in examining the LC x BE treatment data throughout the remainder of this thesis. Moreover, particular attention will be placed on comparisons between cells and their homologous embryo extracts (i.e., BC x BE and LC x LE). This aspect of this project is recognized to be a serious shortcoming; however, any attempt to correct this problem would have required repetition of the entire research program in order to critically control the variable.

The fusion curves seen in Figures 3 and 4 quantitatively

Figure 4. Fusion kinetics in Experimental Series 2 of myogenic tissue obtained from 12 day embryonic chick skeletal muscle. Muscle cell cultures were grown over a ten day period. Percentage fusion was measured as described in Materials and Methods.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PERCENTAGE FUSION
CULTURE AGE, days
BC X BE
BC X LE
LC X LE
LC X BE

Table 2. Mean responses of Experimental Series 2 for percentage fusion and myotube nuclei per culture[a]

Treatment[d] Group	Percentage Fusion	Myotube Nuclei per Culture $x10^{-6}$
BC x BE	59.19	3.23[b]
BE x LE	59.26	2.35[c]
LC x LE	59.18	3.64[b]
LC x BE	60.05	3.47[b]

[a]Cell cultures were examined on days 1, 2, 3, 4, 6, 8 and 10. Values represent the mean response over the entire experimental range.

[b,c]Means in the same column with unlike superscripts differ significantly ($P < .05$).

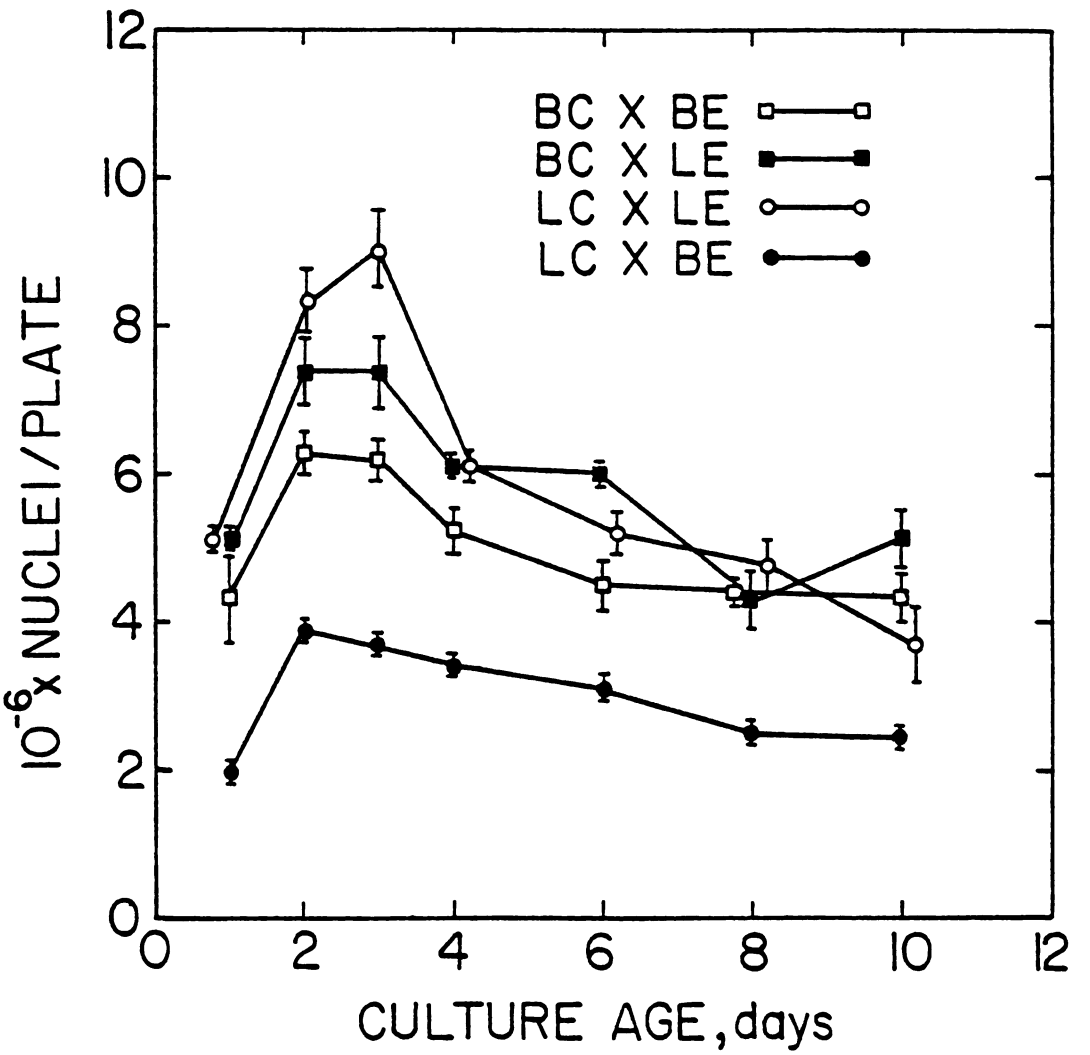
[d]BC = Broiler Cells; LC = Layer Cells; BE = Broiler Extract; LE = Layer Extract

parallel fusion kinetics of similar cultures described by others (White et al., 1975), in which about 75% of the nuclei were contained within Mt of mature cultures. Using quail muscle, fusion percentages of up to 95% have been reported (Lipton and Schultz, 1979).

There tended to be a slight decline in the percentage fusion in all treatments in both experimental series (Figures 3 and 4). The decline may have been due to an acquired resistance to the FdU by mononucleated cells, resulting in their replication and consequent dilution of the Mt by mononucleated cells. However, the number of mononucleated cells actually decreased in number during this time as did the Mt nuclei. Therefore, the decline in fusion percentage must have been the result of larger proportions of fused nuclei disappearing from the cultures as a result of Mt death. For reference, the death of one Mt containing 50 nuclei would have to be accompanied by the death of 50 mononucleated cells for there to be no net change in percentage fusion. The varied results seen between the first experimental series (Figure 3) and the second experimental series (Figure 4) further demonstrates the variability in myogenic expression resulting from the FdU inhibition of DNA synthesis.

