INVITRO PROTEIN SYNTHESIS AND DEGRADATION AND CATHEPSIN D ACTIVITY IN THE MUSCLES OF SELENIUM-VITAMIN E DEFICIENT SHEEP

by

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Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Animal Science

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August, 1985
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ACKNOWLEDGMENTS

The author would like to express his appreciation to Dr. F.D. McCarthy for his guidance, assistance and friendship without which this project could not have been completed.

Appreciation is also extended to Dr. S.H. Umberger, Dr. K.E. Webb and Dr. D.R. Notter for serving on the author's graduate committee and providing additional guidance and suggestions over the past two years.

Appreciation is extended to for his technical assistance with various parts of the laboratory analysis and to Dr. Dennis Bloddgett for performing some of the laboratory analysis and his interst in this project.

A special thanks is extended to his fellow graduate students whose advice, help and friendship provided a pleasant and stimulating environment.

Most of all the author expresses the most heart felt appreciation to his parents for their love, support and sacrifice which made this graduate program possible.

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Chapter I

INTRODUCTION

Selenium and Vitamin E responsive diseases have received considerable attention by researchers over the past two decades. Of particular interest has been the muscular atrophy that results when a deficiency of one or both of these compounds exists. Nutritional Muscular Dystrophy has been shown to be very similar to genetic Muscular Dystrophy in both man and animals. The ability to induce an animal to undergo skeletal muscle degradation provides a unique opportunity to study the processes involved in muscle protein degradation. These observations can also be related to normal degradative processes as well as pathological conditions in animals and humans.

Chapter II

REVIEW OF LITERATURE

2.1 THE ROLE OF SELENIUM AND VITAMIN E IN ANIMAL PRODUCTION

Nutritional muscular dystrophy (NMD), or white muscle disease (WMD) of is one disease associated with vitamin E (E) and selenium (Se) deficiencies in lambs and calves. Vitamin E and Se deficiency diseases can occur not only in cattle and sheep but also in swine, poultry, horses and fish. (McMurray and Rice, 1982). Other diseases such as mulberry heart disease, hepatosis dietetica, exudatitive diathesis and encephalomalacia are also observed in poultry and swine (NRC, 1983). Clinical incidence of these diseases can result in substantial economic losses, but just as striking are the overall improvements in live weight gain and general health when animals known to be subclinically deficient in Se are supplemented (Robertson and During, 1961).

Wolf et al.(1963) demonstrated that the incidence of Se responsive diseases was related to geographical location. Other researchers (Kubota et al., 1967) evaluating the Se concentrations in crops throughout the United States found in general that the Pacific Northwest, the Northeast, and the Southeast are areas where crops are low in Se.

The importance of Se and E in the metabolic processes of animals was recognized early by Schwarz and Folta (1957) who demonstrated that liver necrosis in rats could be prevented by supplying Se and E. At approximately the same time, Patterson et al. (1957) and Schwarz et al. (1957) discovered that Se administration could prevent Exudative Diathesis in poultry.

The earliest work relating E to degenerative disease was conducted by Evans and Bishop (1922) who examined the role of E and reproductive failure in rats. Since that time E deficiency has been related to fetal death, kidney degeneration, vascular degeneration resulting in hemorrhage, erythrocyte hemolysis, and liver and testicular necrosis (Mason 1972).

Many of the early researchers recognized that Se responsive disorders could be prevented by the administration of E (Schwarz, 1965; Thompson and Scott, 1969). During this period, the relationship between E and Se was not well understood since several diseases were shown to be responsive to both E and Se but others only responsive to Se or E alone. Thompson and Scott (1970) conducted an experiment in which chickens that had developed myopathy due to sulfur amino acid deficiency responded to E therapy but not to Se therapy. Mahon and Moxon (1971) reported that hepatosis dieteti-

ca in swine could be treated with Se but did not respond well to E administration. Results obtained were different when pigs were fed a diet deficient in Se, E, and the sulfur amino acids responded to both Se and E treatments. Eggert et al. (1957) and Ewan et al. (1969) presented data demonstrating that liver and muscle degeneration in E deficient pigs could be treated by E or Se administration. Similarly, recent reports have demonstrated that nutritional myodegeneration can occur in lambs of normal Se status but deficient levels of E (Maas et al., 1984; Steele et al., 1980).

