A METABOLIC STUDY OF THE GROWTH AND DEVELOPMENT OF BOVINE SKELETAL MUSCLE

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CHAPTER 1

INTRODUCTION

Because of an increased emphasis placed upon the efficient production of red meat from the bovine, animal scientists have exhaustively investigated gross live animal and carcass parameters. The central idea of such investigations has been to achieve the capacity to control growth patterns for optimum production. Factors such as sire and dam performance have been very effective in estimating progeny performance. Other factors such as birth weight, rate of gain, adjusted weaning weight, and adjusted yearling weight have also been used to estimate the growth potential of an animal and the potential utility value of certain animals in the breeding herd. Certain carcass data such as cutability and quality grade have also been collected on the bovine to describe major changes in tissue (muscle, bone and fat) growth patterns. Chemical composition has also been established in bovine muscle tissue at different chronological ages, allowing an estimate of tissue compositional changes with age.

Considering, however, the vast quantity of work accomplished on the bovine previously, one still cannot find comprehensive data describing changes in certain biochemical entities during growth in bovine muscle tissue. It is of paramount importance that cellular and subcellular changes due to growth be defined in the bovine, because changes at the ultrastructural level dictate changes observed at the

gross structural level.

Data are available which describe growth-associated skeletal muscle tissue biochemical changes in the pig and in certain laboratory mammals and birds. These data are valuable to establish models of growth curves, yet direct inferences are hazardous when made between species.

Due to the above, this investigation was initiated to observe cellular and subcellular changes in bovine skeletal muscle tissue at various ages post-weaning in order to more closely define patterns of biochemical change in that tissue.

CHAPTER II

REVIEW OF LITERATURE

The objective of this review is to outline the metabolic and compositional growth changes at the cellular and subcellular levels in bovine muscle tissue. In many instances, information obtained from laboratory or other domestic animals will be cited due to the limited amount of information available at this time on the bovine.

Metabolism

General

It has long been known that muscle tissue is not homogeneous and that two primary muscle cell or fiber types exist (Lorenzini, 1678 as cited by Cassens and Cooper, 1971). The work of Needham (1926) and Denny-Brown (1929) indicated that these two muscle fiber types, the red type and the white type, may be described according to their degree of redness, their movement in contraction and their genesis of tetanus. Szent-Gyorgi (1953) also stated that metabolic and functional differences exist between red and white muscle fiber types, with the white type generating energy primarily by glycolysis and the red type generating energy primarily by oxidative metabolism. Microscopic and histological investigations have shown that most mammalian muscle tissue contains at least three types of fibers (Stein and Padykula,

1962; Ogata and Mori, 1964; Moody and Cassens, 1968), the white, red and intermediate fiber.

Gauthier (1970b) has described these fiber types ultrastructurally, emphasizing that the red fiber exhibits extensive subsarcolemmal aggregates of large, closely packed mitochondria containing abundant cristae and located in longitudinal rows among the myofibrils. Gauthier (1970b) also indicated that the white fiber has pairs of filamentous mitochondria, with cristae sparsely evident, located at the I band. Gauthier (1970b) explained that the Z line was approximately one half the width in white fibers as compared to that of the red fibers. Goldspink (1970) and Gauthier and Padykula (1966) further indicated that size differences exist between the fibers, with the red being smallest in diameter, the white being largest in diameter and the intermediate being midway between the two. The intermediate type has also been shown to reflect ultrastructural architecture midway between that described for the red and the white fiber types.

Metabolically, red fibers have a high mitochondria content, are rich in oxidative enzymes (Dubowitz and Pearse, 1960), contain quantitatively greater amounts of myoglobin (Lawrie, 1950; Chinoy, 1963; James, 1968), greater lipase activity (Piantelli and Rebollo, 1967) and greater amounts of lipids and triglycerides (George and Jyoti, 1965; George and Naik, 1958; Adams et al., 1962; Lawrie, et al., 1964; Beecher et al., 1965) than white fibers. White fibers have a higher glycogen content (George and Naik, 1958; Ogata, 1960) and higher myofibrillar adenosine triphosphatase, phosphorylase, and glycolytic enzyme activity (Engle, 1962; Dubowitz and Pearse, 1960; Opie and Newsholme, 1967; Bar et al., 1965) than red fibers. Generally,

intermediate fibers exhibit a metabolic state midway between the red and white muscle fibers (Ogata and Mori, 1964; Stein and Padykula, 1962; Romanul, 1964; Dawson and Romanul, 1964).

Thus, as indicated above, skeletal muscle tissue of the mammal is not homogeneous in fiber type. Functional, metabolic and compositional characteristics exist between individual muscles within the same muscle of differing species and in muscles at various maturity levels within a specie. These characteristics are a reflection of the major fiber types present in the muscle. It is the objective of this review, not to outline differences in fiber types but to outline changes in composite muscle tissue during growth and development in the mammal, assuming the parameter observed reflects the predominate fiber type(s) contained within.

Enzyme Activity

Glycolysis. The glycogen catabolic pathway is the principal means by which carbohydrates enter the cell's metabolic pool. Two enzymes, lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase, were selected in the present metabolic study to be representative of glycolytic metabolism; consequently, they will be reviewed.

Lactate dehydrogenase (LDH), (L-lactate; NAD oxidoreductase, E.C. 1.1.27) catalyzes the following reaction:

$$\text{L - (or D-) Lactate} + NAD^+ \longleftrightarrow \text{Pyruvate} + NADH+H^+$$

It should be noted that in muscle tissue from higher order animals (Xenopus vs Bovine), the L - isomer of lactate is produced by the reduction of pyruvate.

Thunberg (1920, as cited by Schwert and Winer, 1963) recognized an enzyme in heart muscle which oxidized lactate and which depended on a water soluble cofactor for its activity. After much work with cell free extracts showing LDH activity, LDH was successfully crystallized from bovine heart muscle. This initial isolation opened the door to the preparation of pure LDH in subsequent years from skeletal muscle tissue from the rat, rabbit and pig, from human and pig heart and from rat liver.

Crystalline preparations have allowed workers to define molecular properties such as molecular weight of LDH. Miester (1950) and Long and Kaplan (1968) have reported molecular weight information on LDH; their data indicated that the molecular weight of LDH was approximately 140,000, or 35,000 per subunit indicating that LDH is a tetrameric enzyme.

Neilands (1952) discovered that tissue LDH had more than one component. Markert and Moller (1959) termed these components isoenzymes, for they appeared to be proteins showing different molecular forms yet catalyzing the same biological reaction. Weiland and Pfleiderer (1957) as cited by Schwert and Winer (1963) first demonstrated that from one to six components exhibiting lactate dehydrogenase activity could be separated by high-voltage electrophoresis from extracts of liver, heart, spleen, skeletal muscle, kidney, brain and erythrocytes derived from a single specie. They noted that the same number of components were not always found when the same organs from different species were examined. Many other workers agreed with Weiland and Pfleiderer's conclusions that heterogeneity existed in LDH components in various organs between species. Markert and

Moller (1959) also indicated that LDH component differences existed in various organs between the embryonic and the adult pig.

When most vertebrate tissue extracts are subjected to electrophoresis on starch gel, polyacrylamide gel, or any other suitable medium and the electrophoresed gel subsequently stained as indicated by VanBogaert et al. (1967), a total of five active bands are usually observed. The nature of these bands has proved to be due to two parent types and three hybrids, all of which are tetrameric (Cahn et al., 1962). The two parent types have been referred to as the muscle or M type and the heart of the H type; the M type having been isolated from skeletal muscle and the H type isolated from the heart muscle. The homotetramers would thus be H_4, H_3M_1, H_2M_2, HM_3 and M_4. Clausen (1970) further indicated that the H type is also referred to as LDH-A and the M type as LDH-B. Dietz and Lubrano (1972) emphasized that a characteristic pattern is evident after electrophoresis of LDH with the five bands distributed along the electrophoresed medium. In their work the isoenzyme or "isozyme" migrating most rapidly, anodically, was referred to as LDH-1, and the one migrating the least rapidly was referred to as LDH-5. Table I reflects a summary of the LDH nomenclature used thus far in the literature.

Although five electrophoretically separable bands are evident in vitro, various physical and/or chemical factors affect the number, quantity and migration of the LDH isoenzymes. It has been shown by Chilson et al. (1965a and b) that "hybrids" of the two parent bands can occur after freezing and thawing. Everse and Kaplan (1973) pointed out that hybrid formation is a consequence of dissociation of homotetramers into subunits followed by reassociation into tetrameric

hybrids. They also indicated that various factors affect the rate of hybridization during freezing and thawing. Some of these factors include: temperature, pH, the presence of halides, ions, coenzymes or certain denaturing agents. Chilson et al. (1965a) stated that when a mixture of chicken H_4 and M_4 LDH is frozen and thawed in potassium phosphate buffer, no hybridization occurs. Yet when the enzymes were frozen and thawed in sodium phosphate buffer, hybridization occured. This was attributed to the fact that at the eutectic point, the pH of the sodium phosphate buffer decreased upon freezing, whereas the pH of the potassium phosphate buffer increased upon freezing. These results suggested that a lowering of the pH is essential for hybridization. Chilson et al. (1965b) also indicated that high salt concentrations apparently induce the dissociation of the tetramers when the solutes are concentrated during freezing. Levi and Kaplan (1971), Markert and Massaro (1968) and Clausen and Hustrulid (1968) reported that coenzyme presence such as NAD+ and NADH inhibited the formation of hybrids by preventing dissociation. Hybridization is also a result of the addition of halide ions or thiocyanate during freezing and thawing. The order of reaction is SCN^-, $I^- > Br^- > Cl^- >>> F^-$.

Many studies have been made on hybridization of LDH, and as a result of these, some speculation has been made as to the nature of the forces that hold the subunits together in the native enzyme state. Results indicate that a number of interactions are possible, involving various types of intermolecular forces. The effects of urea and guanidine on LDH tend to show that ion-pair bonding is present to some extent. The dissociation of the tetramer in low concentrations of sodium dodecylsulfate (SDS), indicates the presence of hydrogen

TABLE I

NUMBERING AND MONOMERIC COMPOSITION OF LDH ISOENZYMES[e]

Isoenzyme Number	Isoenzyme Letter	Monomers Present	LDH-M %	LDH-H %
LDH-1[a,c]	A[a,c]	HHHH	0	100
LDH-2		HHHM	25	75
LDH-3		HHMM	50	50
LDH-4		HMMM	75	25
LDH-5[b,d]	B[b,d]	MMMM	100	0

[a] Pyruvate specific isoenzyme

[b] Lactate specific isoenzyme

[c] Most rapidly migrating band

[d] Slowest migrating band

[e] Obtained from Dietz, A. A. and H. M. Rubinstein. 1972. LDH Isoenzymes. In Standard Methods of Clinical Chemistry Vol. 7, pp 49-61.

bonding. Jaenick et al. (1971) emphasized that hydrophobic bonding may not be of prime importance in the maintaining of subunit integrity; yet, that coulombic attractions enforced stabilization in the tetrameric form.

It has been questioned whether or not the tetrameric form of LDH is the sole active form. The work of Millar (1962), as well as Reithel (1963), has investigated this and indicated that dimers and monomers were the most active units. On the other hand Griffin and Griddle (1970) concluded that the tetrameric form must be the active species. It is noted that Griffin and Griddle (1970) used much more sophisticated techniques of isozyme separation in their studies and the author tends to identify with this more recent and quantitative work.

As far as the overall catalytic reaction is concerned, it has been shown that skeletal muscle tissue LDH is specific for the L form of lactate and that no detectable activity was noted with the D-isomer. It should be mentioned, however, that Meister (1950), Nisselbaum and Bodansky (1961), Markert and Moller (1959), Winer and Schwert (1958), Neilands (1954), Sakawi and Yamada (1966), Romano and Cerra (1969), Warren (1970), Schatz and Segal (1969) and Lane and Dekker (1969) have found other substrates that are utilized by LDH. The simple fact is that the maximum velocities achieved with substrate analogs are much lower (about one tenth to one hundreth as rapid) than those of lactate or pyruvate. Thus, in crude preparations, the artifacts produced by the presence of these analogs would be very insignificant. As is well known, NAD^+ is the required cofactor for the reduction of lactate to pyruvate and is about 170 times

as effective as the $NADP^+$ molecule (Mehler et al., 1948). Also it has been shown by Meister (1950) that if substrate analogs are present NAD^+ is approximately 100 to 380 times as effective as $NADP^+$. Consequently, these investigators have suggested that in assessing LDH activity in crude extracts, the lactate or pyruvate should be considered to be the specific substrate and NAD^+ the most effective coenzyme.

According to results reported in the literature it is apparent that the function of LDH in glycolysis of skeletal muscle is to provide for reoxidation of NADH originating from the oxidation of glyceraldehyde phosphate. This reoxidation of NADH in muscle tissue appears to be a cyclic phenomenon for the LDH enzyme and is needed only when a sudden demand of energy or ATP production is required. It appears that skeletal muscle has evolved a potent glycolytic system to accomplish this function. The lactate produced in such a cycle cannot be efficiently reoxidized in muscle tissue because of the overriding quantity of the pyruvate specific M_4 isozyme. Lactate can, however, be reoxidized in aerobic tissues such as the heart, kidneys, and liver. These tissues contain a high percentage of the H-type isozyme and allows lactate to be reoxidized to pyruvate, with the generation of NADH. These aerobic tissues may function as scavengers for lactate produced during glycolysis either for resynthesis to glucose as occurs in the liver or to be further degraded via the TCA cycle to produce ATP energy as in the heart. Since most of the energy in aerobic tissues, such as the heart, is generated by oxidative phosphorylation, a constant requirement of NADH is present in the mitochondria. Therefore, it can be assumed that (Everse and Kaplan 1973) a significant

part of the NADH generated in heart muscle is oxidized by the mitochondria to yield ATP and thus NADH is obtained from the oxidation of lactate to pyruvate. Hence, a threefold role of LDH is observed: (1) LDH generates the NAD coenzyme for glyceraldehyde phosphate oxidation in glycolysis, (2) LDH allows the oxidation of lactate to pyruvate with pyruvate used in ATP production or gluconeogenesis in aerobic tissue and finally, (3) LDH produces the reduced coenzymes for use in oxidative phosphorylation in the mitochondria of aerobic tissue.

Glyceraldehyde phosphate dehydrogenase (D-Glyceraldehyde-3-Phosphate: NAD^+ oxidoreductase, phosphorylating), EC 1.2.1.12, was first crystallized from yeast by Warburg and Christian (1939). Prior to the purification of glyceraldehyde phosphate dehydrogenase (GPD), it was noted by Needham and Pillai (1937) that the oxidation of glyceraldehyde-3-phosphate in crude systems was associated with a coupled phosphorylation of adenine nucleotides. Warburg and Christian (1939) indicated that the clue to the phosphorylation mechanism was that orthophosphate ions were stiochiometric participants in the oxidation system. Cori et al. (1948) were of the first investigators to crystallize the enzyme from mammalian sources. It was noted also by these authors that GPD from mammalian sources could be prepared easily and in abundant quantities.

Harris and Perham (1965) showed decisively that native GPD, with a molecular weight of 144,000, was composed of four similar and probably identical polypeptide chains, with a molecular weight of about 36,000 each. Allison and Kaplan (1964) characterized GPD according to the following criteria: Molecular weight, 144,000; extinction

coefficient (0.1%, 1cm, 280 mμ), 1.00 ± 0.05; absorbance 280:260 mμ, 1.07 ± 0.03; specific activity of 115 ± 5 μm NADH formed per minute per mg protein; 20 cysteine residues per mole L-GPD, and four moles of bound NAD^+ per mole L-GPD. Davidson et al. (1967) found that L-GPD is composed of four identical polypeptide chains, each containing a cysteine residue at position 148. They also mentioned that it was this cysteine residue that was acylated by the substrate during catalysis. Watson and Banaszak (1964) stated that native L-GPD forms crystals approximately 1 mm in diameter and are orthorhombic in shape.