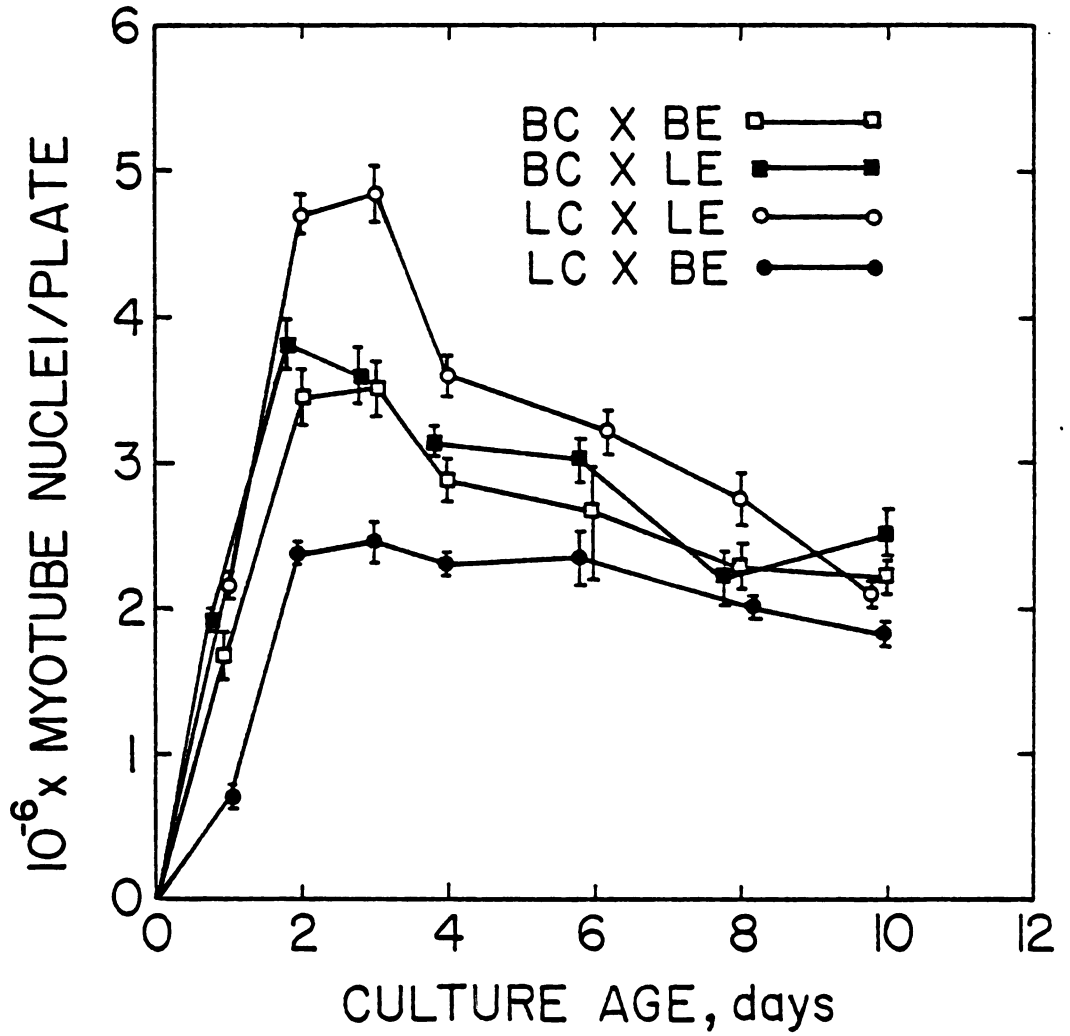
Figure 5 is a diagramatic representation of the change in Mt nuclei per culture, which reached maximal levels at day 3. After day 3 there was gradual decay of the cultures.

Figure 5. Comparison of total Mt nuclei per 10 cm culture dish in Experimental Series 1. Muscle cell cultures were grown over a ten day period. The numbers of nuclei were microscopically evaluated from Giemsa stained cultures as detailed in Material and Methods.


BC X BE
BC X LE
LC X LE
LC X BE
10^{-6} x NUCLEI/PLATE
CULTURE AGE, days
0
2
4
6
8
10
12

This trend is in general agreement with the previous observations of Young et al., (1978a), except that the decline in number of LC x LE nuclei per culture is more exaggerated. In addition to the contribution of Mt detachment from the culture dishes, it should also be noted that it becomes progressively more difficult to enumerate Mt nuclei in older cultures because of their increased thickness. Figure 5, with the exception of the LC x BE, treatment demonstrates the reproducibility of establishing cultures with equal populations of muscle-forming cells. At day 1 (Figure 5), all treatments except LC x BE contained essentially equal numbers of Mt nuclei. The treatment LC x BE underwent less proliferation of myogenic cells than the others (Figure 5), as evidenced by the lower number of Mt nuclei per culture dish at each time point throughout growth. This was probably due to lower viable myogenic cells available for tissue culture initiation (Figure 6), even though all treatments were theoretically begun with equal number of cells per plate. Interestingly, even though the initial number of Mt nuclei was similar in the BC x BE and LC x LE treatments, the number of Mt nuclei in the LC x LE cultures was consistently and significantly higher than in the BC x BE cultures (Fig. 5, Table 1). The same observation was apparent for total nuclei (Fig. 6, Table 1), suggesting that myogenic cells in the LC x LE treatment may have undergone more rapid initial proliferation.

Figure 6. Comparisons of total nuclei per 10 cm culture dish with increasing culture age. Muscle cell cultures were grown over a ten day period. The number of Mt and unfused nuclei were microscopically evaluated from Giemsa stained cultures.


BC X BE
BC X LE
LC X LE
LC X BE
10^{-6} x MYOTUBE NUCLEI/PLATE
CULTURE AGE, days
0
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12

The layer cells, when under layer hormonal or growth factor control, may have been embryonically of a younger physiological age than the broilers at 12 days. Once the muscle cultures were produced from the 12 day embryos, a developmental pattern similar to what would normally occur *in vivo* may take place. Mizuno and Hikami (1972) concluded from their analysis of layer and meat-type chicks that there is no difference in nuclei/muscle content between broilers and layers at hatching. If at day 12 the layers were of a younger physiological age than broilers, the proliferation rate of myogenic cells would have to be greater in layers than in broilers in order for there to be equal quantities Mf nuclei per muscle in layer and broiler chicks at hatching. Mizuno and Hikami (1972) found there were more Mfs per muscle in broilers, but muscle weight and physiological unit size (mg N/mg DNA) were equal for both types. Therefore, based on work by Cheek (1971) there must have been equal numbers of Mf nuclei in muscles in the two chick types prior to hatching.

There is evidence in the literature that muscle mass is related to Mf content (Cheek, 1971); indicating that increases in muscle mass are proportional to and concomitant with increasing numbers of Mf nuclei. Measuring the numbers of nuclei within a Mf could be utilized as a useful tool for the quantitation of muscle growth. Examination of

entire Mt population of the different treatments was not possible because Mt lengths were far greater than the diameter of each microscopic field (c.f., Figs. 1 and 2). Therefore, both Mt segments per 10 cm culture dish and nuclei per Mt segment are relative values, and they may not be an accurate representation of the actual Mt population of each treatment. These values were obtained by quantitation of only the portions of Mt and nuclei within those Mt portions found in each of the 10 random microscipic fields. Relative estimation of Mt numbers in this manner required two assumptions: 1) uniform Mt numbers and size within cultures and 2) portions of Mt extending out of a particular field must be compensated by equal extensions of others into the visible field.

From this study of embryonic skeletal tissue, it was evident that the layer myogenic cell populations ultimately formed larger Mt (more Mt nuclei per Mt segment) rather than greater numbers of Mt segments (Figures y and 8 and Table 1, 2 and 3). Why this occurs is unknown. Possibly the cell densities were similar enough that differences in Mt numbers could not become manifested in the culture system. The larger Mt size in the layer treatment (LC x LE) may have been the result of more replication cycles between the time of plating and 2 days in culture, when replication was terminated.

The layer treatment LC x LE contained larger fused nuclei populations and more nuclei were found in these Mt than were

Figure 7. Quantitation of the average number of Mt nuclei per Mt segment. Muscle cell cultures were grown over a ten day period. The numbers of Mt nuclei and Mt segments were microscopically evaluated in Giemsa stained cultures.