The synergistic relationship of E and Se has been investigated thoroughly with several investigators suggesting that E functions as an antioxidant (Glavind, 1973; Tappel, 1962). This antioxidant activity of E is thought to protect the polyunsaturated fats of the cell from oxidation. Support for this view is derived from the fact that certain E deficiency diseases can be associated with high levels of dietary fat (Green and Bunyan, 1969). The intake of polyunsaturated fatty acids (PUFA) from pasture has also been shown to be a possible causitive factor in the occurance of NMD in ruminats (McMurray and McEldowney, 1977; McMurray and Rice, 1982). However, researchers had questioned this hypothesis when several E deficient conditions were examined and no evidence of increased lipid peroxidation was observed

(Green et al., 1967; Diplock et al., 1967). Diplock et al. (1971) were the first researchers to link the antioxidant property of E to Se. This group suggested that E acted to protect a class of proteins that contained Se located in oxidation sensitive areas of membranes. These oxidation sensitive areas were shown to contain higher concentrations of PUFA. Dillard and Tappel (1971) demonstrated that as lipid peroxidation increased, the uptake of oxygen increased. The amount of peroxidation and oxygen uptake was found to be dependent upon the amount of PUFA in the tissue. Also, the amount of peroxidation and subsequent oxygen uptake was found to be inversely related to the E content, thus implicating further the role of E as an antioxidant. Other reports have shown lipid peroxidation to be a generally deterorative reaction that occurs under a variety of conditions, (Tappel, 1968; Diluzo et al., 1967; Haugaard, 1968).

The roles of E and Se appear related since they both play a part in protecting the cell membrane fron oxidative damage. Witting and Horwit (1964) demonstrated that the susceptibility of membranes to oxidative damage was positively related to the number of double bonds present in the phospholipid portion of the membrane. Diplock (1981) later confirmed that the PUFA of the membrane phospholipids were the major site of oxidative attack. The greater the number of

double bonds in the membrane lipids, the more susceptable to oxidative damage it appeared. This theory was supported by Horn et al. (1973) who demonstrated that rabbit erythrocytes could be caused to hemolyze when the rabbits were provided with a high level of unsaturated fats in the diet. Mills (1957) first reported that the enzyme glutathione peroxidase could protect membranes from oxidative denaturation. It was later discovered that Se could prevent hemolysis and that this activity was related to the concentration of reduced glutathione (Rotruck et al., 1972). These findings led to the discovery that Se was a main component of the enzyme glutathione peroxidase and it was this enzyme which provided the protection against oxidative damage (Rotruck et al., 1971; Hafeman, 1972). From this information, researchers began to realize Se and E had very separate roles in protecting cells from peroxidative denaturation. Further research also suggested independent roles for Se and E when glutathione peroxidase was found to be associated with the aqueous phase of the cell and E located within the membrane as a lipid soluble component (Noguchi et al., 1973; Krishnanmurthy and Beri, 1962).

Factors that may contribute to peroxidation of membranes could conceivably involve an E or Se deficiency as well as the presence of polyunsaturated fats in the diet. Hoekstra