According to Velick and Furfine (1963) the oxidative phosphorylation of glyceraldehyde occurs in two reaction steps in which the enzyme is a stiochiometric participant. The first reaction occurs as:

$$R - CHO + NDA^+ + Enz \longleftrightarrow R\text{-}CO\text{-}Enz + H^+$$

When an orthophosphate ion is added, there is another NAD^+ reduction, acyl phosphate is formed by acyl transfer from the enzyme acceptor site to the orthophosphate ion and the overall oxidative phosphorylation reaction is established.

$$R\text{-}CO\text{-}Enz + HPO_4^{=} \longleftrightarrow R\text{-}CO\text{-}OPO_3^{=} + Enz$$

Velick and Furfine (1963) also emphasized that the experiments, summarized above, on GPD provided the first direct evidence for a covalent compound to exist between enzyme and substrate.

Velick, Hayes and Harting (1953) indicated that high affinity NAD^+ binding sites are also the catalytic sites. This was based on the observation that the rate of oxidation of D-glyceraldehyde increased linearly in the presence of the GPD enzyme, the arsenate ion

and varying levels of NAD^+. They demonstrated that the rate of oxidation reached a constant maxima when the NAD^+ concentrations equalled the NAD^+ binding capacity. Recent studies have strengthened these conclusions.

Velick (1958) indicated that NADH binding constants and the kinetic parameters of NADH are in close agreement and the NADH binding is strictly competetive with the strongly bound NAD^+. Hilvers and Weenen (1962) observed that one apparent requirement for NAD^+ is the catalysis by GPD of the acetylphosphate. It was observed by Dagher and Hultin (1973) that GPD is a "loosley" bound enzyme in the muscle tissue particulate fraction. Melnick and Hultin (1973), realizing the location of the enzyme, became involved with muscle tissue GPD kinetic studies. They pointed out that inhibitor constants showed that the soluble enzyme was considerably more subject to inhibition by NADH than the bound form. Melnick and Hultin (1973) further stated that a portion of GPD of some muscle tissues is bound in the intact muscle.

Hultin et al. (1972) suggested that both recent studies and his work showed that modifications of the kinetic properties of GPD can occur by changing the enzyme from the soluble to the bound state and vice versa. They indicated that the lower Michaelis Constant of the bound enzyme for the substrate compared to the soluble form enables the bound enzyme to operate near its maximal performance at low substrate concentrations. Hultin et al. (1972) further suggested that in resting muscle, low glycolytic activity exists, but in the working state it increases. This increase in glycolysis leads to higher concentrations of glycolytic intermediates including glyceraldehyde-3-phosphate. If this compound is involved in the solubilization of GPD,

the enzyme would thus become more soluble. In this form it would have a higher velocity maxima and would be more efficiently utilized in high substrate concentrations. The soluble enzyme is also more sensitive to NADH, thus allowing greater control of the "forward only" energy generating system.

Krebs Cycle. The Krebs Cycle is an extremely important metabolic pathway in skeletal muscle tissue. Most all ATP energy is derived as a result of Krebs Cycle coupling with oxidative phosphorylation. This pathway is solely oxidative and can exist only in periods of aerobiosis. If a state of decreased oxygen or anoxia exists, this pathway is inhibited until oxygen once again enters the system. Many investigators have worked with various enzymes, as well as metabolites of this cycle, to assess qualitative and quantitative activity indices of aerobic metabolism.

It is not the objective of the manuscript to review all aerobic enzymes, yet to review those which were felt to be of key importance.

Succinate dehydrogenase (succinate: (acceptor) oxidoreductase, E.C.1.3.99.1) has been isolated from animal tissues as a soluble, essentially homogenous flavoprotein, the prosthetic group of which is FAD bound to the protein via the isoalloxazine ring (Kearney 1960). The nonprotein constituents of succinate dehydrogenase (SDH) are nonheme iron, labile sulfide, and flavin; the latter covalently bound to the peptide chain (Kearney 1960). Also, according to Singer et al. (1956), SDH contains four iron atoms per molecule of 20,000 Daltons. Singer et al. (1962) indicated that the covalently bound flavin is of particular importance in measuring the concentration of the enzyme since it provides an unambiguous chemical measurement for SDH.

Because of this fact, the use of various dyes, ferricyanide or N-acetylphenozonium salts, such as phenazine methosulfate, as acceptors in assays, allow convenient methods of quantitating SDH activity.

Subunit composition of SDH has been studied extensively. SDH resolution into two catalytically inactive, nonidentical subunits by SDS-polyacrylamide gel electrophoresis has been obtained by Davis and Hatefi (1971). One component has a molecular weight of 70,000 and a flavin-iron-labile sulfide molar ratio of 1:1:4. The second component contains iron-sulfur, but not flavin, and has a molecular weight of 27,000-30,000. Since the flavin is covalently linked to the 70,000 molecular weight subunit, it was suggested by Davis and Hatefi (1971) that this component of the enzyme is essential for catalytic activity, although there is no assurance that the catalytic binding site is also located on this subunit.

It has been suggested (Davis and Hatefi, 1971 and Righetti and Cerletti, 1971) that SDH has a molecular weight of approximately 100,000. Molecular weights other than this, as recorded in the literature, may be a result of dimering during preparation, producing artifacts and promoting varying conclusions (Singer et al., 1973). Some research has shown however that SDH does not exist in solution as a monomer but undergoes dimerization (Bennett and Scott, 1971 and Cox 1969). Evidence for dimerization was obtained from measurements of temperature dependence on the sedimentation coefficient in the ultracentrifuge (Coles et al., 1972). Results from this and similar studies indicate the apparent molecular weight to be approximately 200,000. Considering SDH to be in the dimered state, this is in very close agreement with the data of Davis and Hatefi (1971) and Righetti and

Cerletti (1971).

Activation and deactivation of SDH by various reagents has been studied most extensively. The tendency of this enzyme to undergo activation-deactivation under the influence of a variety of reagents and experimental conditions has posed problems in routine assays. Results have shown that activation is not an all-or-none phenomenon, but that partially activated forms of the enzyme may exist (Zeylemaker et al., 1970). Some of the reagents that have been shown to promote activation include: the substrates succinate and fumarate; the competitive inhibitor malonate (Kearney 1957); reduced coenzyme Q_{10} (Gutman et al., 1971a); NADH (Gutman et al., 1971b); ATP (Gregolin and Scallela 1965); ITP and IDP (Gutman et al., 1971a); pH decreases (Kearney et al., 1972); and various anions in the order of ClO_4^-, HCO_2^-, NO_3^-, $I^- > Br^- > Cl^- > SO_4^=$ (Zeylemaker et al., 1970). Deactivation of SDH has been shown to occur in a number of ways. It can result from the removal of the activator, from elevated pH with low concentrations of substrate (Singer et al., 1973) or from dicarboxylic acids and oxalacetate (DerVartanion and Veeger 1965).

In aerobic cells, SDH has been found to be membrane bound and associated with the respiratory chain. Its primary function is to catalyze the oxidation of succinate to fumarate, which proceeds much more rapidly than the reverse reaction. The kinetic properties are such that fumarate accumulation does not inhibit the oxidation of succinate, because the inhibition constant for fumarate is not low. Moreover, the affinity of the enzyme for succinate is high, that is, the affinity is high enough to result in the efficient oxidation of succinate at <u>in situ</u> concentrations (Walker et al., 1971). As

mentioned earlier, FAD^+ is covalently bound to the enzyme. FAD^+ serves to raise the redox potential of the flavin couple to a level above that of the relatively high potential succinate/fumarate couple, and in so doing favors the oxidation (Singer et al., 1973).

Citrate Synthetase (Citrate oxalacetate-lyase (CoA-acetylating)), E.C.4.1.3.7, was first described by Stern and Ochoa, (1951). This enzyme catalyzes the first reaction of the Krebs cycle, and leads a prominent role in cellular aerobic metabolism. The reaction

$$\text{Oxalacetate} + \text{Acetyl CoA} + H_2O \leftrightarrow \text{Citrate} + \text{CoA} + H^+$$

is reversible <u>in vitro</u>; yet <u>in vivo</u>, the reaction appears to be catalyzed in one direction only, toward citrate synthesis.

Citrate synthetase (CS) occurs naturally in two molecular forms, a large and a small. The large form is found primarily in lower order animals such as the gram negative bacteria; whereas the small size is found in eukaryotes and higher animals (Weitzman 1969). Wu and Yang (1970) have shown that pig heart CS, a very representative small enzyme, has a molecular weight of 100,000 and is dissociable into two inactive, physically indistinguishable subunits in six molar guanidine hydrochloride. Also, according to Wu and Yang (1970), a pig heart CS consists only of amino acids. Stern (1961) has indicated that CS has no prosthetic group in its structure and no divalent metal ion is required for activity. Amino acid analysis of CS (pig heart) by Sreie et al. (1965) indicated that a molecule of the enzyme possessed six to eight sulfhydryl groups. It has been concluded by Sreie et al. (1965) that the sulfhydryls of the enzyme are occupied internally to maintain proper conformation of the active site and that

the active site is devoid of sulfhydryls. His conclusions were based on his work, as well as work of others, on sulfhydryl activators and chelators. Iodoacetamide and N-ethlymaleimide do not inhibit, just as ferricyanide fails to inhibit (Srere 1965). No activation was observed in the presence of glutathione, cysteine or mercaptoethanol. It should be mentioned that CS is inhibited by monovalent cations in the order, $Li^+ > K^+ > Na^+ > Cs^+$. Thiocyanate has been found to be a very potent anionic inhibitor (Wu and Yang 1970), just as magnesium ions are powerfully inhibitory (Kosicki and Lee 1966).

With CS being the "gate" to the Krebs cycle, it might be expected to be a regulatory type of enzyme (Garland et al., 1969). If this is true, CS is subject to inhibition by the products of the TCA cycle; NADH, ATP and α ketoglutarate (Weitzman and Jones 1968). It should be noted that the CS from lower order organisms is sensitive to allosteric control by NAD, whereas, the CS from higher animal systems is sensitive to NADH. Mammalian CS is extremely sensitive to ATP via the Pasteur Effect (Flechtner and Hanson 1969). Also citrate synthetase of animals is insensitive to α ketoglutarate due to the directionality of the pathway (Taylor 1970).

Malate dehydrogenase (L-malate: NAD oxidoreductase E.C.1.1.1.37) is found in both the cytoplasm and the mitochondria of many animal tissues. Malate dehydrogenase (MDH) of the mitochondria (M-MDH) differs from that found in the cytoplasm (C-MDH). Differences have been reported in catalytic (Grimm and Doherty, 1961), physiochemical (Thorne and Cooper, 1964) and chromatographic (Thorne and Cooper, 1964) behavior. Thorne and Kaplan (1963) indicated in their electrophoretic studies that M-MDH is often a "family" of catalytically-active

components. It should also be noted that Davidson and Cortner (1967) have shown that M-MDH and C-MDH are under different genetic control which determines their specific molecular properties.

This family of enzymes catalyzes the reaction:

$$\text{L-malate} + NAD^+ \longleftrightarrow \text{Oxalacetate} + NADH + H^+$$

Studies dealing with the specificity of NAD^+ linked MDH have indicated that L-malate was the only substrate. However, work by Davies and Kun (1957) demonstrated that MDH can catalyze six different reactions, each containing a different substrate. Davies and Kun (1957) emphasized that all catalyses were accomplished by the same protein as evidenced by sedimentation properties and electrophoresis of MDH. Davies and Kun (1957) pointed out kinetically, only two substrates were of any significance, L-malate and oxalacetate (OAA). Coenzyme specificity studies by Mehler et al. (1948) suggested that the rate of oxidation of NADH by OAA was 17 to 34 times as efficient as NADPH at physiological pH.

Molecular properties of MDH have been studied by several workers since the enzyme was first purified. The first enzyme preparation of acceptable molecular purity was obtained by Wolfe and Neilands (1956) from pig heart and had a molecular weight of approximately 40,000. However, this was whole heart MDH; for Thorne and Kaplan (1963) isolated MDH from pig heart mitochondria which exhibited a molecular weight of 70,000. Devenyi et al. (1966) indicated that pig heart mitochondria MDH is composed of similar subunits of 35,000 Daltons each. Also noteworthy is that for each mole of MDH there exists two moles of coenzyme bound to it and 14 sulfhydryls contained within it.

As indicated by Seguin and Kosicki (1967), two sulfhydryls are involved per mole of MDH in substrate binding. Since these sulfhydryl groups are exposed, sulfhydryl inhibition of MDH is quite strong (Wolfe and Neilands, 1956).

Various other inhibitory agents or physical conditions are capable of decreasing or stopping MDH catalytic activity. The agents include various chelating reagents, urea, guanidine hydrochloride, lithium chloride and acid (Chilson et al., 1966). Physical effects such as freezing and thawing caused loss of MDH activity at various rates, depending on the chemical environment of the solution and the rates of freezing (Joyce and Grisolia, 1961; Blonde et al., 1967). In general, it seems to be more appropriate to study MDH activity on fresh rather than on frozen tissue.

Pentose Phosphate Pathway. The metabolism of glucose, in mammalian tissues, through glycolysis is not the only means of hexose oxidation. Hexose monophosphate oxidation can occur with the generation of two moles of NADPH and one mole of ribulose 5-phosphate and CO_2 per mole of hexose phosphate utilized. Ribulose 5-phosphate can, after isomerization to ribose-5-phosphate, be utilized for the resynthesis of nucleic acids or recycled to synthesize fructose 6-phosphate, an intermediate in glycolysis. Also, noteworthy is that the reduced NADP's are available for use in fatty acid synthesis, thereby establishing a second very important function of the pentose phosphate pathway. Probably the most important and certainly one of the regulating enzymes of the pentose phosphate pathway is glucose-6-phosphate dehydrogenase.

Glucose-6-phosphate dehydrogenase (G6PD) (D-glucose-6-phosphate: NADP oxidoreductase, 1.1.1.49) catalyzes the first step of the pentose phosphate pathway:

D-glucose-6-phosphate + NADP $\leftrightarrow$ D-glucono- δ-lactone-6-phosphate + $NADPH_2$

This enzyme was noted as early as 1931 when Warburg and Christian (as cited by Bruns and Werners, 1962) referred to an enzyme which catalyzed the reduction of NADP by glucose-6-phosphate as "Zwischenferment". Later, Ogston and Green (1935) proposed the name glucose-6-phosphate dehydrogenase to identify it according to its hexosemonophosphate dehydrogenase activity. Many workers isolated the enzyme from various sources and in 1959 Noltmann and Kuby prepared the classical enzyme from brewers yeast. Also at about this time, G6PD was prepared from bovine mammary gland by Julian et al. (1961).

Studies of the physical properties of mammary G6PD in a 10,000 fold purification has shown a specific activity of 420 units per milligram protein. This enzyme contained no bound $NADP^+$. Levy et al. (1966) have indicated that the enzyme exists as a dimer, with a molecular weight of 241,000 at protein concentrations above 0.4 milligrams per milliliter. In these studies, Levy et al. (1966) indicated that its formation was pH dependent. There are two monomers each having an apparent molecular weight of approximately 130,000. The monomers exist in two forms, X and Y and are in rapid, mobile equilibrium (Levy et al., 1966). According to Levy et al. (1966), the catalytic activity of the dimer is still undefined; yet current data indicate that either the X or Y form can be reversibly dissociated

into inactive subunits and either X or Y form can be redimerized at high protein concentrations. The formation of Y from X is promoted by the addition of $NADP^+$ or NADPH; whereas, glycerol associated NAD^+ promotes the formation of X from Y. The NADP-linked activity of Y appears to be greater than that of X, but X possesses greater NAD-linked activity than Y.