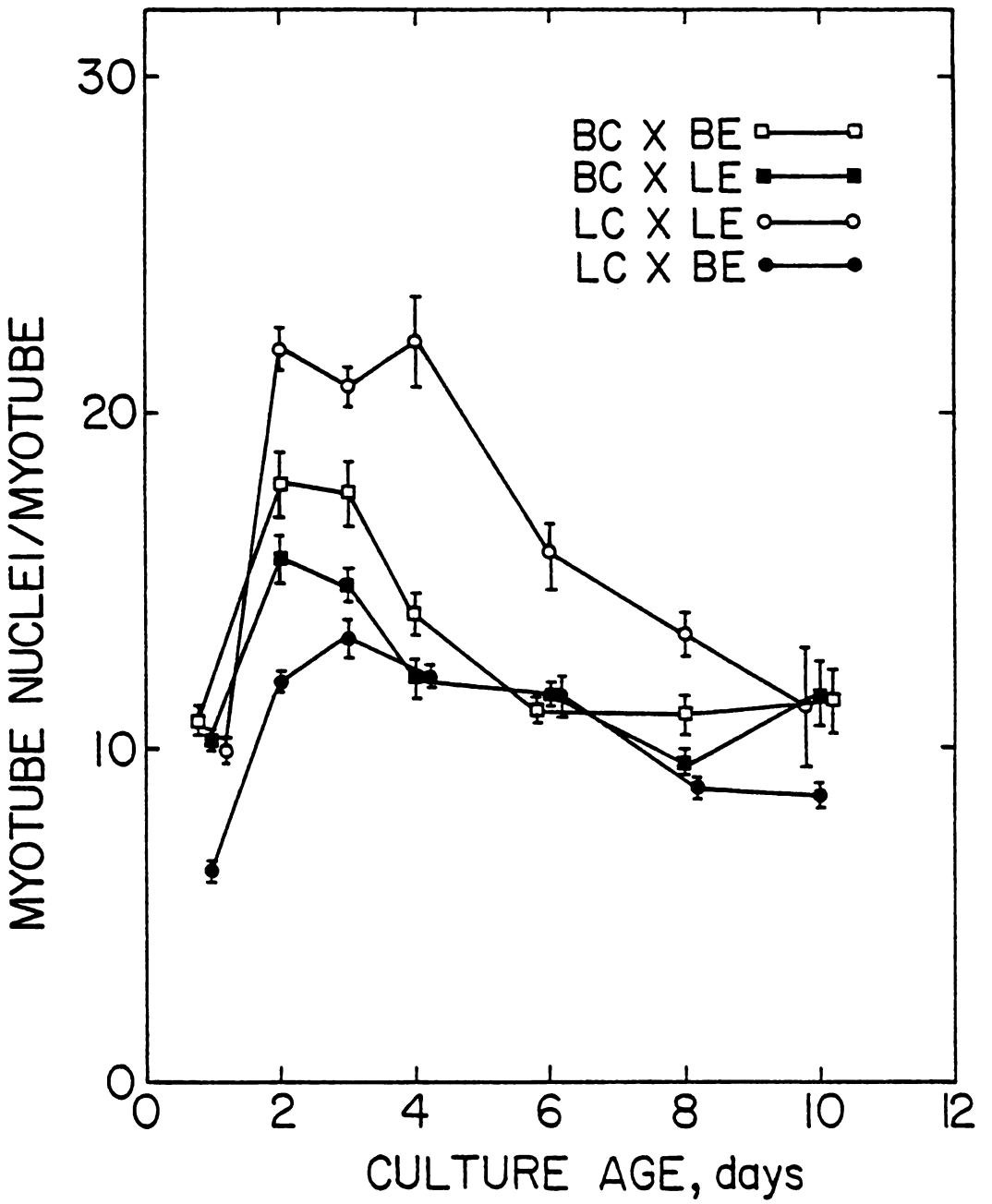
BC X BE
BC X LE
LC X LE
LC X BE
MYOTUBE NUCLEI/MYOTUBE
CULTURE AGE, days
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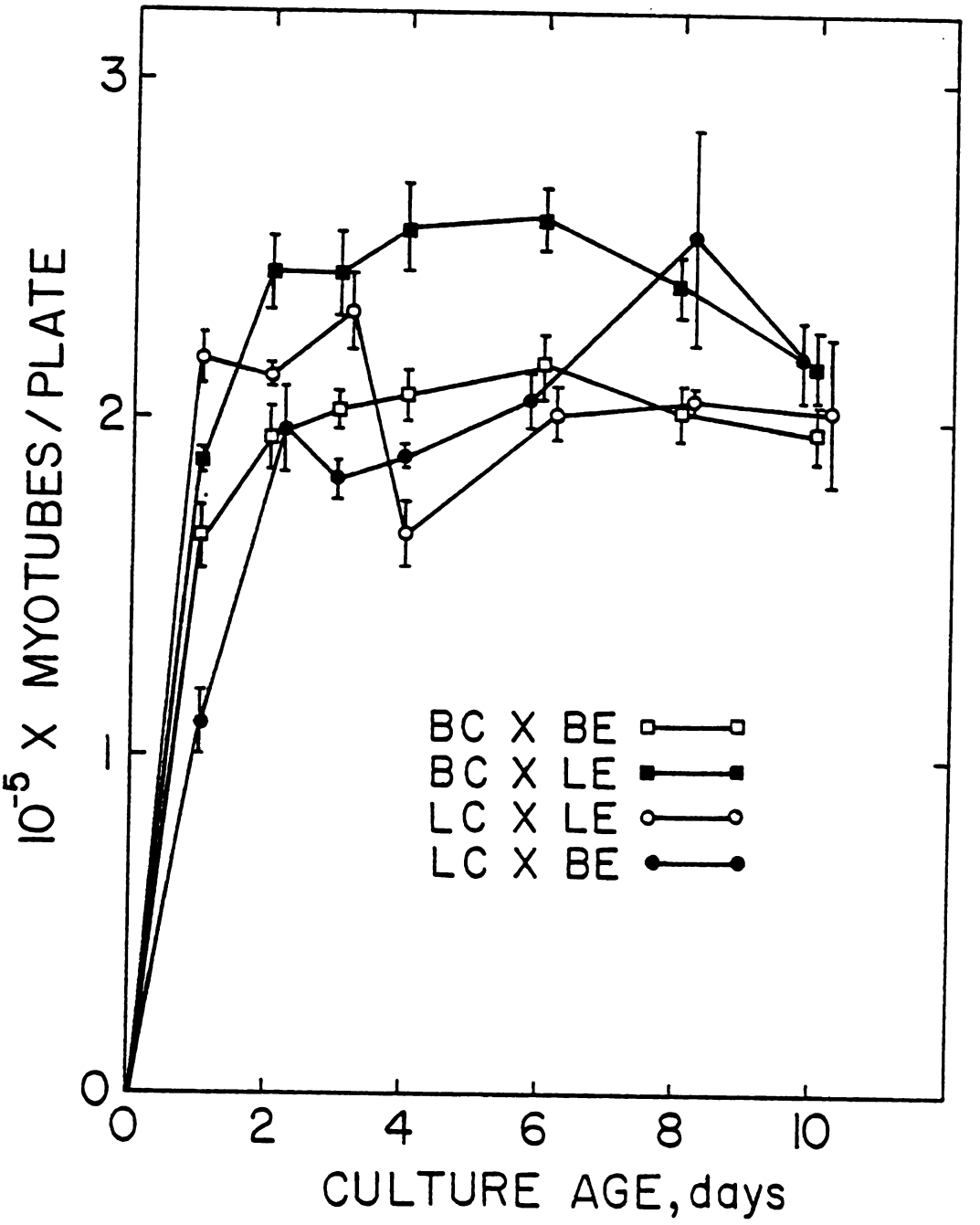
Figure 8. Quantitation of Mt segments per 10 cm culture dish. Muscle cell cultures were grown over a 10 day period. The number of Mt nuclei and Mt segments were microscopically evalueated and Giemsa stained cultures.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10^{-5} X MYOTUBES/PLATE
CULTURE AGE, days
BC X BE
BC X LE
LC X LE
LC X BE

Table 3. Mean responses of Experimental Series 1 for myotube nuclei per myotube segment and myotube segments per culture

Treatment	Myotube Nuclei per Myotube Segment	Myotube Segments per Culture $x10^{-5}$
BC x BE	13.39^{c}	1.97^{c}
BCx LE	12.20^{c}	2.36^{b}
LC x LE	16.50^{b}	2.06^{c}
LC x BE	10.40^{d}	1.93^{c}

[a]Cell cultures were examined on days 1, 2, 3, 4, 6, 8 and 10. Values represent the mean response over the entire experimental range.

[b,c,d]Means in the same column with unlike superscripts differ significantly ($P < .05$).