(1974) proposed a mechanism by which such cell membrane damage could occur. In this mechanism, PUFA undergo peroxidation to hydroperoxides. The hydroperoxides are then degraded with free radicals produced. The free radicals produced from this reaction damage the membranes. Other reasearchers have proposed that oxygen, when metabolized, can give rise to toxic oxygen metabolites such as hydrogen peroxide, superoxide, hydroxyl radicles, and singlet oxygen all of which can elicit damage to cell membranes (Chance et al., 1979; Badwey and Karnovsky, 1983). This sequence of events can be interupted at two steps, one by the removal of the hydroperoxides by glutathione peroxidase and the other by the free-radical-quenching action of E. It is in this way that E and Se are related to membrane protection. This mechanism for chemical pathogenesis was supported by the work of Scott et al. (1974) on exudative diathesis in the chick. The question remains as to what mechanisms initiate this reaction and under what conditions the reacton is allowed to produce free radicals and subsequently damage membranes. Obviously, dietary deficiencies in Se or E contribute to damage via this pathway. Additionly, high levels of PUFA in the diet as suggested by McMurray et al. (1983) could also result in hydroperoxide and free radical production beyond that which could be neutralized by glutathione peroxidase

and E. Several experimental models have been developed in which high levels of PUFA were fed in conjunction with Se and E deficiency with clinical cases of NMD resulting (McMurray and McEldowney 1977; Oh et al., 1976).

2.2 BLOOD AND TISSUE LEVELS OF SELENIUM AND VITAMIN E

Vitamin E consists of two subfamilies, the tocols and the trienols. These differ in the degree of unsaturation of the phytol side chain. These two famalies are further subdivided into alpha, beta, and gamma depending on the amount of methyl substitution of the cromanol ring (McMurray et al., 1982). The d isomer of a-tocopherol is the form found most often in plants and is the form that has the most biological activity in animal tissues. In a study by Hidiroglou and Jenkins (1972) in which tritiated d-l-tocopherol was administered to sheep, the highest concentrations of the vitamin were found in the liver, adrenals, spleen, depot fat and the lungs. The authors felt that the higher concentrations of E in these sites was related to the physiological role of E as an antioxidant. McMurray and Rice (1982) reported on the tocopherol content of swine tissues with liver containing more E than muscle and blood containing less than either liver or muscle.

Various concentrations of E and Se in tissue have been reported over the last twenty years in livestock suffering from Se and Vit E deficiencies. Hidiroglou et al. (1972) reported E values of 8 to 35 mg•100 ml^{-1} of plasma in lambs suffering from NMD. These values were lower than the levels of 64 mg•100 ml^{-1}, (Welch et al., 1960) and 48 $mg^{-}100\ ml^{-1}$ (Whiting et al., 1949) reported earlier. Gabbedy et al. (1977) reported plasma tocopherol levels of 62 mg•100 ml^{-1} in NMD affected sheep. Maas et al. (1984) reported plasma tocopherol concentrations of 81 to 267 mg•dl^{-1} in lambs that had shown clinical signs of NMD, such as lameness, death, and gross or microscopic lesions of the skeletal muscles. Hoelscher (1978) stated that a plasma tocopherol concentration of 20 mg•dl^{-1} would be diagnostic of E deficiency in sheep and cattle while Maas et al. (1977) suggested that further research may show, as is evident from the values presented here, that levels much higher than 20 mg•dl^{-1} would be associated with Vit E deficient diseases. McMurray and Rice (1982) reported values of 60 mg•dl^{-1} in normal pigs. In a study of clinical cases of NMD in lambs conducted by Maas et al. (1977) normal sheep were found to have blood Vit E concentrations of 20 to 38 mg•dl^{-1}. Hidiroglou et al. (1972) reported values of 49 to 59 mg•dl^{-1} in lambs which had not been supplemented with Vit E. At this level

they did see a 50 to 60 % incidence of the disease with deaths from NMD declining significantly at plasma concentrations of 157 to 189 $mg \cdot dl^{-1}$. Values of 45 to 130 $mg \cdot dl^{-1}$ in plasma in calves that had been housed indoors and fed a diet of hay and rolled barley were reported by McMurray and McEldowney (1977). From this discussion it follows that NMD can occur over a wide variety of E concentrations. Of course, the wide range reported can be due to various experimental conditions and analytical techniques but nevertheless one can not disregard the possibilities of other physiological processes influencing the protective role of Vit E thus resulting in a large amount of individual animal variation.