It has been suggested (Pontremorli and Gruzi, 1969) that slight variations in $NADP^+$ and NADPH concentrations provide a very sensitive regulation of G6PD activity. Adenosine triphosphate (ATP) has also been shown (Avigad, 1966) to have a potent regulatory effect on G6PD; for data indicate that inhibition of its activity is evidenced with increasing quantities of ATP. In addition Levy et al. (1966) have shown AMP to have an effect on the enzyme, apparently promoting the conversion of the dehydrogenase from the low affinity $NADP^+$ to the high affinity $NADP^+$ linked enzyme. Long chain acyl-CoA derivatives also inhibit G6PD activity at very low concentrations (below one millimolar) (Neufeldt et al., 1965). Although the above factors all affect G6PD activity, the principal factor influencing its activity seems to be the availability of $NADP^+$. It has been shown repeatedly (Cahill et al, 1958) that the addition of systems which generate $NADP^+$ in vitro increases the utilization of glucose through the hexose monophosphate oxidation pathway. Therefore, it becomes clear that the rate of this oxidative pathway is linked to the rate of all of the processes which utilize NADPH.

Various cellular reactions require NADPH including fatty acid biosynthesis (Langdon, 1957), amino acid synthesis through the reductive amination of α-ketoglutarate to glutamate (Pontremorli and Gruzi, 1969),

proline synthesis (Yuza and Vogel, 1959), hydroxylation of aromatic compounds such as phenylalanine to tyrosine (Kaufman, 1958) and hydroxylation of steroids (Grant 1956). Pontremorli and Gruzi (1969) noted that ribose-5-phosphate was the most important structural material of this cycle, as it is one of the primary building blocks for nucleic acids synthesis.

Fatty Acid Oxidation. β-hydroxyacyl CoA dehydrogenase (β-HACoAD) (L-3-hydroxyacyl-CoA:NAD oxidoreductase, EC1.1.1.35) catalyzes the reversible oxidation of L(+) - β hydroxyacylCoA by NAD^+ to the corresponding β-ketoacyl CoA as indicated in the following reaction:

$$L(+)RCHOHCH_2COSCoA+NAD^+ \longleftrightarrow RC(=O)CH_2COSCoA + NADH + H^+$$

The BHACoD has a strict specificity for the L isomer, but can utilize hydroxyacyl derivatives of a variety of chain lengths (Stern, 1957). The enzyme is relatively specific for NAD^+, since $NADP^+$ is utilized at much lower rates than NAD^+ (Wakil, 1963).

The properties of the enzyme appear to be quite similar, regardless of the source of its preparation. Stern in 1957 has shown that BHACoAD had a specific activity of 420 micromoles of acetoacetyl CoA reduced per minute per milligram of enzyme at pH 7.0 at 25°C. Also, it has been shown that enzyme preparations are colorless and have a typical protein absorption spectrum.

The ability of fatty acids to induce the β-oxidation systems is dependent on the chain length of the fatty acid. Long chain fatty acids are capable of supporting normal growth while short chain (C_4 to

C_{12}) fail to support growth. Shorter length fatty acids are oxidized by the resting cell.

Muscle Tissue Development

Myogenesis and Muscle Growth

The primitive embryonic cell type that gives rise to most muscle cells is the myoblast (Boyd, 1960). The myoblasts that give rise to skeletal muscle apparently originate from the progenitor mesenchymal cells as indicated by Holtzer and Bischoff (1970). Stromer et al. (1974) outlined a sequence of events accepted by most muscle biologists describing the differentiation of primitive germ cells to the mature muscle cell. The mesoderm or middle germ layer is the first stage in the events of myogenesis. The second stage involves a mononucleated cell committed to becoming a muscle cell, but incapable of fusion or of synthesizing contractible proteins. This is the presumptive myoblastic phase and it is very difficult to distinguish this cell type at this point by ultrastructural examination alone. The third stage or myoblastic stage describes a mononucleated cell capable of fusion and of synthesizing contractible protein. The myoblasts are thought to elongate and eventually fuse to form cylinder-like structures. During the fourth stage, nuclei do not divide mitotically and are centrally located. These fused myoblasts are referred to as myotubes; having the capability to synthesize myosin and actin at the periphery of the cell. In the final phase, the mature skeletal muscle cell exists and is referred to as a myofiber. The cell is filled with myofibrils and the nuclei are located just inside the sarcolemma. At

this point neither the myofibers, nor the nuclei within them, divide mitotically.

Assuming that the myofiber stage has been attained or is in the process of maturation, muscle development can be studied or assessed prenatally and postnatally. Prenatal development in mammalian muscle follows the general pattern as described above. The myoblasts and myotubes cluster and fuse (Fischman 1967), each being mononucleated, to form a multinucleated myofiber; the number of nuclei in the myofiber being indicative of the number of myoblasts which fused. The myofiber then increases in size by synthesis of myofibrillar protein (Goldspink 1972), thereby establishing two methods for which skeletal muscle tissue may increase in quantity prenatally; by an increase in the number of myofibers (hyperplasia) through fusion of committed muscle cell precursors, and by increases in size of the myofiber (hypertrophy) through contractile protein synthesis.

Mammals are born at a relatively late stage in their development in comparison to other animals such as marsupials. The number of muscle cells in mammals either do not increase after birth or increase only slightly up to a few weeks of age. In a variety of species including man, rabbits, sheep, pigs, rats and chickens the number of cells in a muscle bundle is premanently established by several weeks after birth (Moss 1968) and subsequent growth is a result of increases in cellular size (hypertrophy). The work of Goldspink (1972) also indicated that postnatal increases in skeletal muscle size is primarily a result of hypertrophy. Goldspink (1972) also pointed out that hypertrophy is due almost entirely to an increase in the number of individual myofibrils within the cell and that hypertrophy

due to accretions in actual cytoplasm or sarcoplasm was very minor.

It has become obvious through investigations by workers such as Winick and Noble (1965), MacConnachie et al. (1964), Marchok and Herrmann (1967), Cheek et al. (1971), and Williams and Goldspink (1971) that just as the skeletal muscle cell increases in size, so do the nuclei contained within that cell increase in number. Cheek et al. (1971) and Goldspink (1972) alluded to the limitations existing on the quantity of cytoplasm that can be maintained by a nucleus. They suggested that an increase in the number of nuclei is necessary for a continual increase in the size of the fiber. The actual mechanism of increment in nuclear number will not be discussed. Suffice to say that whether it be due to a result of nuclear mitosis (MacConnachie et al., 1964) or a result of satellite cell incorporation (Moss and Leblond, 1971), there is an increase in nuclear number per increment of increase in total cell mass.

Another factor to consider is how the myofibrillar proteins increase in number and length within the myofiber. Denovo synthesis of fibrillar proteins and their subsequent organization into sarcomeres is the accepted method of prenatal myofibrillar growth (Stromer et al., 1974). Nevertheless, convincing evidence for myofibrillar growth postnatally has been advanced by Goldspink (1970, 1971, 1972). He indicated that longitudinal splitting of one or more of the existing myofibrils form two daughter myofibrils. The method as summarized by Stromer et al. (1974) has never been actually observed by electron microscopy, yet the hypothesis of Goldspink has been demonstrated in electronmicrographs. Goldspink (1971) offered a mechanistic approach for this splitting and indicated that oblique or diagonal stress of

the thick and thin filaments during contracture on the Z disc material caused this type of fibrillar separation.

Increases in length of muscles have been observed as an animal matures. The basis for this increase in muscle length is an increase in number and length of the functional unit of the muscle cell, the sarcomere. Goldspink (1968) has shown that existing sarcomers increase in length as much as 0.5 microns. He indicated that this increase is due to a decrease in the overlap of the interdigitating thick and thin filaments. In addition, Goldspink (1972) and Griffin et al. (1971) and Muhl and Grimm (1974) have shown that a dramatic increase in length of muscle cells was a result of the addition of new sarcomers, usually at the ends of the myofibril. Legato, (1970) advocated a similar theory which she termed "sarcomerogenesis". This author demonstrated, by repetetive electronmicrographs of growing muscle cells, that Z disc material cleaves centrally and transversely and that new fibrillar proteins could be deposited between each successive "half" Z disc. This would result in the creation of new sarcomeres. These theories, although experimentally documented, need further work before definite conclusions can be drawn.

Biochemical Growth

Nucleic Acids. As indicated above, skeletal muscle nuclei have been shown to increase in quantity during growth in mammals. Cheek (1968) as well as others have offered an estimate of the ratio existing between the nuclear number and the quantity of fibrillar and cytoplasmic proteins.

At this time, a brief review will be made concerning how RNA and protein content in the muscle cell change relative to nuclear number as measured by DNA quantities.

Devi et al. (1963) investigated muscle growth in rats. They found that the RNA concentration declined with increasing body weights and age. They reported that the largest percentage of decrease in RNA occurred at a young age. At weaning age, an almost consistant RNA level was attained. Srivastava and Chaudhary (1969) presented similar results, but indicated that although total RNA declined with growth, the protein: RNA ratio increased at a rapid rate in early life and tended to plateau with increased maturity. A discussion of the kinds of RNA and their distribution during growth is not within the limits of this review; only quantitating changes of total RNA.

As indicated earlier, muscle nuclei increase with maturity inutero and exutero until the declining increase in cellular area requires no further nuclear addition. As pointed out, nuclei are usually quantitated, via DNA measurements. Therefore, it may be generally summarized that DNA quantity increases with age per total muscle, decreases with age per unit of muscle weight, and is very constant with age per unit of fiber area.

Metabolism. Sink and Judge (1971) indicated that biochemical development of skeletal muscle involved the growth of the contractile system, the storage of various metabolic fuels and the development of enzyme systems adequate to convert these fuels into ATP energy at the required rate demanded by the contractile system. In terms of metabolic pathways, some fibers demonstrate a distinct dependence upon either the aerobic or anaerobic metabolic pathways. Some fibers show a

substantial capacity to generate ATP by either of the pathways depending upon the metabolic environment at the time. However, when investigating a tissue system such as muscle, the sum of the metabolic processes must be taken into consideration, for as outlined earlier, muscle tissue is a heterogeneous mixture of red, white and intermediate fibers, each with differing metabolic capacities.

Many advances in biochemical investigations in developing skeletal muscle tissue have been made in the last decade. Investigators have utilized changes in enzyme activity or isoenzyme patterns as tools to ascertain finite developmental changes. It was noted in many of the research articles reviewed that enzymic and isoenzymic patterns of skeletal muscle tissue reflected opposing, although nonproportionally opposing, trends in aerobic and anaerobic enzyme activity.

The enzyme probably most studied in work concerned with developmental changes is lactate dehydrogenase (Cassens et al., 1969). As indicated earlier, there are five electrophoretic forms of LDH representing two distinct types and three hybrids. Fine et al. (1963) demonstrated in their growth studies on human skeletal muscle that the H subunits (representing the aerobic specific isoenzyme) decreased in percent of the total isoenzymic quantity from 99 percent in the six week fetus to 26 percent in the adult. Clausen and Hustrulid (1969) studied several enzymes including LDH in human skeletal muscle, and their results reflected a continued increase in the synthesis of M subunits during growth. In 1962 Cahn et al. in their chick embryo studies reported data similar to the above and concluded that immature muscle tissue relies more on aerobic than on anaerobic metabolism. This also emphasizes that less mature muscle contains larger quantities

of aerobic enzymes than anaerobic enzymes and that the aerobic enzymes are more active than the anaerobic enzymes. This apparently reverses with age.

Reiterating Clausen and Hustrulid's (1969) work on human skeletal muscle, there was a steady, linear increase in total LDH activity and similar decreases in the H:M type subunit ratio during fetal development. They mentioned that their findings were consistant with the accepted hypothesis that in skeletal muscle development of the fetus, white fibers which are primarily glycolytic cells, reflect the primary growth impetus. Singh and Kanungo (1968) reported similar results in rat muscle and emphasized that in general, LDH activity increased up to 30 weeks of age and then decreased during senescence. They also indicated that the ratio of H:M type subunits was higher in the aged (96 weeks) than in the adult (30 weeks), or in the young rat.

Ramponi et al. (1968) studied the activity of several glycolytic enzymes including glyceraldehyde phosphate dehydrogenase (GPD) in pre- and post-natal rat skeletal muscle tissue. They noted that in general, GPD and other glycolytic enzyme levels were low in fetal muscle, but increased throughout growth. As pointed out in the review by Mersmann et al. (1972), the enzymes involved in glycolysis are generally developed during late fetal life and increase after birth. These results are in line with conclusions reached by Goldspink and Rowe (1968) and Goldspink (1970b). They concluded that less oxidative enzyme activity was reflected in mature than in immature skeletal muscle. They also mentioned that the decrease in oxidative activity was accompained by a concomitant increase in glycolytic activity.

Oxidative activity has often been indexed in skeletal muscle tissue by determination of succinate dehydrogenase (SDH) as well as several other key Krebs cycle enzymes. Succinate dehydrogenase activity in developing chick muscle was investigated by Cosmos and Butler (1966). They emphasized that during the first week exovo, SDH activity showed a rapid increase. However, further development was characterized by a continuous decline in SDH activity. Nystrom (1968) studied skeletal muscle of the cat from the embryo to the adult. He suggested a decrease in activity of the oxidative muscle cell type with maturation. Results such as these were also reported by Germino et al. (1965) in chick muscle. They concluded that SDH activity decreased with development. Greenfield and Boell (1968), experimenting with skeletal muscle in the chick during development by monitoring two oxidative enzymes, SDH and cytochrome oxidase, concluded that the specific activity of both enzymes were similar during development and that the ratio of these enzymes remained constant. They advocated, based on their data, that the enzyme activity did not decrease, only the quantity of the mitochondria that was present to support them. Other workers (Sacktor and Shrimada, 1972) explained that in developmental changes of the oxidative capacity in the aging blowfly skeletal muscle, a loss in functionality of the mitochondria resulted in a decrease in oxidative metabolic activity. Whatever the mechanism, it appears that with advanced postnatal age, oxidative capacity decreases and glycolytic capacity increases.

Chemical Composition. According to Helander (1966) certain age periods seem to be easily discernible in the life span of an animal

with those periods being prenatal, neonatal, adolescence, adult and senescence. He alluded to the fact that the first two age periods have definite physical factors associated with them and are therefore easily defined. He stressed, however, that the last three age groups were not so easily defined and are also very difficult to compare between the species.

It has been known for quite some time that the percentage of water in muscle tissue falls during development (Needham, 1926). Horvath (1945) indicated this in rat growth studies in which he showed decreases of moisture in skeletal muscle from three days of age to 768 days of age with most of the decreases having occurred by the 18th day. Dickerson and Widdowson (1960) noted in their work that the percentage of water in skeletal muscle decreased from 94 percent in the early stages of fetal life to 74 percent in the adult animal. Norris et al. (1963) have indicated in their work with human skeletal muscle that decreased muscle tissue water was evidenced during development. More recent results of Zinn (1967) and Carr (1975) also emphasized that decreases in moisture content of skeletal muscle paralleled increasing age. It should be noted also that development is associated with an increase in the proportion of intercellular water (Briskey, 1969). Dickerson and Widdowson (1960) indicated that in the 46 day pig fetus, 80 percent of the water was extracellular. In the 90th day of gestation, extracellular water accounted for about 50 percent of the total, and by parturition, only 40 percent. At three weeks postpartum, extracellular water had fallen to approximately 33 percent, and by the adult state only 23 percent. This, of course, assumes intercellular water comprises the remainder of the tissue moisture

thereby indicating an increase in intercellular water with development.