[e]BC = Broiler Cells; LC = Layer Cells; BE = Broiler Extract; LC = Layer Extract

found in the BC x BE complementary treatment (Table 1 and 3). However, this may not have been a true representation of *in vivo* cellular activities, because Mizuno and Hikami (1972) found more muscle fibers at hatching in chicks of broiler origin. When comparing the two independent studies, the conclusion can be made that the mechanisms involved in the production of greater Mf numbers may occur prior to 12 days *in vivo*, and after 12 days conditions may be such that the means utilized to increase muscle fiber numbers have been lost. Of course, a second possible explanation is that muscle cells in culture are removed from cytological controls which regulate the quantity of muscle fibers per muscle.

The larger Mts found in layer cultures (LC x LE) were probably the result of greater proliferative activity of the LC x LE cells. It is not clear whether the greater proliferative activity seen in the layer cultures (LC x LE) was an intrinsic capability of the myogenic tissue of 12 day old layer muscle tissue or a manifestation of experimental inadequacies of the cell culture system. The LC x LE cells in Experimental Series 2 also exhibited a tendency toward greater proliferative activity; however, there was no statistical evidence of any proliferative advantage (Table 1 and 2).

The mechanism which determines the number of Mts per muscle is unknown, but it may simply involve specific time intervals that are controlled by numbers of cell divisions of specific primitive myogenic cell types. Thus, after

a given number of replications or a specific time interval, cells derived from this primitive compartment may have the capability of fusion, thereby forming a relatively finite number of Mts. This mechanism would be similar to the mechanisms involved in the aging process; whereby cells of a specific cell compartment (i.e. Fb) are capable of replication for only a finite number of generations (Hayflick, 1980). This mechanism is probably dictated by a genetic mechanism (Hayflick, 1980). All mononucleated cells which become capable of fusion after that critical time, have only the option to incorporate into the existing Mfs or Mts. Since there are fewer muscle fibers per muscle in layer-type chicks (mizuno and Hikami, 1972), layer origin myogenic tissue may undergo fewer replicative cell cycles prior to the critical point at which muscle fiber number is determined. A second possibility is that, during the replicative cycles, greater proportions of nonmyogenic cells may form (i.e. rather than a mothercell dividing to form two PMb daughter cells; a mother cell would replicate forming one Fb and one PMb) in layer tissue. However, this project did not lend support to this scheme because there were equal fusion percentages in both the BC x BE and LC x LE treatments (Tables 1 and 2). This would not have occurred had proportionately more Fb been generated in the LC x LE cultures.

There are approximately 60 percent more nuclei per muscle in broiler type muscle tissue than in layer type muscle tissue at 8 weeks (Mizuno and Hikami, 1972). But, the numbers of

nuclei per muscle are equal in the two strains at hatching, with layer Mfs containing more nuclei than the broilers. Layer Mts in cultures derived from embryonic muscle also contain more nuclei per Mf (Fig. 7). By eight weeks *in vivo* more nuclei are contained in the muscles of the broiler muscle. For this to occur either the proliferative activity of broiler satellite cells could be greater (assuming there are equal numbers of satellite cells per muscle fiber in both chick strains), there could be more active satellite cells per muscle fiber in broiler muscle tissue or there could be similar proliferative activity of satellite cells between the two chick strains. The present study involved only embryonic tissue. Extrapolation of results of the culture analysis to post-hatching tissue is difficult, at best. However, if cell activities in cell culture are indicative of post-embryonic tissue, then the two studies are in conflict, provided proliferative activity of broiler satellite cells is indeed greater (Mizuno and Hikami, 1972, Figure 5 and Table 2).

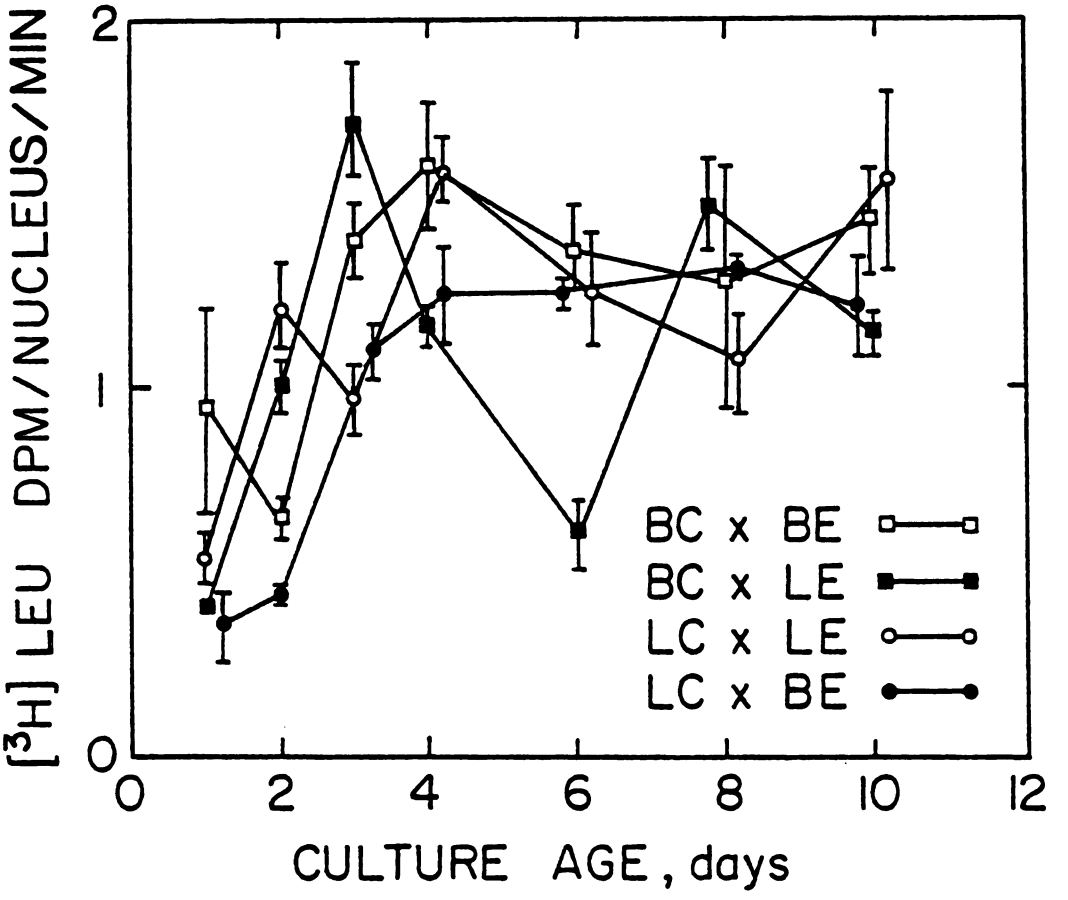
The differences in muscle fiber number between the two strains may be responsible for the majority of the differences observed in muscle mass between meat-type and layer-type chickens. However, as discussed below, other factors such as the ability to synthesize and deposit protein per muscle DNA unit may play an important role in contributing to muscle growth.

Cells were prepared for protein synthesis rate studies on days 1, 2, 3, 4, 6, 8 and 10 as outlined in Materials and

Methods. After the 4 hr pulse labeling period, cultures were harvested and processed. Total protein synthesis rate (dpm/plate/min) was divided by the number of nuclei per plate, obtaining the rate of protein synthesis per nucleus per min. The contribution to total protein synthesis by the contaminating mononucleated cells was tolerated for this portion of the study. Figure 9 and Table 4 demonstrate no distinct differences in rates of total protein synthesis in any of the treatments. The percentage Mt nuclei per culture were equal in treatments BC x BE, BC x LE and LC x LE (Figure 3 and Table 1). Therefore, within each of these treatments there would be equal total protein synthesis rates. Since the percentage fusion was higher in the treatment LC x BE (Figure 3 and Table 1), the proportion of protein synthesis contributed by multinucleated cells in treatment LC x BE would therefore be larger than for treatments BC x BE, BC x LE and LC x LE. These data indicate the presence of equal protein synthesis per Mt nucleus in all four treatments (Figures 3, 5 and 9 and Table 4).