Compositionally, lipids reflect opposing trends to moisture quantities in muscle tissue during growth and development in animals. Helander (1966) noted in human skeletal muscle tissue that fat increased with age just as moisture decreased with age. Briskey (1969) stated that there was more intramuscular lipid in muscles of older barrows than in younger barrows. Lee and Kaufman (1974) emphasized in their work that lipid synthesis enzyme activities of intramuscular lipids showed increases through 24 weeks in the pig. Anderson (1972) in his review of composition research, indicated that in all cases skeletal muscle-associated lipids showed increasing quantities during growth in laboratory animals, domestic animals, and humans. Briskey (1969) explained that generally a decrease in lipid catabolism enzyme activity resulted in an increasing quantity of lipid to be deposited in muscle tissue. He explained that deposition of lipids into muscle tissue occurs through the circulatory system. As oxidative metabolic capacity decreased with age (became less efficient), lipid quantities increased because the expanding anaerobic system cannot catabolize the lipids as a fuel for ATP synthesis as efficiently as the aerobic system. Thus marbling increased with age.

According to Widdowson (1970) it is well known that the concentration of extracellular electrolytes such as sodium (Na) and Chloride (Cl) declines with development. Conversely, concentrations of the intracellular electrolytes potassium (K) and phosphorous (P) increase with development. Of course, the concentrations of these ions fluctuate with the distribution of intracellular and extracellular water. This was well illustrated by the work of Dickerson and

Widdowson (1960) with pig skeletal muscle sampled at five different stages of development from the 46 day fetus to the mature hog. These conclusions were also documented by the work of Forbes (1962) with human skeletal muscle. Forbes not only illustrated the same trends at three different stages of development (500 gram fetus, neonate, adult), but also that calcium (Ca), a major divalent cation, reflected increases during growth. Similar conclusions were reached by Widdowson, Dickerson and McCance (1960) in their work with pig muscle.

In general, increases in the concentration of myoglobin are evident during development in skeletal muscle (Whipple 1926). Morita et al. (1970) investigated pig growth via histochemical procedures. They found that there was essentially a very small quantity of myoglobin in the one day old pig, but this quantity increased through three weeks of age, at which time near adult levels were attained. Lawrie (1952) demonstrated that as skeletal muscle increased in its capacity to undergo oxidative metabolism, the concentration of myoglobin increased. This corresponds with statements of Dubowitz (1970) that a positive correlation existed between activity of mitochondrial enzymes and myoglobin concentration. Lawrie has made many studies (Lawrie, 1950; Lawrie, 1952; Lawrie, 1961, Lawrie et al., 1963) on myoglobin concentrations in skeletal muscle during animal development yet no mention was made concerning the efficiency of the myoglobin nucleus and its relationship to oxygen transport. In later work, however, Lawrie (1950) pointed out that there might be a "break down" in the capacity for oxygen exchange as an animal increased in age. Briskey (1969) indicated that an increase in fat deposition with age may also interfere with blood distribution and thus require a need for

greater quantities of myoglobin.

It has been the conclusion of most workers that skeletal muscle protein increases in quantity from early fetal life to maturity. Helander (1957) and Lawrie (1961) concluded that muscle tissue obtained from the bovine depicted increments in total protein during growth and development. Dickerson and Widdowson (1960) and Sink and Judge (1971) also advocated similar results of protein increases during growth in the pig. Many other workers have reflected similar conclusions in protein studies on skeletal muscle in various laboratory animals and humans. Goldspink (1962a) pointed out that there were increases in total protein in skeletal muscle during development, however, he indicated that muscle protein could be broken down into an intracellular and an extracellular fraction; the former increasing during development and the latter showing slight decreases. The primary constituents of the intracellular fraction are the sarcoplasmic and myofibrillar proteins, both reflecting increments during growth and both being vitally important in growth and hypertrophy of the cell. The extracellular fraction is composed almost entirely of connective tissue type proteins which lend a supportive role to the cell and as a percentage of total quantity either remains the same or decreases during growth.

CHAPTER III

MATERIALS AND METHODS

General

Twenty-four Hereford and Charolais crossbred steer calves were used to investigate biochemical growth patterns in bovine muscle tissue. The Hereford and the Charolais crossbred calves were selected for the study according to their genetic background and similar body types.

The calves were obtained immediately after weaning and placed on a standard growing and finishing ration at the OSU beef center. The calves were randomly allotted to three slaughter weight groups designated as 227, 318, and 409 kilograms. As the individual calves reached their respective slaughter weights, they were taken off feed for 24 hours and were slaughtered and dressed according to standard procedures at the OSU Meat Laboratory.

Procedures

Sample Procurement

Muscle tissue was removed from the left Longissimus dorsi at the 13th thoracic region within 10 minutes of exsanguination. The tissue was placed immediately on ice and separated into two portions, the first of which was frozen immediately in an acetone-dry ice bath

(-55° C) and then stored at -20° C, in plastic, moisture proof, Whirl-Pak freezer bags obtained from Nasco. This portion was used for fiber diameter studies. The second and largest portion was minced through the small (1.6 millimeter) plate of a prechilled laboratory grinder. Approximately 20 grams of thoroughly mixed tissue were used for extraction and the remainder (approximately 100 grams) was frozen in an acetone-dry ice bath, placed in plastic, moisture proof, Whirl-Pak freezer bags and stored at -20° C for future analysis.

Care was taken during sampling to insure that the tissue remained on ice. After freezing, the tissue samples remained frozen at -20° C until the desired biochemical analysis was performed. At such time, the tissue was "chipped off" and assayed with the remainder of the tissue, resealed and replaced in the -20° C storage freezer.

Analytical Procedures

Moisture. Tissue moisture was determined by weight loss of homogenized, duplicate two gram tissue samples after drying for 24 hours at 110° C.

Total Muscle Nitrogen. Tissue nitrogen was determined by modified macrokjeldahl procedures (Escoubas, 1973) on homogenized, duplicate one gram muscle tissue samples.

Ash. Muscle tissue ash was ascertained by weight of residue obtained after a 12-hour, 625° C muffle furnace incineration.

Ether Extractable Lipids. Ether extract was determined via Goldfinch (AOAC, 1965) on duplicate two-gram, homogenized tissue samples.

Mineral Analysis. Duplicate one-gram muscle tissue samples were placed in 50 milliliter beakers. Ten milliliters of concentrated, analytical grade nitric acid were added to these beakers. This mixture was allowed to stand for 12 hours. Five milliliters of analytical grade 72% perchloric acid (PCA) were added to the nitric acid-muscle tissue mixture and was mixed well. The samples were then placed on medium heat (90° C hot plate) and evaporated to near dryness in an approved hood system. The residue was resuspended and made to 50 milliliters with deionized, glass distilled water. Aliquots were taken for potassium, calcium, magnesium, phosphorous and sodium determinations via the Perkin Elmer 403 Atomic Absorption Spectrometer. The results were expressed as parts per million and converted to milligrams per 100 grams tissue.

Soluble Protein. Protein determinations were made by the Lowery method (Lowery et al., 1951). When necessary the protein aliquots were diluted to appropriate concentrations within the range of the standard curve and these dilutions were made with 0.25 molar sucrose. The optical density of the aliquots was determined at 750 mμ (red filter) on a Gilford 240 Spectrophotometer. Bovine serum albumen was used as the protein source for the standard curve.

Enzyme Activity. Fresh tissue samples were extracted in ice cold, buffered sucrose (containing 250 mM sucrose), pH 7.4, 100 mM EDTA, 42 mM Tris-HCl, 8 mM Tris-Base, and 50 units of heparin (sodium salt) per milliliter of media. The muscle tissue was accurately weighed, immersed in five volumes of the ice cold media and extracted. Extraction was accomplished in the Potter-Elvehjem glass homogenizer which was fitted

with a serrated teflon pestle. Extraction was made at a medium speed utilizing six strokes of the pestle. Clearance between the pestle and vessel was approximately one millimeter. The homogenate was decanted into prechilled plastic tubes and centrifuged in a Sorvall RC2-B refrigerated centrifuge for seven minutes at 500 X G. The resulting supernatant was decanted and retained and the precipitate re-extracted in five volumes of media using the same extraction and centrifugation procedures as outlined above. The two supernatants were combined and filtered through glass wool into prechilled centrifuge tubes. The supernatants were centrifuged at 14,500 X G for 10 minutes to sediment the mitochondria. The supernatant was decanted and retained while the mitochondrial pellet was resuspended in 10 milliliters of media. The suspension was centrifuged at 2500 X G for seven minutes and the supernatant decanted and discarded. The mitochondrial pellet was re-suspended in six milliliters of media using a prechilled, glass, hand homogenizer. Thus, from the overall extraction, a supernatant and a mitochondrial suspension were obtained. The supernatant was filtered, for enzyme analysis, through five micrometer diameter pore size Millipore filters.

Enzyme activities were determined as indicated in Tables II, III and IV, via the Gilford 240 Spectrophotometer at a constant temperature of 37^{o} C using the Haake constant temperature circulation system.

Succinate oxidase activity was determined by the YSI Model 53 Biological Oxygen Monitor System using the Clarke electrode and measuring micromoles oxygen consumed per minute per milligram of protein.

All assays were performed on fresh muscle extracts. The time interval between exsanguination and the initiation of the assays was approximately one hour and forty minutes. Where appropriate, oxidation and reduction of substrates by enzymes were monitored by Niacin Adenine Nucleotides, with units of enzyme activity based on the micromolar extinction coefficient of 6.22 $cm^2 \cdot \mu m^{-1}$ for NADH and $NADPH_2$ (Long, 1961). In the estimation of succinate dehydrogenase, a modification of the procedure of King (1963) was implemented using 1.5 millimolar ferricyanide (as indicated in Table III) and the activity was expressed as μM of succinate oxidized per minute per milligram of enzyme protein.

Lactate Dehydrogenase Electrophoresis. Filtered muscle supernatant was diluted in chilled, freshly prepared, 250 millimolar sucrose (1:9, supernatant:sucrose). The diluted supernatant was then mixed in a 1:1 ratio with 40 percent, freshly prepared, chilled sucrose and layered atop five-day polymerized, five percent, polyacrylamide pre-electrophoresed gels. The gels were electrophoresed for 70 minutes in a 0^{o} C environment with a current per tube of four millamperes. A total of three gels were electrophoresed for each extraction, two of which were stained according to modified procedures of reduction of tetrazolium salts as explained by Van Bogaert et al. (1967) and Dietz and Lubrano (1972). The incubation system of the gels contained 27.5 millimolar trishydroxymethylaminomethane buffer, pH 8.6, 101.7 millimolar sodium lactate, 0.25 milligram per milliliter nicotinamide adenine dinucleotide (NAD), 0.85 milligram per milliliter Nitro Blue Tetrazolium (Sigma Chemical Company) and 0.13 milligram per milliliter phenasine methosulfate (PMS). The incubation time for the gels was

five minutes at a temperature of 37° C and the reaction was stopped by washing the gels in five milliliters of seven percent acetic acid. The gels were rinsed and stored in deionized glass distilled water.

The unstained of the electrophoresed gels was aligned with the stained gels and sectioned with a scalpel according to the LDH isozyme pattern exhibited on the stained gels. The sections were appropriately labeled and were then extracted in 2.5 milliliters of ice cold 250 millimolar sucrose for 15 minutes at a low speed in the Sorvall Omni-mixer, micro attachment, apparatus. The resulting suspension was filtered through five micrometer pore size Millipore Filters. The resulting solution was assayed for LDH enzyme activity as noted in Table II. Protein concentrations were estimated as indicated above by the Lowery procedure (Lowery et al., 1951).

Total RNA and DNA. Nucleic acids were isolated and quantitated by modified procedures of Schneider (1945), Ceriotti (1955), and Burton (1956). Duplicate muscle tissue samples (0.4 to 0.6 grams) were sectioned from frozen muscle homogenates, weighed and immersed immediately into five volumes (two to three milliliters) of ice cold deionized, glass distilled water. The samples were homogenized at 0° C for 15 minutes at a low speed in the Sorvall Omni-mixer, micro attachment, apparatus. The resulting suspension was acidified with appropriate quantities of 0.6 N perchloric acid (PCA) to a final concentration of 0.2 N PCA. The acidified suspension was mixed thoroughly on the Vortex mixer and centrifuged for 15 minutes in a clinical centrifuge at a Variad setting of 75. The supernatant was discarded and the precipitate re-extracted in 0.2 N PCA and centrifuged as above

TABLE II

CONDITIONS FOR GLYCOLYTIC ENZYME ASSAYS

Enzyme[a,b,c]	Protein Dilution	Assay pH	Assay Component Concentrations
Lactate Dehydrogenase	3000X	7.0	4.3 mM Potassium Phosphate Buffer, 0.77 mM Sodium Pyruvate, 0.1 mM NADH, Muscle Extract.[d]
Glyceraldehyde Phosphate Dehydrogenase	300X	7.5	90.8 mM Triethanolamine Hydrochloride Buffer, 1.5 mM ATP, 2.3 mM Phosphoglyceric acid, 50 mM NADH, 2.5 mM EDTA, 2.0 mM Magnesium Sulfate, 33.3 µg/ml 3-Phosphoglycerophosphokinase, Muscle Extract.[d]

[a] Enzymes were assayed using the supernatant as the protein source.

[b] Incubation time was five minutes.

[c] Activity was determined at wavelength of 340 mµ.

[d] Quantity of muscle extract varied with concentration of protein.

TABLE III

CONDITIONS FOR TCA ENZYME ASSAYS

Enzyme[a,b]	Protein Dilution	Assay pH	Assay Component Concentrations
Succinate[c] Dehydrogenase	60X	7.8	100 mM Potassium Phosphate Buffer, 40 mM Sodium Succinate, 0.1 percent Bovine Serum Albumen, 1.5 mM Potassium Ferricyanide, Muscle Extract.[e]
Malate[d] Dehydrogenase	30X	7.5	86.7 mM Potassium Phosphate Buffer, 0.3 mM NAD, 0.5 mM Malic Acid, Muscle Extract.[e]
Citrate[d] Synthetase	30X	7.9	102.7 mM Tris-hydrochloride Buffer, 2.5 mM EDta, 5.0 mM NAD, 60 mM Malate, 0.22 mM Acetyl CoA, Muscle Extract.[e]

[a] Enzymes were assayed using the mitochondrial extract as the protein source.

[b] Incubation time was five minutes.

[c] Activity was determined at wavelength of 420 mμ.

[d] Activity was determined at wavelength of 340 mμ.

[e] Quantity of muscle extract varied with concentration of protein.

TABLE IV

CONDITIONS FOR OTHER PATHWAY ENZYME ASSAYS

Enzyme	Protein Dilution	Assay pH	Assay Component Concentrations
B-HydroxyAcyl CoA Dehydrogenase[a,c,d]	15X	7.2	110 mM Triethanolamine Hydrochloride Buffer, 8.3 mM EDTA, 0.25 mM NADH, 0.13 mM AcetoacetylCoA, Muscle Extract.[f]
Glucose-6-Phosphate Dehydrogenase[b,c,d]	3X	7.5	31.7 mM Triethanolamine Hydrochloride Buffer, 0.5 mM NADP, 0.67 mM Glucose-6-Phosphate, Muscle Extract.[f]
Succinate Oxidase[a,e]	5.6X	7.4	170 mM Sucrose, 15 mM Potassium Chloride, 15 mM Dipotassium Monohydrogen Phosphate, 3.8 mM Magnesium Dichloride, 0.038 percent Bovine Serum Albumen, 17.9 mM Sodium Succinate, 0.14-0.29 mM ADP, Muscle Extract.[f]

a Enzymes were assayed using the mitochondrial extract as protein source.
b Enzyme was assayed using the supernatant as a protein source.
c Incubation time was 10 minutes.
d Activity was determined at wavelength of 340 mμ.
e Activity was determined polargraphically using the Clark Electrode.
f Quantity of muscle extract varied with concentration of protein.

for 10 minutes. This procedure was performed twice. The resulting precipitate was extracted in the following solvents, in the indicated order and centrifuged in the clinical centrifuge at a Variad setting of 75 after each extraction.

1. 95 Percent Ethanol: Extracted 10 minutes, Centrifuged 15 minutes
2. Ethanol:Chloroform (3:1): Extracted 10 minutes, Centrifuged 10 minutes
3. Ethanol:Ether (3:1): Extracted 10 minutes, Centrifuged 10 minutes
4. Ether: Extracted 10 minutes, centrifuged 10 minutes

The resulting precipitate from the ether extraction and centrifugation was extracted in 0.2 N PCA and centrifuged, after the extraction, for 10 minutes as noted above. This procedure was performed twice. The final precipitate was air dried under convection in the laboratory hood for 20 minutes. Note should be made that all extractions, mixings and centrifugations were accomplished in a 1.1° C environment.

The air dried precipitate was incubated at 90° C for 46 minutes in seven milliliters of 0.2 N PCA with constant mixing. After incubation, the tubes and contents were chilled in icewater for 10 minutes and centrifuged at 1.1° C in a clinical centrifuge at a Variad setting of 75. The supernatants obtained were decanted through glass wool into 10 milliliter acid washed volumetric flasks. The residues were washed with 1.5 milliliters, each, of 0.2 N PCA and the suspensions centrifuged according to the above indicated method for 10 minutes. The supernatants were decanted through glass wool into the appropriate volumetric flasks and the glass wool washed with several drops, each,

of 0.2 N PCA. The combined supernatants and washes were finally made to 10 milliliters.

RNA concentrations were determined on the solutions by a modified procedure of Ceriotti (1955) at a wavelength of 660 millimicrons using the orcinol system (Table V). DNA was also quantitated from these solutions by modified procedures of Burton (1956) using a wavelength of 600 millimicrons and utilizing the diphenylamine system (Table VI). Standard stock solutions were prepared at a concentration of 1.0 milligram DNA and RNA per milliliter in 0.01 N potassium hydroxide.

Working standards of 100 microgram per milliliter DNA and RNA were prepared from the stock solutions using 0.01 N potassium hydroxide. The DNA was obtained from Sigma Chemical Company as the highly polymerized sodium salt from calf thymus and the RNA was also obtained from Sigma Chemical Company as baker's yeast core RNA, Type II C. Calculations for the quantification of RNA and DNA were made based on an assay curve of the standard stock solutions described in Tables V and VI. It was determined that at the stock solution concentrations used, the linear, repeatable portion of the curve was maintained. Calculations were made as follows, based on a three milliliter nucleic acid system (Tables V and VI):

Optical Density	Volume of 100g/ml Nucleic Acid	Dilution factor	Adjusted Optical Density
O.D.	3.0	1	O.D.
O.D.	2.5	1.2	O.D.
O.D.	2.0	1.5	O.D.
O.D.	1.5	2.0	O.D.
O.D.	1.0	3.0	O.D.
O.D.	0.5	6.0	$\frac{\text{O.D.}}{\text{X}}$

where X = Summed adjusted O.D.'s $\frac{X}{6} = Y$

Y = Average adjusted O.D.'s $\frac{Y}{100} : \frac{\text{O.D. of unknown}}{Z}$

Z = Concentration in µg/ml of tissue extract.

Fiber Diameters. Intact tissue sections were immersed while frozen in ice cold 10 percent formalin fixative. The tissue was held in this fixative at 0° C for 48 hours and then placed in fresh, ice cold, fixative for another 48 hours. Approximately three to six fasciculae were removed and placed in a Waring blender equipped with reversed blades and mixed at a low speed for two minutes. The fibers were then stored at 1.1° C for 24 hours. Following this period, 50 fibers were measured for their diameters after a one hour acclimation period at room temperature via ocular micrometer using light microscopy. A standard reading pattern of horizontal and vertical sweeps was implemented.

TABLE V

PROTOCOL FOR DETERMINATION OF RNA BY ORCINOL PROCEDURE

Tube Number	Volume RNA (ml)	Volume H_2O (ml)	Volume Acid Reagent[c] (ml)	Volume Orcinol Reagent[d] (ml)	Mixing Time (min.)	Autoclave Incubation (100°C) (min.)	Tap H_2O Bath[e] (min.)
1	3.0[a]	0.0	6.0	0.4	2	20	10
2	2.5[a]	0.5	6.0	0.4	2	20	10
3	2.0[a]	1.0	6.0	0.4	2	20	10
4	1.5[a]	1.5	6.0	0.4	2	20	10
5	1.0[a]	2.0	6.0	0.4	2	20	10
6	0.5[a]	2.5	6.0	0.4	2	20	10
7	0.0	3.0	6.0	0.4	2	20	10
8	3.0[b]	0.0	6.0	0.4	2	20	10

[a] The RNA contained in these tubes was pipetted, quantitatively, from the working standard solution.

[b] The RNA contained in these tubes was pipetted, quantitatively, from the tissue extract.

[c] The assay concentration of this reagent contained 0.032 percent $FeCl_3 \cdot 6H_2O$ in hydrochloric acid.

[d] The assay concentration of this reagent contained 0.26 percent orcinol.

[e] After cooling in running tap water, the optical density was recorded at 660 mμ (red filter).

TABLE VI

PROTOCOL FOR DETERMINATION OF DNA BY DIPHENYLAMINE PROCEDURE

Tube Number	Volume DNA (ml)	Volume H_2O (ml)	Volume Diphenylamine[c] (ml)	Mixing Time (min.)	Autoclave Incubation (100°C) (min.)	Tap H_2O Bath[d] (min.)
1	3.0[a]	0.0	6.0	2.0	20	10
2	2.5[a]	0.5	6.0	2.0	20	10
3	2.0[a]	1.0	6.0	2.0	20	10
4	1.5[a]	1.5	6.0	2.0	20	10
5	1.0[a]	2.0	6.0	2.0	20	10
6	0.5[a]	2.5	6.0	2.0	20	10
7	0.0	3.0	6.0	2.0	20	10
8	3.0[b]	0.0	6.0	2.0	20	10

[a] The DNA in these tubes were pipetted quantitatively from the working standard solution.

[b] The DNA in these tubes were pipetted quantitatively from the tissue extract.

[c] The assay concentration of this reagent contained one percent diphenylamine (W/V) 1.83 percent sulfuric acid (V/V) in glacial acetic acid.

[d] After cooling in running tap water, the optical density was recorded at 600 mμ.

Total Muscle Pigments. Quantification of total muscle pigments was performed according to modified procedures of Drabkin and Austin (1932) and Rickansrund (1966). Tissue samples were sectioned (4.5 - 5.5 grams) in duplicate from frozen muscle homogenates and immersed in five volumes of ice cold, deionized, glass distilled water. This mixture was extracted for five minutes at low speed on the Sorvall Omni-mixer apparatus. Care was taken to insure that the extraction temperature remained at zero to one degree Centigrade. The homogenate was centrifuged at 2500 X G for 15 minutes in the RC2-B refrigerated centrifuge and the supernatant filtered via Buchner funnels through Whatmann number 41 filter paper. The precipitate was re-extracted and re-centrifuged three times as indicated above and following each extraction the supernatants were filtered. The filter paper was rinsed with a few milliliters of deionized, glass distilled water and the supernatants combined and made to 100 milliliters with deionized, glass distilled water. Potassium ferricyanide (0.025 grams) and potassium cyanide (0.005 grams) were quantitatively weighed and placed in a 100 milliliter volumetric and made to volume with the combined supernatant. This solution was gently but thoroughly mixed and an aliquot was centrifuged at 2500 X G for 15 minutes. This centrifuged aliquot was examined spectrophotometrically and the optical density measured at 540 millimicrons against a deionized distilled water blank prepared just as the sample.

The calculations made assumed a molar extinction coefficient of myoglobin of 11.3 mM per liter and a myoglobin molecular weight of 17,000. Thus, the equations

$$\frac{17000 \cdot 0.2}{11.3} = K$$

$$\frac{O.D. \cdot K}{\text{sample weight}} = \text{Concentration of pigments in milligrams per gram of tissue.}$$

were used in pigment quantitation.

Statistical Analysis. Data analysis were made in accordance with appropriate procedures outlined by Steele and Torrie (1960).

CHAPTER IV

RESULTS AND DISCUSSION

Tissue Preparation

The muscle tissue obtained from the cattle in this study was utilized in both the fresh and frozen states. Results of Escoubas et al. (1974) emphasized that certain isoenzyme electrophoretic patterns were altered due to freezing and thawing stresses, thus enzymatic analysis required the use of fresh muscle tissue, whereas nucleic acid and mineral analysis, fiber diameter measurements, muscle pigment quantitation and chemical composition analysis permitted the use of frozen muscle tissue. Therefore, the tissue to be frozen for future analyses as stated above was immersed rapidly in a dry ice-acetone bath, frozen solid, and stored at -20^{o} centigrade.

Tissue Enzyme Preparation

Various proteins in muscle tissue differ in their capacity to withstand the effects of freezing and thawing stresses. Many enzymes are rather unstable and exhibit a tendency to denature upon storage, even at low temperature (Chilson et al., 1965a). Blonde et al. (1967) studied the effects of freezing and thawing on mitochondrial and supernatant associated malate dehydrogenase in pig heart and found that these stresses had detrimental effects on the in vivo activity as

observed in fresh extracts. Chilson et al. (1965a, 1965b and 1966) identified similar results in their work with lactate dehydrogenase (LDH) in both chicken and beef muscle tissue. Markert et al. (1963) suggested that the freezing and thawing of two electrophoretically distinct forms of LDH caused formation of multiple forms to appear. This was brought about by a complete dissociation and random recombination of the multiple subunits which appeared to form in a binominal distribution. Similar results were obtained by Escoubas et al. (1974), suggesting that freezing and thawing were detrimental to electrophoretic properties as well as the resulting enzyme activity. Therefore fresh tissue removed immediately post exsanguination was utilized in all enzyme analysis.

According to Colowick and Kaplan (1955) isotonic sucrose (0.25M) has been shown to be an excellent medium for tissue extractions. However, Ernster and Nordenbrand (1967) emphasized that sucrose and other nonelectrolytes alone make poor homogenizing mediums by themselves in skeletal muscle tissue because of quality and quantity factors. They mentioned that high concentrations of calcium in muscle tissue adsorbs to the mitochondria during homogenization. This can be eliminated, as indicated by Slater and Cleland (1952), by addition of calcium chelating substances to the medium. Yield problems arise in nonelectrolyte mediums because muscle tissue assumes a gelatinous consistancy creating difficulty in obtaining a sufficient disintegration of the myofibrils (Chappell and Perry, 1954). Chappell and Perry (1954) thus devised a medium to extract muscle tissue to obtain quantitative, quality yields of mitochondrial and supernatant fractions. This medium has been used rather widely in muscle tissue extractions to date.

The Chappell-Perry medium would have been used in this study were it not that electrophoretic separation of aliquots of the supernatant fraction was to be accomplished. Electrophoretic separation of proteins, including enzymes, suspended in salt solutions, produce inconsistant results and/or artifacts. Therefore, an extraction procedure was developed to obtain maximum yields of quality mitochondria that would allow immediate resuspension for electrophoretic analysis which would preclude lengthy dialysis procedures.

A modified procedure for tissue extraction was developed from the procedures of Max et al. (1972) and Dow (1967). The procedure of the present study contained a media of 250 millimolar succrose, 100 millimolar EDTA (tetrasodium salt), 42 millimolar tris-HCL, 8 millimolar tris-base and 50 units of Heparin per milliliter media, pH 7.4. This tris-buffered sucrose acted as an excellent medium for mitochondrial extraction without resulting in variances due to ionic or pH shifts. The excess EDTA chelated the calcium liberated from the skeletal muscle tissue during homogenization and eliminated the adsorbing of calcium to the mitochondria. The heparin added in the above quantities prevented the agglutination of the myofibrils as described by Ernster and Nordenbrand (1967) and resulted in an acceptable yield of mitochondria.

Tissue Nucleic Acid Preparation

The ubiquitous presence of enzymes capable of degrading nucleic acids into smaller molecules in animal tissues makes it imperative to protect the nucleic acid while the tissue is being excised, stored, and prepared for nucleic acid analysis (Hutchison and Munro, 1961). To

prevent significant enzymatic degradation of nucleic acids in the present study, the tissue was removed within 10 minutes post-exsanguination. The tissue was placed immediately on ice and transported to the analytical lab. The tissue was minced through the fine plate (0.063 millimeter) in an ice-chilled head of a laboratory grinder. This was done as rapidly as possible and the tissue placed in Whirl-Pak (Nasco) plastic freezer bags and frozen quickly in an acetone-dry ice mixture. The tissue was stored below -20° centigrade and extracted immediately upon removal from the freezer.

Enzyme Analysis

The data obtained from enzyme analysis were determined as indicated in Tables II, III, IV and VII. Units of enzyme activity were expressed as specific activity (SA) or International Units (IU) of the enzymes, per gram of protein, per gram of extracted tissue. Protein was determined by the Lowery method as indicated earlier. The activity of the enzymes is reflected in Tables VIII, XIX, X, XI, and XII according to their respective slaughter weight classifications. The number of cattle sampled and assayed for metabolic growth patterns is shown in parenthesis beside the indicated activity in the respective slaughter weight.

Average ages should be noted here to project a better idea of the cattle utilized. The average ages were seven, twelve and sixteen months for the 227, 318 and 409 kilogram slaughter weights, respectively. The first weight group contained weaned calves whereas the last weight group contained typical market age, A maturity cattle.

It should also be noted that an analysis of variance has been accomplished on the data collected during each enzyme analysis. The source of variation used to test the null hypothesis was among steers within weight groups, thus this was referred to as the error term. Due to small numbers of animals in this experiment, the major benefit of the analysis of variance was the estimation of variation between animals within slaughter weight groups.

Anaerobic Metabolism

As reflected in Figure 1 and Table VIII, lactate dehydrogenase (LDH) enzyme activity made variable changes during growth of the steers. The change in activity from the first to the second slaughter weight group was 130.8 IU of activity, indicating an apparent decrease in glycolytic capacity. However, an increase of 52.5 IU was noted from the second to last slaughter weight group presenting a total decrease from the first to the last slaughter weight group of 18.0 percent. It would be unreasonable to estimate activity and changes in activity of LDH prior to or after this study. However, it appears that data obtained in this study do not follow patterns suggested in previous literature.

Cosmos and Butler (1966) reported that phosphorylase activity in chicken breast muscle in early ex ovo life was low and increased during growth. They reported similar trends for all glycolytic enzymes and indicated that there appeared to be an approximate 50 fold increase in total glycolytic activity during growth. Cosmos and Butler (1966) suggested that the increase in anaerobic metabolic activity was a result of anaerobic, or white, fiber differentiation. Other workers

TABLE VII

CALCULATIONS FOR DETERMINATION OF ENZYME ACTIVITY FROM BOVINE MUSCLE TISSUE

Enzymes Assayed	Calculation of Activity
Lactate Dehydrogenase, Glyceraldehyde Phosphate Dehydrogenase, Glucose-6-Phosphate Dehydrogenase, Malate Dehydrogenase, Citrate Synthetase, β-Hydroxy Acyl CoA Dehydrogenase	$\frac{3.0^{a}}{6.22^{c} \cdot 1.0^{b} \cdot \text{Volume}^{g}} \cdot \Delta OD^{f}/\text{minute}$ Grams Protein / Grams Tissue
Succinate Dehydrogenase	$\frac{3.0^{a}}{0.485^{d} \cdot 1.0^{b} \cdot \text{Volume}^{g}} \cdot \Delta OD^{f}/\text{minute}$ Grams Protein / Grams Tissue
Succinate Oxidase	$\frac{\text{m}\mu\text{M Oxygen Consumed}^{e}}{\text{Minute}}$ Milligrams Protein / Grams Tissue

[a]Volume of assay in milliliters.

[b]Length of light path (cuvette) in centimeters.

[c]Molar extinction coefficient for NADH and NADPH (6.22 $cm^2 \cdot \mu moles^{-1}$).

[d]Maximum velocity units for the electron acceptor ferricyanide expressed as micromoles oxidized per minute.

[e]Oxygen consumption determined polarigraphically.