Total protein content per Mt nucleus was measured for each group over the 10 day culture period. Individual protein determinations were corrected for collagen content (collagen was used as a matrix for cell attachment) and for protein content contributed by mononucleated cells. Protein content per mononucleated cell (Fb) was 76 pg/nucleus (Young et al., 1979). Use of this value was justified because cultures in the present study and those of Young and coworkers (1979) were identical.

Figure 9. Total protein synthesis rate ([^{3}H] dpm/nucleus/min) in muscle cell cultures. Muscle cell cultures were grown over a 10 day period. The number of nuclei was microscopically evaluated in Giemsa stained cultures. Total protein synthesis was measured as described in Materials and Methods.


[3H] LEU DPM/NUCLEUS/MIN
2
1
0
0
2
4
6
8
10
12
CULTURE AGE, days
BC x BE
BC x LE
LC x LE
LC x BE

Protein content per Mt nucleus was similar in treatments BC x BE, BC x LE and LC x LE during the first four days in culture, but BC x BE tended to have more protein between days 6 and 10 and the LC x BE treatment was lower throughout the experiment (Figure 10 and Table 4). There are three explanations for this difference: 1) there may have been a cell density effect which could be related to physiological unit size; 2) the stronger FdU in treatment LC x BE may have inhibited protein synthesis or facilitated protein degradation; and 3) ther may have been a cell-embryo extract interaction in LC x BE treatments. The plausible explanation for the lower protein accumulation in LC x BE treatments was population size. However, no reason could be pinpointed, since no reexaminations were made. If this were the case, protein accumulation (at least in cell cultures) could be a function of cell density. Miller (1977) alluded to this situation in his cell culture examinations of muscle growth of mouse satellite cell cultures.

The protein accumulation per Mt nucleus between treatments was shown to be similar (Table 4) and reasons for the extremely low values of the LC x BE treatment were discussed. However, it may be inferred from the data that there is a tendency for more accumulation of total protein per fused nucleus in the BC x BE and BC x LE treatments in older, more mature cultures (Figure 10). Even though, statistically, these treatments were of equal protein content per fused nucleus as that of the LC x LE treatment (Table 4), the broiler cell treatments in-

Figure 10. Comparison of total protein content per Mt nucleus in muscle cell cultures. Protein was measured as described in Materials and Methods. Muscle cell cultures were grown over a 10 day period. The number of Mt nuclei was microscopically evaluated in Giemsa stained cultures. Each line represents one of four different treatment groups. Each value represents the mean of five experiments in which all measurements were obtained in triplicate. Error bars represent $\pm$ 1 SEM.

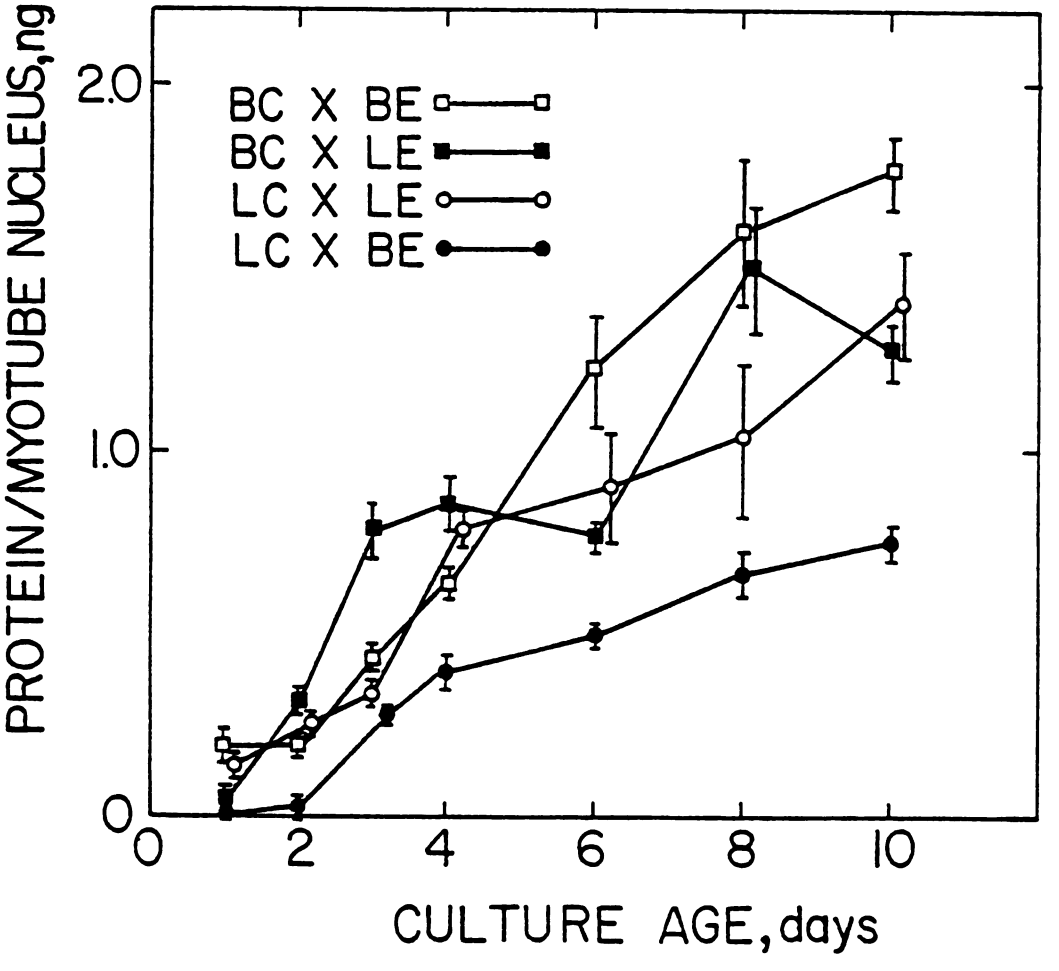

PROTEIN/MYOTUBE NUCLEUS,ng
2.0
1.0
0
0
2
4
6
8
10
CULTURE AGE, days
BC X BE
BC X LE
LC X LE
LC X BE

Table 4. Mean responses of Experimental Series 1 for total protein synthesis rate and total protein content[a]

Treatment Group[e]	Rate of Total Protein Synthesis (dpm/nucleus/min)	Total Protein Content (ng/nucleus)
BC x BE	1.24[b]	0.899[b]
BC x LE	1.08[b]	0.771[b]
LC x LE	1.18[b]	0.689[bc]
LC x BE	0.99[c]	0.443[3]

[a]Cell cultures were examined on days 1, 2, 3, 4, 6, 8 and 10. Values represent the mean response over the entire experimental range.

[b,c,d]Means in the same column with unlike superscripts differ significantly ($P < .05$)

[e]BC = Broiler Cells; LC = Layer Cells; BE = Broiler Extract; LC = Layer Extract

creased in total protein (on a per fused nucleus basis) more rapidly and maintained a constant advantage throughout growth (Figure 10). These data indicate that muscle cells of broiler origin may be capable of accumulating more protein per physiological unit due to inherent genetic information. However, methods for analysis were such that differences between protein content per Mt nucleus could not be delineated. The accumulation of total protein was obviously greater in the BC x BE treatment on days 6 through 10 in cell culture. But, due to each time point having equal statistical importance, differences in the overall responses could not be found because there were no differences in the growth paramenters of any of the treatments during the first 4 days of culture. There are probably great differences in some of the parameters associated with muscle growth in the latter stages of development in cell culture between the two chick strains. If this is true, embryonic muscle tissue of broiler origin may have the capability of maintaining a larger physiological unit in terms of total protein per Mt (Mf) nucleur than does layer embryonic tissue. Reasearch by Mizuno and Hikami (1972) demonstrated that this does not seem to carry over to the live animal at hatching or at maturity. However, this area may require some reexamination in the near future.