[f]Enzyme activities determined spectrophotometrically.

[g]Volume of protein aliquot.

TABLE VIII

GLYCOLYTIC METABOLIC ENZYME ACTIVITY IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Lactate Dehydrogenase Activity[a] (IU)	Glyceraldehyde Phosphate Dehydrogenase Activity[a] (IU)
227	434.880 (5)[b]	2.080 (1)
318	304.050 (4)	0.685 (2)
409	356.500 (5)	0.275 (4)
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	19439.528	0.214
D.F. (Error Mean Square)	11	4
Observed Significance Level	P = 0.40	P = 0.06
Coefficient of Variation	39.3 %	42.5 %

[a]Enzyme activity expressed in International Units as the activity resulting in an initial rate of oxidation or reduction of one micromole of nicotinamide adenine dinucleotide per minute per gram protein per gram of tissue at 25° C.

[b]Number of cattle assayed per slaughter weight group is noted in parenthesis.

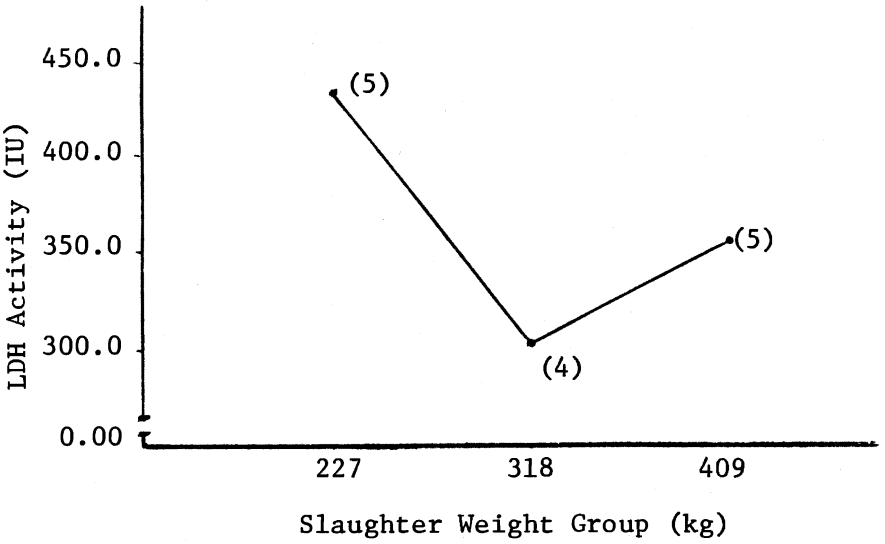

Figure 1. Lactate Dehydrogenase Enzyme Activity in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Number of Animals Assayed per Weight Group is Noted in Parenthesis.

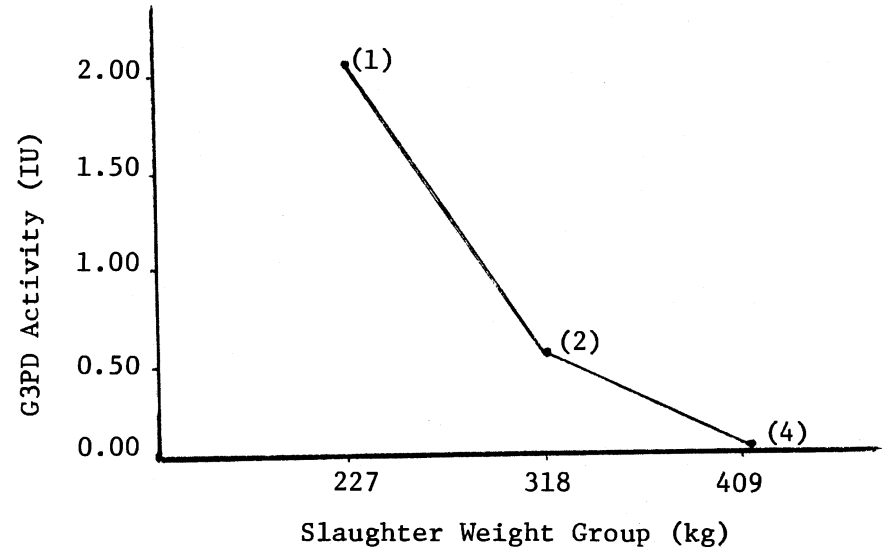

Figure 2. Glyceraldehyde Phosphate Dehydrogenase Enzyme Activity in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Number of Animals Assayed Per Slaughter Weight Group is Noted in Parenthesis.

(Dawson and Kaplan, 1965 and Gresham, 1973) have also presented data which suggested increases in glycolytic capacity during growth.

Glyceraldehyde phosphate dehydrogenase (GPD), as reflected in Figure 2 and Table VIII, also decreased in activity during growth. Moreover, the decreases noted were continuous throughout growth. The percent decrease from the first to the last slaughter weight groups was 86.8 percent, a much stronger decrease than was noted in LDH activity.

The overall anaerobic (glycolytic) activity, as suggested by the general patterns of LDH and GPD, appeared to have decreased with growth (with increasing age). This is not at all consistant with other work as referenced above. However, as presented by Dalrymple et al. (1973), there are undulating patterns of glycolytic metabolic enzyme activity during the mammalian growth process. The glycolytic enzyme activity changes noted in this study appear to be, however, more than a decreasing undulation of an overall increasing pattern. The glycolytic metabolic enzyme activity patterns observed here reflect low observed significance levels (Table VIII). Also, according to Table VIII, there are moderately high coefficients of variation between the observations made. These results yield inconclusive data on glycolytic metabolic enzyme activity in muscle tissue during bovine growth.

Aerobic Metabolism

Previous work has shown that decreases in oxidative enzyme activity exist in mammalian skeletal muscle during growth from the neonate to the adult. Goldspink (1972) indicated that oxidative enzyme activity in mature, adult muscle tissue was less than that in

immature, young muscle tissue. Kistler and Weber (1974) presented similar results in their work in skeletal muscle tissue of Xenopus during larval development but the changes that were evident were small. Greenfield and Boell (1968) worked with chick skeletal muscle tissue and reported that oxidative enzyme activity (succinate dehydrogenase and cytochrome oxidase) showed slight increases during the growth period. Moreover, Beatty et al. (1967) in their growth studies advocated that increases were noted in oxidative enzyme activity from the fetal stage to the adult in mammalian skeletal muscle. Thus, it can be seen that some variation exists in the literature as to the direction and rate of change of oxidative enzyme activity during growth in skeletal muscle tissue.

Results of the aerobic (oxidative) enzyme analyses in the present study are presented in Figure 3 and 4 and in Table IX. Succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) enzyme activity depicted statistically significant ($P < .05$) increasing trends from the first through the last slaughter weight groups. These trends represented a 5.1 and 2.7 fold increase in SDH and MDH activity, respectively. Similarities of oxidative enzymic trends between the present work and Beatty et al. (1967) results are noted, however their data were derived from muscle tissue of different species than those used in the present study. Legitimate comparisons may be made, however, because obvious differences between these species are a result of differing points of inflections in growth curves of the soft and hard tissues.

Citrate synthetase (CS) and succinate oxidase (SO) activity, are noted in Figure 4 and in Table IX. The activity of these enzymes

TABLE IX

OXIDATIVE METABOLIC ENZYME ACTIVITY IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Succinate Dehydrogenase Activity[a] (SA)	Citrate Synthetase Activity[b] (IU)	Malate Dehydrogenase Activity[b] (IU)	Succinate Oxidase Activity (mμM O_2 consumed)
227	6.268 (5)	2.550 (2)	0.633 (3)	1.880 (2)
318	27.795 (4)	1.277 (3)	1.123 (4)	1.420 (3)
409	38.424 (5)	2.217 (3)	2.363 (4)	2.158 (4)
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	93.006	1.164	0.471	0.564
D.F. (Error Mean Square)	11	5	8	6
Observed Significance Level	P = 0.001	P = 0.56	P = 0.02	P = 0.52
Coefficient of Variation	43.3%	65.1%	43.0%	21.6%

[a]Specific activity expressed as micromoles of succinate oxidized per minute per milligram protein per gram of tissue.

[b]Enzyme activity expressed in International Units as the activity resulting in an initial rate of oxidation or reduction of one micromole of nicotinamide adenine dinucleotide per minute per gram protein per gram tissue.

[c]Activity expressed as millimicromoles of oxygen consumed per minute per milligram protein per gram tissue using succinate as substrate.

Number of cattle assayed per slaughter weight group is indicated in parenthesis.

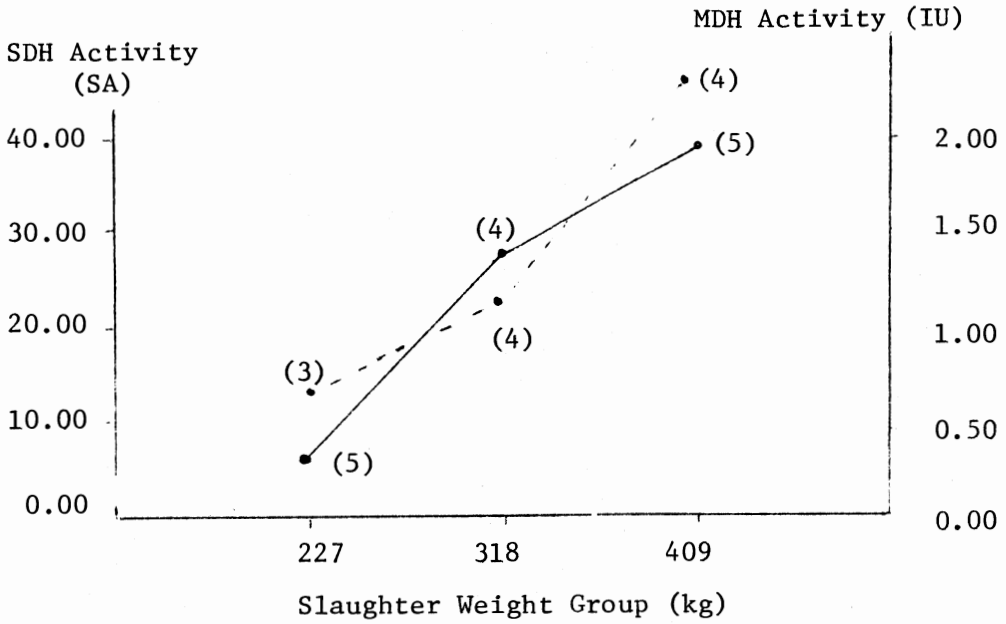

Figure 3. Succinate Dehydrogenase (-) and Malate Dehydrogenase (--) Activity in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Number of Animals Assayed Per Slaughter Weight Group is Noted in Parenthesis.

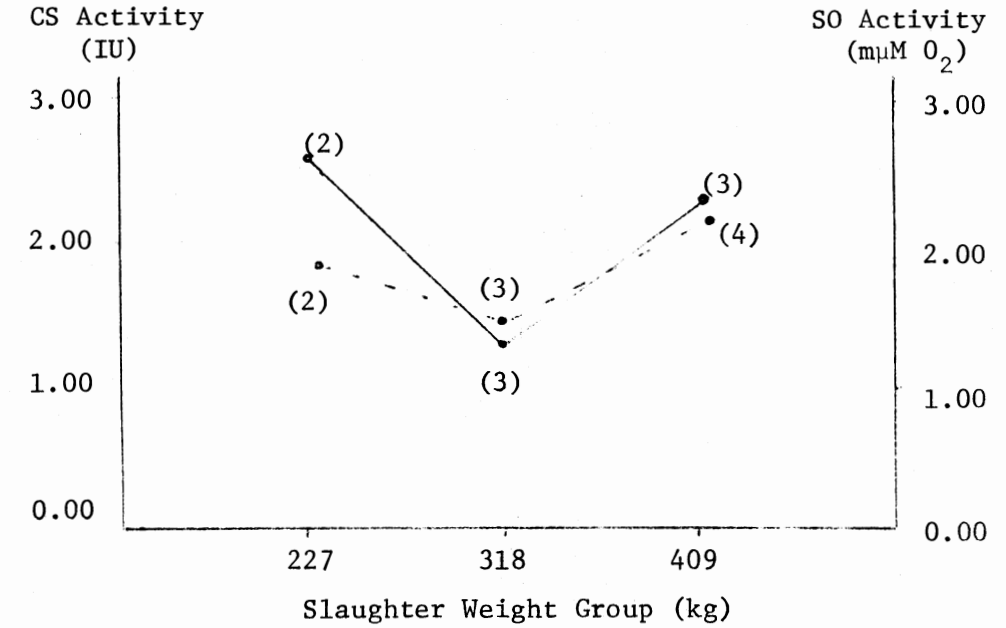

Figure 4. Citrate Synthetase (-) and Succinate Oxidase (--) Activity in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Number of Animals Assayed Per Slaughter Weight Group is Noted in Parenthesis.

displayed varying changes during growth. A decrease in activity was noted for both enzymes from the first to second slaughter weight group and was followed by increases from the second to the third slaughter weight group. The activities of CS, a vital krebs cycle enzyme, and SO, an indicator of mitochondrial functionality, were not expected to display such variable changes. Moreover, the patterns of change noted in SO and CS activity represent low observed significance levels (Table IX). Also, the coefficient of variation of CS activity readings was very high (Table IX) which suggested that experimental error may have adversely affected the data obtained.

Hexose Monophosphate Oxidation

Results for glucose 6-phosphate dehydrogenase (G6PD), as described in Figure 5 and in Table X showed a decreasing trend during this growth study. As noted in Table X, the activity of G6PD was low as has been shown in past work. Muscle tissue has very low quantities of G6PD relative to other tissues such as liver. However, G6PD performs a vital role in muscle metabolism in the fact that it is the branch point enzyme of the pentose phosphate pathway. This pathway is utilized to generate reduced nicotinamide adenine dinucleotide phosphate cofactors for fatty acid synthesis as well as pentose phosphate moieties (phosphoribosylpyrophosphate) for nucleic acid biosynthesis. Bruns and Werners (1962) have shown that G6PD activity increases with age from the fetal stage to the neonate in liver tissue slices. Also Dawson and Romanul (1964) presented data that suggested an increase in G6PD activity during growth in rat skeletal muscle tissue. Thus, data obtained from liver and rat skeletal muscle studies have shown that

G6PD activity increased during growth. The abatement in bovine skeletal muscle G6PD activity in this study did not resemble patterns established for other tissues and species. No statistically significant changes were noted (Table X) in G6PD activity from the first to the last slaughter groups probably as a result of the amount of variation observed in the data.

Fatty Acid Oxidation

Fatty acid oxidation enzyme activity has been shown to increase with increasing pigmentation in skeletal muscle of the rabbit and chicken (Bass et al., 1969). Increasing pigmentation in skeletal muscle has been identified with increasing age (Sink and Judge, 1971). Very little data other than that of Bass et al. (1969) have been presented on fatty acid oxidation enzyme activity changes during growth. Thus, this author is unable to make specific comparisons between the data of this study and pertinent literature values.

As can be observed in Figure 6 and in Table X, beta hydroxyacyl coA dehydrogenase activity increased during the entire course of this study although the observed significance level for these changes was low. This trend suggests that there was an increased lipid oxidation capacity in the muscle tissue with growth.

Lactate Dehydrogenase Electrophoresis

Lactate dehydrogenase (LDH) is composed of five molecular forms (Chapter II), two of which are homogeneous tetramers and three of which are heterogeneous mixtures of the two pure forms. The five LDH isozymes are very similar in physical properties in that they catalyze

TABLE X

AUXILLARY METABOLIC ENZYME ACTIVITY IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS.

Slaughter Weight Group (kg)	Glucose 6-Phosphate Dehydrogenase Activity[a] (IU)	β-Hydroxy Acyl CoA Dehydrogenase Activity[a] (IU)
227	0.044 (3)	0.607 (3)
318	0.025 (4)	1.283 (4)
409	0.015 (5)	1.852 (5)
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	0.001	0.450
D.F. (Error Mean Square)	9	9
Observed Significance Level	P = 0.09	P = 0.09
Coefficient of Variation	99.5 %	51.6 %

[a]Enzyme activity is expressed in International Units as the activity resulting in an initial rate of oxidation or reduction of one micromole of nicotinamide adenine dinucleotide per minute per gram protein per gram tissue.

Number of cattle assayed per slaughter weight group is noted in parenthesis.

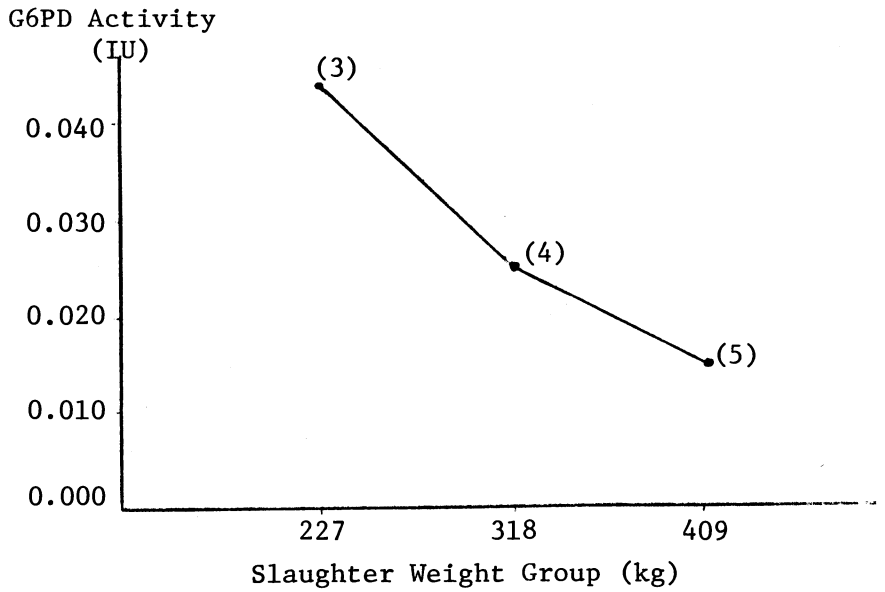

Figure 5. Glucose 6-Phosphate Dehydrogenase Activity in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Number of Animals Assayed Per Slaughter Weight Group is Noted in Parenthesis.

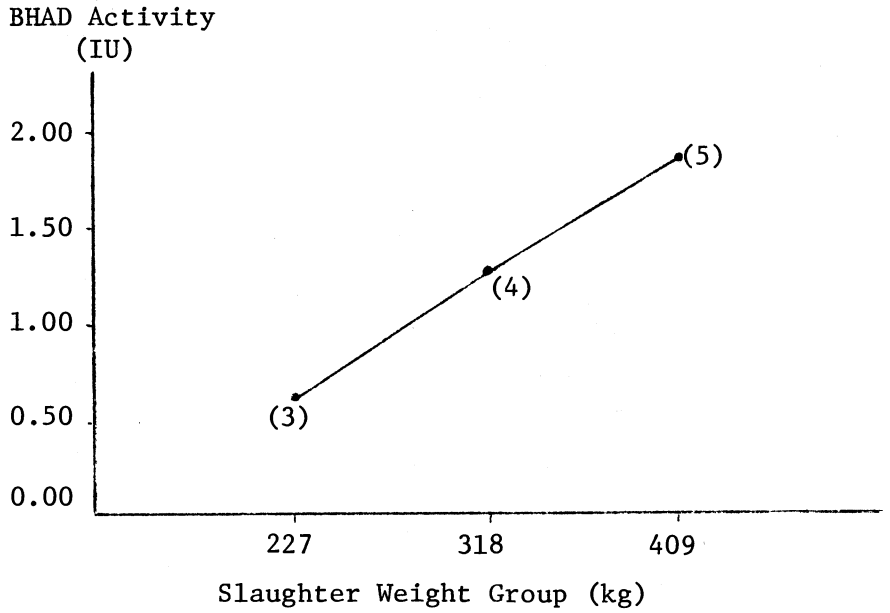

Figure 6. Beta Hydroxy Acyl CoA Dehydrogenase Activity in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Number of Animals Assayed Per Slaughter Weight Group is Noted in Parenthesis.

the same general reaction, but have different specificities for lactate and pyruvate. The M or muscle form is highly specific for pyruvate, whereas the H or heart form is highly specific for lactate; the three hybrids showing differential specificities depending upon the tetramer makeup.

According to Blanchaer and Van Wijke (1962), Fritz and Jacobson (1965) and Dietz and Lubrano (1967), the various isoenzymes can be separated by gel electrophoresis. In the present study, a total LDH activity assay was accomplished using the crude tissue preparation, however the distribution and activity of the various isozymes were determined by assaying each of the electrophoresed enzymic bands.

As indicated by Brody and Engel (1964), slow moving LDH isoenzymes predominate in the aqueous sarcoplasm of anaerobic fiber types. They also indicated that the fast moving LDH isoenzymes predominate in the aqueous sarcoplasm, mitochondria and lipid complexes of aerobic fiber types. Thus, the slow moving, or muscle type, band would be expected to exhibit greater activity in an anaerobic muscle system than would the fast moving, or heart type, band. Data of Fine et al. (1963) have verified this LDH isoenzyme and metabolic activity relationship.

The results of the present study are presented in Tables XI and XII. Total LDH activity, representative of total glycolytic capacity, showed a decrease (though not statistically significant) from the first to the last slaughter weight group in this study (Table VIII). As observed in Table XI, the LDH isoenzyme activity patterns (isoenzyme bands 1, 2, 3 and 5) appeared to reflect an increase in activity at the second slaughter weight period and decreased at the last slaughter

TABLE XI

LACTATE DEHYDROGENASE ISOENZYME ACTIVITY[a] IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS.

Slaughter Weight Group (kg)	Isoenzyme Band 5[b] (IU)	4 (IU)	3 (IU)	2 (IU)	1[c] (IU)
227 (3)	83.333	1.850	20.223	12.843	30.287
318 (2)	215.750	9.310	69.700	56.450	46.250
409 (5)	77.100	14.258	17.060	41.642	39.402
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	1613.090	215.814	393.288	534.880	374.810
D.F. (Error Mean Square)	7	7	7	7	7
Observed Significance Level	P = 0.01	P = 0.55	P = 0.04	P = 0.16	P = 0.67
Coefficient of Variation	40.4 %	144.1 %	63.0 %	51.2 %	40.6 %

[a]Enzyme activity expressed in International Units as the activity resulting in an initial rate of oxidation or reduction of one micromole of nicotinamide adenine dinucleotide per minute per gram protein.

[b]Isoenzymic band migrating least rapidly anodically has been referred to as the muscle specific subunit.

[c]Isoenzymic band migrating most rapidly anodically has been referred to as the heart specific subunit.

Number of cattle assayed per weight group are noted in parenthesis.

TABLE XII

LACTATE DEHYDROGENASE ISOENZYME ACTIVITY[a] IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS.

Slaughter Weight Group (kg)	Isoenzyme Band 5[b] (%)	4 (%)	3 (%)	2 (%)	1[c] (%)
227 (3)	56.2	1.2	13.6	8.6	20.4
318 (2)	54.4	2.3	17.5	14.2	11.6
409 (5)	40.7	7.5	9.0	22.0	20.8

[a]Enzyme activities denoted are expressed as a percent of the total isoenzyme activity determined by oxidation of nicotinamide adenine dinucleotides.

[b]Isoenzymic band migrating least rapidly anodically has been referred to as the muscle specific subunit.

[c]Isoenzymic band migrating most rapidly anodically has been referred to as the heart specific subunit.

Number of cattle assayed per weight group is noted in parenthesis.

weight. This pattern of change proved to be statistically significant in the third and fifth isoenzymic bands only. Lactate dehydrogenase isoenzyme five, the muscle specific and anaerobic band, decreased as a percent of the total isoenzyme activity (Table XII). These results are similar to, but do not agree completely with, data in the literature as indicated above. It should also be noted that two of the oxidative enzymes investigated in this bovine growth study, CS and SO, Table IX, reflected similar trends as presented by the fast moving, aerobic or heart specific band (Table XII). Therefore the electrophoretic enzyme investigation complimented, in general, the data obtained in individual enzyme analysis in this study.

Muscle Pigments

Past work has shown that skeletal muscle tissue not only increases in pigmentation with age (Morita et al., 1970) but also increases in pigmentation as mitochondrial activity increases (Dubowitz, 1970).

In the present study, it was shown (Table XIII) that total muscle pigments, of which myoglobin is in greatest concentration, increased (though not significantly) during growth. This increase is very similar to increases of muscle pigments in skeletal muscle during growth (Sink and Judge, 1971). When these pigment data are compared to the enzyme activity data in this study, two aerobic enzymes, SDH and MDH, are found to display similar trends (Table IX).

Anaerobic enzyme activity reflect opposite trends as were noted by SDH and MDH. Thus, increases in pigment concentration tended to parallel increases in SDH and MDH activity and opposed decreases in glycolytic activity. These results suggest an increasing aerobic

TABLE XIII

MUSCLE PIGMENT CONCENTRATION IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Pigment Concentration[a] (mg/g)
227	1.134
318	1.393
409	1.614
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	0.150
D.F. (Error Mean Square)	21
Observed Significance Level	P = 0.07
Coefficient of Variation	21.5 %

[a]Pigment concentration expressed as milligrams of total muscle pigments per gram of tissue.

Number of cattle assayed per weight group is noted in parenthesis.

skeletal muscle system in the experimental steers with increases in postweaning age or suggests that there was a compensation for a decreased total aerobic capacity by increased pigment concentration and increased specific activities of the aerobic enzymes so as to support hypertrophy.

Fiber Growth

Guenther (1974) has shown that fiber diameter increased with increasing age in bovine muscle tissue. Guenther (1974) also indicated that the greatest impetus of muscle growth occurred between seven and 12 months of age. After 12 months of age, the rate of muscle fiber hypertrophy appeared to diminish. Muscle fiber diameter in the present study depicted increasing trends ($P < .05$) throughout growth (Table XIV) with the greatest impetus of hypertrophy noted at the second slaughter weight group. This weight group represented an average age of 12 months, thus, the present data are very similar to muscle fiber growth data as described in the literature.

Nucleic Acid Analysis

Winick and Noble (1965) have shown that the total quantity of deoxyribonucleic acid (DNA) increased with age. When expressed on a unit of tissue basis, Robinson and Lambourne (1970) pointed out that the quantity of DNA either became static in its changes during growth or declined relative to increasing age. This suggested that although DNA increased in quantity with age, it did not increase at the same rate as skeletal muscle tissue deposition. Further evidence for a muscle tissue: DNA differential rate of increase was offered by

Hubbard et al. (1974).

According to Winick and Noble (1965) and Enesco and Leblond (1962), there was a definite numerical relationship between the quantity of DNA and the number of nuclei in muscle tissue. These investigators presented the following relationships.

$$\text{Number of Nuclei (millions)} = \frac{\text{Total Organ DNA (mg)} \times 10^{3}}{6.2 \times 10^{-12}}$$

where 6.2 is the quantity of DNA in picograms in a single diploid rat nucleus. Also:

$$\text{Weight per Nucleus (mg)} = \frac{\text{Total Organ Weight (gm)} \times 10}{\text{Number of Nuclei (millions)}}$$

According to Hubbard et al. (1974) there was an increase in the number of nuclei per muscle and in the weight supported per nucleus with increased age in rat skeletal muscle tissue.

As can be observed in Table XV, there was a decline ($P < .05$) in DNA concentration per 100 milligrams of wet tissue weight. However, the data in Table XVII suggests that there was an increase in total longissimus dorsi (LD) section DNA during growth. Total LD section DNA was estimated from the LD section weight, Table XVI. These data are very consistant with data in the literature on skeletal muscle tissue in other mammalian species.

Based on LD section weight (Table XVI), muscle tissue growth increased ($P < .01$) with age in the experimental steers. Also, total section DNA (Table XVII), total LD section nuclei, quantity of tissue supported per nucleus and quantity of protein supported per nucleus (Table VIII) increased in similar, rapid rates during growth. These

TABLE XIV

MUSCLE FIBER DIAMETER AND AREA FROM THE LONGISSIMUS DORSI OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS.

Slaughter Weight Group (kg)	Fiber Diameter[a] (μM)	Fiber Area[b] ($\mu M^2 \times 10^2$)
227 (8)	62.93	31.104
318 (8)	67.89	36.201
409 (8)	70.16	38.662
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	19.743	
D.F. (Error Mean Square)	21	
Observed Significance Level	P = 0.01	
Coefficient of Variation	6.9 %	

[a]Fiber diameter expressed in micrometers.

[b]Fiber area expressed as 1 X 10^2 micrometers and assumes circular fiber shape, thus implementing the formula, πr^2.

Number of animals assayed per weight group is noted in parenthesis.

TABLE XV

NUCLEIC ACID CONCENTRATIONS IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	DNA[a] (μg/100 mg)	RNA[a] (μg/100 mg)	$\frac{RNA}{DNA}$	$\frac{DNA}{RNA}$
227 (8)	57.57	101.63	1.77	0.57
318 (8)	55.66	108.31	1.96	0.51
409 (8)	48.56	106.01	2.20	0.46
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	43.87	154.28	0.053	0.004
D.F. (Error Mean Square)	21	21	21	21
Observed Significance Level	P = 0.03	P = 0.56	P = 0.005	P = 0.007
Coefficient of Variation	12.5 %	12.7 %	12.0 %	12.9 %

[a]Nucleic acid content expressed as DNA or RNA in micrograms nucleic acid per 100 milligrams muscle tissue weight.

Number of cattle assayed per slaughter weight group is noted in parenthesis.

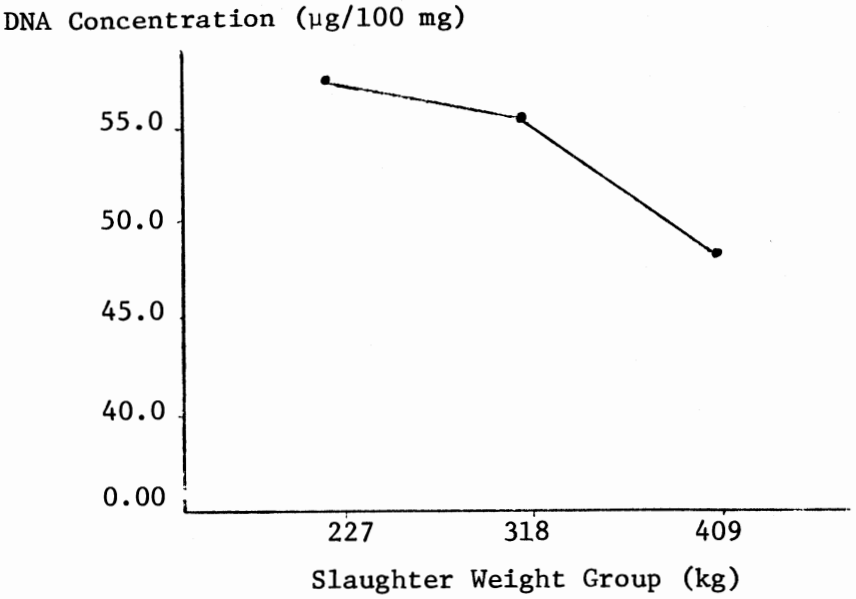

Figure 7. Deoxyribonucleic Acid Concentration in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Each Point is an Average of the Analysis of Eight Animals.