It cannot be inferred from these data that broiler m muscle tissue has the endogenous tendency toward greater efficiency in terms of protein accumulation. But, data pres-

ented later in this thesis will show that there may indeed be greater protein conservation in terms of protein metabolism in broiler muscle cultures.

The term physiological unit describes the quantity of cytoplasm controlled by a cell nucleus (Cheek 1971). Physiological unit size generally refers to a given quantity of protein per Mt nucleus. Moss et al., (1963) found no differences in the physiological unit size (g muscle/nucleus) in chicks of varying breeds. Mizuno and Hikami (1972) found larger physiological units in only the M. sartorius and M. gastrocnemius muscles of layer rather than meat-type chickens at eight weeks of age. But, these researchers found no differences in protein/nucleus between layer and meat-type chicks prior to eight weeks of age. If no difference in protein content could be found prior to 8 weeks in vivo, then differences between the two strains probably would not be manifested in a cell culture system. However, as discussed earlier, subtle differences in protein content during embryonic and early post-natal growth as seen in Figure 10 and 11, could produce the larger divergence in muscle mass seen in the two chicken types.

Muscle cells are distinguished from other tissue by several distinctive characteristics; formation of contractile units and multinucleated Mts are the two most distinctive. Proteins within contractile units are specific for muscle fibers and also comprise the majority of the cytoplasm, with myosin comprising approximately 55% of the total contractile proteins in adult muscle. Ease of myosin extraction on a quantitative basis was

the major rational behind selecting myosin as a marker protein for measuring accumulation, synthesis and degradation of contractile proteins. Rubinstein et al., (1975) determined that the synthesis and degradation of contractile protein were coordinated in muscle cells. Therefore, generalizations concerning contractile proteins could be drawn from the changing parameters of one protein (i.e. myosin). It is difficult to quantitatively separate myosin from other salt-soluble proteins; however, dissociation of the myosin into component subunits, followed by electrophoresis into polyacrylamide matrices, allowed component separation. The protein subunit MHC which comprises the largest portion of the myosin molecule, was used to quantitate myosin turnover, synthesis (molecules per min per Mt nucleus) and total content. Crude myosin extracts were prepared and electrophoresised as described in Materials and Methods. MHC bands were removed from each polyacrylamide gel, the absorbance at 560 nm determined, and picograms (pg) MHC calculated. Accumulation of MHC was not significantly different in any of the treatments (Figure 11 and Table 5). However, MHC content paralleled that of total protein content in that there was a tendency for higher MHC content per Mt nucleus in the broiler treatments (Figure 11), particularly between days 6 and 10. MHC content declined after day 8 even though total protein content per Mt continued to increase during the same period (Figures 10 and 11).

These results indicate that there were no substantial

Figure 11. Comparisons of MHC (pg) per Mt nucleus. Quantitation of MHC was carried out as described in Materials and Methods. Mt nuclei quantitation was microscopically evaluated from Giemsa stained cultures. Muscle cell cultures were grown over a 10 day period.

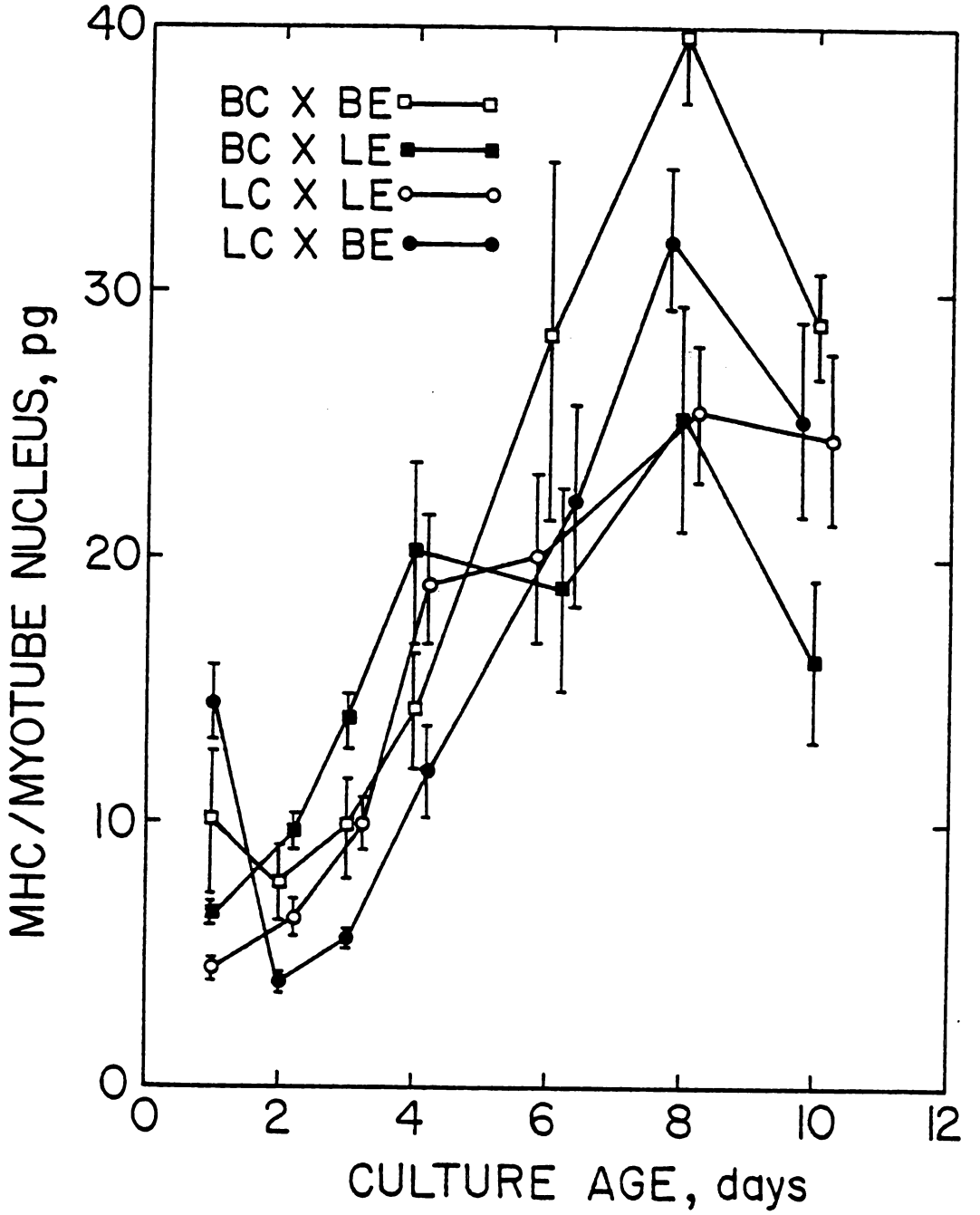

BC X BE
BC X LE
LC X LE
LC X BE
MHC/MYOTUBE NUCLEUS, pg
CULTURE AGE, days
0
10
20
30
40
0
2
4
6
8
10
12

Table 5. Mean responses of Experimental Series 1 for MHC content and MHC synthesis rate

Treatment Group	MHC Content (pg/Mt nucleus)	MHC Synthesis Rate (MHC molecules/min/Mt nucleus)
BC x BE	17.29	6131[c]
BC x LE	15.71	4962[c]
LC x LE	15.81	9917[b]
LC x BE	16.59	5754[c]

[a]Cell cultures were examined on days 1, 2, 3, 4, 6, 8, and 10. Values represent the mean response over the entire experimental range

[bc]Means in the same column with unlike superscripts differ significantly ($P < .05$).

[d]BC = Broiler Cells; LC = Layer Cells; BE = Broiler Extract; LC = Layer Extract.

differences in physiological unit size in terms of both total protein and myosin between any of the treatments (Tables 4 and 5), when entire accretion responses are considered. Had only the time points day 6 through day 10 been considered in the statistical analysis differences would have been found in both myosin content and total protein between broiler and layer-type chickens. Again, whether this is indicative of intact chickens of varying maturity is unknown. But the general conclusion which can be drawn from this study is that heterogeneity in muscle size would be the direct result of one or both of two mutually exclusive manifestations which are known to occur in muscle: 1) more muscle fibers per muscle and 2) greater numbers of nuclei per muscle fiber but not due to other contributory factors such as more protein per muscle nucleus in the more muscular animals.

MHC content per Mt nucleus decreased with respect to total protein per Mt nucleus as the cultures increased in age (Table 6). The large initial MHC content was the result of fusion-associated initiation of MHC synthesis, which occurred prior to that of the other muscle specific proteins. But, as the Mts matured, the accretion of other proteins outpaced that of myosin, thereby diluting myosin content.

This portion of the project demonstrated that qualitatively muscle tissue in cell culture and <u>in vivo</u> reacts to the growth stimulus in like manner. This study also demonstrated that muscle tissue could be grown in an artificial system to a rather advanced state without the need for neural or hormonal

Table 6. Percentages of total protein accretion comprised by MHC.

Day	Treatment Group[a] BC x BE	BC x LE	LC x LE	LC x BE
1	6.14[b]	16.68	3.15	-
2	4.04	2.21	2.54	12.35
3	2.30	1.76	2.85	1.01
4	2.20	2.38	2.41	2.96
6	2.31	2.43	2.24	4.35
8	2.49	1.20	2.44	4.74
10	1.55	1.19	1.87	3.38

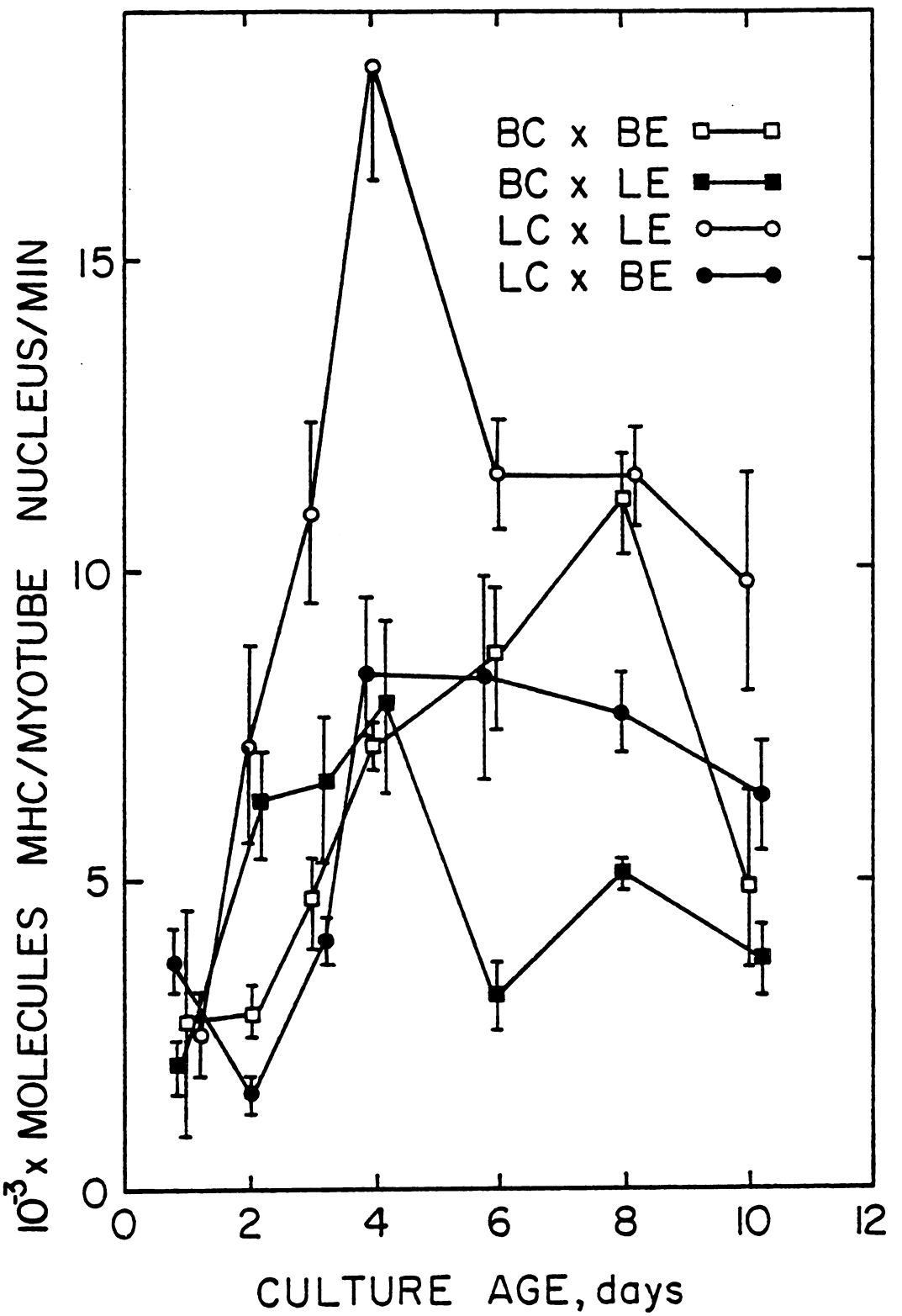
[a]BC = Broiler Cells; LC = Layer Cells; BE = Broiler Extract; LE = Layer Extract

[b]Values represent the MHC content (ng) divided by total protein (ng) times 100.

intervention.

After measurement of Coomassie Brilliant Blue 250R dye binding, the rate of MHC synthesis was calculated for each of the four treatments. Maximal MHC synthesis (molecules/min/Mt nucleus) occurred on day 4 (Figure 12). Maximal MHC synthesis rates were found to be 1.8×10^4, 1.1×10^4, 8.0×10^3 and 8.5×10^3 MHC molecules/min/Mt nucleus in treatments LC x LE, BC x BE, BC x LE and LC x BE respectively. These values were one third to one half that of the values reported in the literature. Both Emerson and Beckner (1975) and Young and co-workers (1978) determined the rate of MHC synthesis to be approximately 3.0×10^4 molecules/min/Mt nucleus in primary cultures derived from chick muscle. It is unknown whether these values are representative of intact muscle. However, the maximal synthesis rates of MHC obtained in this study were greater than that of constitutive myosin of Fb origin (3.2×10^3 MHC molecules/min/ Mt nucleus) (Young et al., 1970) and of Mt cultures derived from mouse satellite cells (6.0×10^3 MHC molecules/min/Mt nucleus) (Miller, 1977). The MHC synthesis rates within each treatment may have been underestimated because there were large populations of mononucleated cells within the cultures, and MHC label attributable to mononucleated cells was not removed from the total MHC. Collection of only myofibrillar MHC would have required immunoprecipitation. Morevoer, the amount of constitutive MHC was of little relative significance in each of the treatments (excluding the LC x BE treatment), because each contained equal proportions of mononucleated and fused nuclei during growth and development. Thus,

Figure 12. Comparisons of rate of MHC synthesis (molecules/myotube nucleus/min) in chick muscle cell cultures over 10 days in cell culture. Synthesis rates of MHC were determined as outlined in Materials and Methods. Mt nuclei quantitation was microscopically evaluated from Giemsa stain cultures.


BC x BE
BC x LE
LC x LE
LC x BE
10^{-3} x MOLECULES MHC/MYOTUBE NUCLEUS/MIN
0
5
10
15
0
2
4
6
8
10
12
CULTURE AGE, days

relative comparisons could be made without complications resulting from Fb contamination.