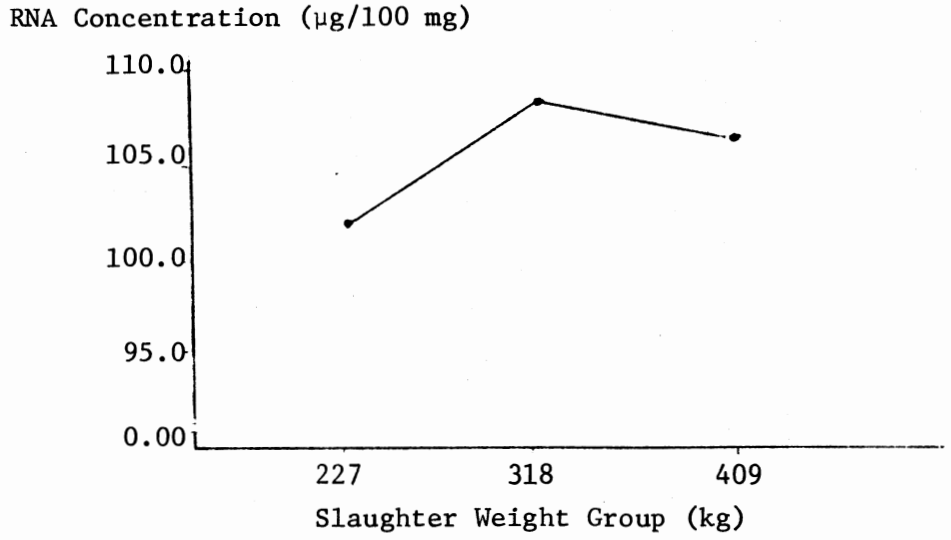

Figure 8. Ribonucleic Acid Concentration in Longissimus Dorsi Muscle in 227, 318 and 409 Kilogram Slaughter Weight Steers. Each Point is an Average of the Analysis of Eight Animals.

results suggest that the muscle tissue of the cattle in this study was still in a phase of rapid, efficient muscle growth at 409 kilograms slaughter weight. As indicated earlier, the animals in this slaughter weight group averaged approximately 16 months of age, an age at which the rate of muscle growth usually starts to diminish. Although the study did not encompass sufficient periods of time to ascertain if this increase in tissue to nuclei ratio would continue to increase or achieve a plateau, it would seem logical to predict that a maximum number of nuclei per fiber would be attained, thereby limiting the potential growth of the fiber. A prediction of nuclear regulation of fiber growth would be acceptable because Cheek et al. (1971) and Goldspink (1972) have indicated that there was a maximum quantity of tissue or cytoplasmic area which could be supported by an individual nucleus in muscle tissue and that limiting nuclear number resulted in the arresting of muscle hypertrophy.

Ribonucleic acid (RNA) has been shown to decrease in skeletal muscle tissue with age when expressed on a unit of tissue basis (Robinson and Lambourne, 1970). Winick and Noble (1965) indicated that, like DNA, RNA increased with age when expressed relative to total muscle mass. They also pointed out that RNA attained its ultimate cellular concentration during early growth although the cell continued to grow. It has also been shown that muscle tissue has high RNA_DNA ratios especially in early life suggesting high levels of protein synthesis were occurring at this point.

Ribonucleic acid analysis in the present study (Table XV) showed evident increases in concentration from the first to the second slaughter weight group but appeared to remain at constant levels from the

TABLE XVI

LONGISSIMUS DORSI SECTION WEIGHT FROM
227, 318 AND 409 KILOGRAM
SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Longissimus Dorsi Section Weight[a] (kg)
227 (8)	1.2
318 (8)	1.5
409 (8)	2.0
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	0.18
D.F. (Error Mean Square)	21
Observed Significance Level	P = 0.0001
Coefficient of Variation	8.8 %

[a]Longissimus dorsi weight is expressed in kilograms and represents the weight of a closely trimmed transverse section excised anterior to the sixth thoracic vertebra and posterior to the twelfth thoracic vertebra.

Number of cattle from which the longissimus section was removed is noted in parenthesis.

TABLE XVII

TOTAL CELL AND TOTAL MUSCLE SECTION NUCLEIC ACID FROM THE LONGISSIMUS DORSI OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Total Nucleic Acid[a] (µg/100 mg)	Total Muscle Section RNA[b] (g^2)	Total Muscle Section DNA[b] (g^2)
227 (8)	159.20	1.219	.691
318 (8)	163.97	1.625	.835
409 (8)	154.57	2.120	.971

[a]Total nucleic acid content is expressed as micrograms of the sum of ribonucleic and deoxyribonucleic acid per 100 milligrams of fresh tissue.

[b]Total muscle section RNA and DNA is expressed in grams and calculated based on total longissimus dorsi section weight.

Number of animals assayed per slaughter weight group is noted in parenthesis.

TABLE XVIII

TOTAL MUSCLE SECTION NUCLEI, GRAMS OF TISSUE SUPPORTED PER NUCLEUS, AND GRAMS OF PROTEIN SUPPORTED PER NUCLEUS IN LONGISSIMUS DORSI OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Total Muscle Section Nuclei[a] (10^{12} million)	Quantity of Tissue Supported Per Nucleus (10^{-5} µg)	Quantity of Protein Supported Per Nucleus[c] (10^{-6} µg)
227 (8)	111.5	1.08	2.34
318 (8)	134.7	1.11	2.54
409 (8)	156.6	1.23	2.81

[a]Total muscle section nuclei is expressed in 10^{12} millions.

[b]Grams of tissue supported per nucleus is expressed in 10^{-5} micrograms based on the longissimus dorsi section weight.

[c]Grams of protein supported per nucleus is expressed in 10^{-6} micrograms based on the longissimus dorsi section weight.

Number of animals assayed per slaughter weight group is noted in parenthesis.

second to last slaughter weight group analysis. Total RNA concentrations, Table XVII, showed almost linear increases with age. These data suggest that although RNA concentration, on a unit of tissue basis, appeared to plateau at the second slaughter weight group, total muscle tissue synthesis capacity had not reached its maximum point at any time during the study. These data are substantiated when RNA is expressed relative to DNA (Table XV). A significant increase ($P < .01$) is noted when the RNA:DNA ratio was determined indicating a continued, elevated, protein synthetic capacity in the muscle tissue during growth.

Mineral Analysis

The literature has shown that several methods exist for the determination of intra- and extracellular areas in muscle tissue. Burr and McLennan (1960) and Boyle et al. (1941) described several methods for doing this, including inulin space analysis and muscle tissue electrolyte analysis. Generally, it appears that tissue electrolyte analysis has been the most reliable and convenient.

It has been generally thought that there was an increase in the intracellular space and a decrease in the extracellular space with growth in muscle tissue (Widdowson, 1970). This has also been indicated by the fiber hypertrophy data of Goldspink (1962a).

In the present study, mineral analysis were accomplished on potassium (K), sodium (Na), phosphorus (P), magnesium (Mg), and calcium (Ca). The data of these various electrolytes are presented in Table XIX.

Muscle tissue potassium concentration in this study showed variable, decreasing trends which refelcted a low observed significance

level (Table XIX). It should be emphasized that although the pattern of change during bovine growth was irregular, the actual variation (Table XIX) in the collected data was very small not only in the K results but in all electrolyte results. Potassium has been shown to be one of the major intracellular ions in muscle tissue (Dickerson and Widdowson, 1960). The concentration of K has been shown to increase during growth. The trend in this study did not follow the pattern suggested in the literature.

Sodium, one of the predominant extracellular ions, depicted slight nonsignificant decreasing trends during growth. These results are very similar to previous results. When Na and K were expressed in relation to each other (Table XX), the K:Na ratio appears to increase slightly and the Na:K ratio appears to decrease slightly.

Phosphorus, a predominant intracellular ion, depicted slightly decreasing trends during this study which proved to yield no statistical significance (Table XIX). When the ratio of the two intercellular ions were calculated, no changes were noted during growth (Table XX). Moreover, when P was expressed relative to Na, constant values were maintained as slaughter weight increased. With the data obtained from K, Na, and P quantification, it may be reasonable to conclude that adult levels of electrolytes had been attained by the first slaughter weight group. Dickerson and Widdowson (1960) have indicated that electrolytes positively or negatively fluctuate during early growth in skeletal muscle. At a point of chemical maturity, very static levels are maintained.

A point of diminishing of the rate of cellular hypertrophy in the last slaughter weight group was suggested considering the muscle fiber

TABLE XIX

ELECTROLYTE CONCENTRATION IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Potassium[a] (mg/g)	Sodium[a] (mg/g)	Phosphorus[a] (mg/g)	Magnesium[a] (mg/g)	Calcium[b] (μg/g)
227 (8)	3.493	.413	2.158	0.248	38.73
318 (8)	3.320	.408	2.031	0.243	62.50
409 (8)	3.379	.376	2.026	0.245	55.92
Mean Square Among Steers Within Slaughter Weight (Error Mean Square)	27.06	1.819	25.09	0.4925	265.8
D.F. (Error Mean Square)	21	21	21	21	21
Observed Significance Level	P = 0.12	P = 0.19	P = 0.19	P = 0.91	P = 0.02
Coefficient of Variation	4.8%	11.2%	8.1%	7.7%	25.0%

[a]Concentration of electrolytes expressed as milligrams per gram wet tissue weight.

[b]Concentration of electrolyte expressed as micrograms per gram wet tissue weight.

Number of animals assayed per slaughter weight group is noted in parenthesis.

growth data and the nucleic acid analysis data. Cellular hypertrophy directly affects the amount of intra- and extracellular space; increases in cell size results in increases in intracellular space and decreases in extracellular space. The assumption that an increased K:Na ratio, or, a decreased Na:K ratio, inferred greater intracellular space and decreased extracellular space, allows a positive relationship to be made between electrolyte, protein synthesis and fiber diameter data in this study.

The concentration of magnesium has been shown to increase through the fourth posthatching week in avian muscle and then decrease slightly through the adult ages (Dickerson and Widdowson, 1960). Similar trends were noted by the same authors in human skeletal muscle and opposite trends in pig skeletal muscle. The results in the present study (Table XIX) suggest that mature levels had been attained prior to the onset of the study due to obvious static values.

Dickerson and Widdowson (1960) and Dickerson and McCance (1964) have shown that the concentration of calcium decreased slightly with increasing age up to a point of chemical maturity. At this point, constant levels were maintained. In the present study, Ca made variable but significant ($P < .05$) changes during growth (Table XIX). The trends noted here are not consistant with trends depicted in the literature. According to Marsh (1966), Ca is required in the tissue for muscle contraction to occur. Considering this requirement, increased deposition of contractile proteins would necessitate increasing concentrations of calcium ions. Such relationships, although suggested in the literature, have not been defined from previous work. Trends, considering this hypothesis, were evident in this study. More work

TABLE XX

RATIOS OF CONCENTRATIONS OF VARIOUS ELECTROLYTES IN LONGISSIMUS DORSI MUSCLE OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Potassium / Sodium	Sodium / Potassium	Phosphorus / Sodium	Sodium / Phosphorus	Potassium / Phosphorus
227 (8)	8.4	0.12	5.2	0.19	1.6
318 (8)	8.2	0.12	4.9	0.20	1.6
409 (8)	9.0	0.11	5.3	0.18	1.6

Number of animals assayed per slaughter weight group is noted in parenthesis.

in this area is required before conclusions can be made.

Chemical Composition

Chemical compositional studies were determined, Table XXI, on the muscle tissue utilized in this study. It was observed that total moisture decreased ($P < .01$), ether-extractable lipids increased, and tissue protein and ash (minerals) changed very little during growth when expressed as a percent of muscle weight. These data, along with the electrolyte data, suggest that muscle tissue chemical maturity had been attained prior to the onset of the study.

TABLE XXI

CHEMICAL COMPOSITION[a] OF THE LONGISSIMUS DORSI SECTION OF 227, 318 AND 409 KILOGRAM SLAUGHTER WEIGHT STEERS

Slaughter Weight Group (kg)	Tissue Moisture (%)	Tissue Protein (%)	Tissue Ether Extractable Lipids (%)	Tissue Ash (%)
227 (8)	75.37	21.7	1.83	0.86
318 (8)	71.61	22.8	3.19	1.03
409 (8)	71.53	22.0	4.32	0.91
Mean Square Among Steers Within Slaughter Weights (Error Mean Square)	3.237	0.695	4.023	0.029
D.F. (Error Mean Square)	21	21	21	21
Observed Significance Level	P = 0.001	P = 0.06	P = 0.07	P = 0.14
Coefficient of Variation	1.6 %	3.7 %	59.9 %	17.8 %

[a]Chemical composition expressed as a percent of the total fresh muscle tissue mass.

Number of animals assayed per slaughter weight group is noted in parenthesis.

Failure for the addition of the components to sum to 100 percent is assumed to be a result of a failure of proximate analysis procedures to quantitate all tissue chemical components.

CHAPTER V

SUMMARY AND CONCLUSIONS

Twenty-four Hereford and Charolais crossbred steers were used to investigate biochemical growth patterns in bovine muscle tissue. The steers were obtained immediately after weaning and fed a standard growing and finishing ration. The steers were allotted randomly to three slaughter groups of 227, 318 and 409 kilograms live weight. Eight steers, four of each breed, comprised a slaughter weight group. As the individual steers attained their respective slaughter weight, they were taken off feed for 24 hours and subsequently slaughtered according to standard slaughter and dressing procedures. Samples were removed from the left longissimus dorsi at the thirteenth thoracic region within ten minutes of exsanguination and handled as necessary for specific biochemical analyses.

Metabolic enzyme analyses reflected decreasing trends in glycolytic enzyme activity (lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase). Succinate dehydrogenase and malate dehydrogenase activity, increased ($P < .05$) during growth however citrate synthetase and succinate oxidase reflected variable trends and low observed significance levels. Glucose 6-phosphate dehydrogenase activity decreased during growth and beta hydroxyacyl CoA dehydrogenase activity increased during growth yet the changes noted were not statistically significant. Thus the data suggests that aerobic capacity increased, anaerobic

capacity decreased, pentose phosphate pathway activity decreased and fatty acid oxidation enzyme activity increased. These results are not entirely consistant with previous work reported on muscle tissue growth data from other species. It should be emphasized however that not only might technique errors be responsible for unexpected trends but also deviations in points of physiological and metabolic maturity between species reported in the literature and the species utilized in this study. Another significant fact which could certainly have affected the experimental variation between animals was the lack of uniformity in ages of the cattle within each slaughter weight group. Further work under more controlled circumstances must be accomplished to elucidate these data.

Muscle tissue fiber (cell) diameter increased ($P < .05$) with growth in the steers. The pattern of fiber hypertrophy noted in this study was consistant with patterns of fiber growth noted in the literature.

Muscle tissue pigment analysis yielded data which reflected constant, increasing trends throughout growth. These data are also very consistant with previous work yet do not correlate well with the enzyme activity results

Deoxyribonucleic acid concentration decreased ($P < .05$) during the entire growth study and ribonucleic acid concentration increased and plateaued as slaughter weight increased. Also reflecting increases were total muscle section RNA, DNA, and nuclear number, quantity of tissue supported per nucleus and quantity of protein supported per nucleus. These data support the conclusion that the muscle tissue in this section of the longissimus dorsi continued to show hypertrophy at

rapid rates during the entire course of the study.

Electrolyte concentrations showed static, consistant patterns during growth. The ratios of intra- to extracellular ions were also constant during growth. These results suggest, along with the results of muscle tissue chemical composition that chemical maturity had been attained prior to the onset of this growth study.

In considering the results presented from this growth and development study, it is obvious that these data are preliminary. Future studies must be initiated including not only investigations of cellular and subcellular growth parameters, but also live animal and carcass growth studies. Yet, methodology and data presented here will allow a more proper tailoring of future projects so as to yield complete data on the growth and development processes of the muscle tissue of the bovine. In order to obtain experimental animals necessary for such studies, foundation stock must be established to yield cattle of proper uniformity in age and physiological and metabolic maturity. Meat scientists and muscle biologists can no longer accept "left over" products from animal breeding and feeding research if significant advances are to be made.

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