The synthesis rate of MHC was much more rapid in the LC x LE cultures. In comparison to other parameters discussed earlier, MHC synthesis rate in LC x LE was higher than in BC x BE throughout the period studied, rather than between days 6 and 10. As will be demonstrated later, this was probably the consequence of more rapid MHC turnover within this treatment. However, which parameter caused the change in the other is unknown. The MHC synthesis rate in the LC x LE treatment was almost twice that of any other treatment. Whether this synthesis rate is minimal in relative terms in <u>in vivo</u> is unknown. Embryologically, one chick type may be more mature than the other at 12 days. The myogenic cells at day 12 which were used for culture initiation were of similar nature, since the fusion curves were similar (Table 1). But, hormonal content and ratios within the the embryo extract derived from each chick type may have varied in such a way so as to manipulate the metabolism of proteins within each treatment in a divergent manner.

Turnover of MHC was measured in cultures which were labeled for 24 hours with medium containing low levels of [^{3}H]-L-leucine, followed by a chase period in the presence of unlabeled medium. Turnover rates were corrected for loss of label resulting from Mt death, since the combination of advancing culture age and radioactivity resulted in cell detachment during the chase

period. The turnover rates of MHC appear distinctly divergent (Table 7); however, large variances of $t_{\frac{1}{2}}$ (half-life) within treatments eliminated most significance (Table 7). In view of the facts that MHC synthesis rate by LC x LE cells was approximately twice as high as the rate of MHC synthesis by BC x BE cell cultures (Fig. 12), and that the quantitiy MHC in both cell types was comparable (Table 5), it would be anticipated that MHC turnover in LC x LE cells would be twice as fast as in BC x BC cells (Table 7). The MHC of the LC x BE treatment was the most stable (Table 7). The broiler extract may impart contactile protein stability by altering protein metabolism. However, further analysis in this area was not carried out. The cell populations established for measurement of MHC turnover were differnnt from those used for measurement of the other growth parameters. There were no differences in population size between treatments established for MHC degradation analysis. Therefore, divergence due to population size and percentage fusion did not apply to this parameter.

One problem associated with pulse chase procedures was the frequent occurrences of greater radioactivity at the first time point after the zero time (which should be of highest radioactivity). This perhaps resulted from label in intracellular amino acid pools eluding dilution by cold amino acids prior to incorporation into protein. The greater protein-bound radioactivity after a 12 hour pulse chase may not have been the consequence of labeled leucine recycling since leucine may be rapidly oxidized in skeletal muscle (Odessey and Goldberg, 1972) and

Table 7. Summary of turnover rates of MHC in fully developed Mt of each of the four treatment groups.

Treatment Group	Half-life (hours)
Broiler Cells x Broiler Extract	$103^{c} \pm 18^{a}$
Broiler Cells x Layer Extract	$133^{c} \pm 60$
Layer Cells x Layer Extract	$45^{c} \pm 12$
Layer Cells x Broiler Extract	$238^{b} \pm 113$

[a]Standard deviation from the mean

[b,c]Means in the same column with unlike superscripts differ significantly ($P < .05$).

since little recycling of amino acids occurs in cell cultures when quantities of fresh (unrecycled) amino acids are prevalent (Schneible et al., 1978).

The experimental evidence indicates that there were strong interactions between cell and embryo extract from the divergent cell origins. This was seen in both cell treatments when embryo extract from opposite cell types was applied to cells; the stability against degradation of the MHC molecule was greatly increased in these experimental situations (Table 7).

The $t_{\frac{1}{2}}$ of MHC of treatment LC x LE was one-half that of the BC x BE. But, this difference was not found to be significant (Table 7). The MHC synthesis rate in LC x LE treatments was more rapid than that of the other three treatments (Figure 12) and Table 5). Paralleling this data with MHC content, a faster degradation would be required to affect the more rapid synthesis of MHC in the LC x LE treatment in order for maintainance of MHC content of magnitude equal that of the other three treatments (Figures 11 and 12 and Tables 5 and 7). These data indicate that broiler cells are more efficient in terms of contractile protein metabolism and this would contribute to the heavier muscling seen in the broiler chick strains.

This project demonstrates that the many parameters associated with muscle growth can be measured at the cellular level using culture systems. However, the degree to which these experimental measurements represent intact muscle and the accuracy of the methods utilized, though the best presently available,

remains unknown. Concerning muscle protein accretion, the mature Mts of broiler cultures (BC x BE) were capable of accumulating larger quantities of both total and contractile proteins than were the layer cells (LC x LE). The layer cells synthesized contractile proteins much faster than did the broiler cells. However, the total content of contractile protein per Mt nucleus was not greatly different between the two chick strains, incicating that degradation of contractile proteins must be more rapid in layer type muscle cells. Statistically there was no difference in the degradation rates of myosin between the two strains. However, had the sample size been larger, the degradation rate of myosin would have been found to be faster in the layer cells. Generally, due to the method of statistical analysis, differences found in muscle growth parameters between the two strains were minimal. This occurred because the early growth period was given equal statistical weight as the later growth periods. During the later portion of the growth periods the cultures appeared drastically divergent as far as protein accretion and synthesis were concerned. But, this difference was masked statistically by the essentially equal growth responses which occurred during the early states of myogenic proliferation and development. Two general interpretations can be drawn from this study: 1) there are no statistical differences in cellular metabolism associated during the initial stages of muscle developments with growth in muscle fibers derived from divergent strains of chicks: and 2) subtle differences may exist during later periods of advanced development of culture growth demonstrate that in

advanced development these differences may be more than subtle.

SUMMARY

Muscle cell cultures derived from mononucleated myogenic cells of hind limb muscles from 12 day old chick embryos of divergent strains were established for comparative examinations of muscle growth. Unknown constituents contained in the embryo extract were required for optimun growth and development of these cultures. Therefore, four different culture treatments were established: 1) Broiler type muscle cells nourished with medium containing 5% broiler embryo extract, 2) broiler cells nourished with medium containing 5% layer origin embryo extract, 3) layer cells nourished with layer embryo extract, and 4) layer cells nourished with broiler extract.

Cell cultures were collected for morphological observation on days 1, 2, 3, 4, 6, 8 and 10 in culture. Parallel cultures were labeled for 4 hours with [^{3}H]-L-Leucine on each of the eight sampling days. From the labeled cultures; total protein content, synthesis of total protein, MHC content and MHC synthesis were obtained for each of the treatments. Separate cultures were established for analysis of MHC turnover.

Layer cells (LC x LE) had greater proliferative activity of both total cells and myogenic cells than broiler cells ($P < .05$). Percentage fusion was not different in any

of the treatments. The number of Mts per 10 cm culture dish was similar in all treatments, but the number of nuclei per Mt was greates in the layer cultures nourished with layer medium ($P < .05$). This was probably the result of greater myogenic numbers per plate in this treatment.

Rate of protein synthesis of total proteins was significantly lower in the LC x BE treatment ($P < .05$) but there were no differences in the synthesis rate of total protein in the remaining three treatments. The problems associated with the LC x BE treatment were discussed and the results obtained from this treatment were discounted for the entire study. Total protein per Mt nucleus increased dramatically from day one through day 10 in all cultures, however, protein content per Mt nucleus was similar in all treatments except the protein content per Mt nucleus was significantly lower in the LC x BE treatment ($P < .05$).

The rate of MHC synthesis was more rapid in the layer cells (LC x LE) ($P < .05$) and the cells within the other three treatments synthesized MHC at similar rates. MHC content per myotube nucleus was similar in all treatments. The quantity of muscle specific proteins (measured in terms of MHC content) decreased proportionately with respect to total protein with advancing culture age.

MHC was most stable in the LC x BE treatment ($P < .05$). However, the stability of MHC subunit in the remaining treatments was similar.

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