Selenium has also been measured under a wide variety of conditions and in various tissues. The normal concentration of Se in the blood of sheep has been reported to be .08 to .20 ppm with plasma concentrations of less than .04 ppm considered diagnostic of Se deficiency (Whanger et al., 1977; Baxter, 1981). Allen et al., (1974) suggested a value of .05 ppm as the critical value for selenium levels in feedstuffs to prevent Se deficiency. Jenkins et al. (1974) reported values of 12 to 26 ppm in the plasma of calves whose dams received no supplemental E or Se and plasma Se concentrations of 31 to 69 ppm in calves nursing supplemented dams. In the same study blood Se levels ranged from 29 to

42 ppm in lambs from unsupplemented ewes and 33 to 131 ppm in lambs born of Vit E and Se supplemented ewes. Paulson (1968) reported values of .03 ppm in the blood of deficient lambs with supplemented normal lambs having blood concentrations of .18 to .29 ppm.

Se levels in other body tissues have also been examined. Jenkins et al. (1974) in an extensive study of Se and E supplementation in cattle and sheep, reported kidney Se levels of 2.16 ppm to 2.06 ppm in calves born of cows supplemented with Se and E. In the same experiment, calf liver to contained .123 and .153 ppm of Se while the quadriceps muscle was reported as having Se concentrations of .050 and .062 ppm for calves of unsupplemented and supplemented dams respectively. Jenkins et al. (1974) and Paulson et al. (1968) have reported values for lamb tissues with liver Se levels for deficient lambs ranging from .119 to .190 ppm and normal lambs having liver Se concentrations of .153 to .930 ppm. Kidney Se concentrations were reported to range from .08 to 2.41 ppm in deficient lambs and 2.01 to 4.41 ppm for supplemented lambs. Muscle in general was observed to have the lowest concentration of Se with deficient levels ranging from .04 to .110 ppm and normal muscle containing .053 to .220 ppm (Jenkins et al., 1974; Paulson et al., 1968; Hartley, 1967).

2.3 INVOLVEMENT OF THE LYSOMAL SYSTEM IN MUSCLE DEGRADATION

In addition to the involvement of Se and E in the etiology of NMD, lysosomal enzymes also play a role in the degeneration of muscle tissue in animals diagnosed as having a Se-E responsive myopathy. Muscle degradation that is related to muscular dystrophy has long been associated with increased cathepsin activity and autolysis. In one of the earlier studies which examined enzyme activity as it related to muscular dystrophy, Weinstock et al. (1958) reported that cytocrome oxidase and cathepsin activities were significantly increased in the muscle of genetically dystrophic mice. These results were similar to the earlier observations of Weinstock et al. (1955) in which cathepsin activity was significantly increased in Vit E deficient dystrophic rabbits. It follows that since both the genetic and nutritional forms of the disease resulted in increased cathepsin activity, it was suggested that the degradation processes brought on by the two conditions were similar. Tappel et al. (1962) reported large increases in the lysosomal enzymes cathepsin, acid ribonuclease, B-glucoronidase, and arylsulfatase in genetically dystrophic mouse leg muscle. Increases in cathepsin, acid ribonuclease, and B-glucoronidase were also observed in genetically dystrophic chickens in the same study. The authors were some of the first to propose that the

lysomal enzymes were specific for the hydrolysis of muscle components and that they could account for the biochemical changes that occur in muscular dystrophy. Zalkin et al. (1962) also reported increased lysosomal enzyme concentrations in muscles of E deficient rabbits. They observed an 11 fold increase in ribonuclease, 15 fold increase in cathepsin and a 61 fold increase in the activity of B-galactosidase. This group addressed the question of lysosomal enzyme involvement as a function of the amount of time it took for the animals to become dystrophic. This was examined in order to determine if the lysosomal enzymes were arising after tissue distruction had begun and functioning to break down muscle proteins after damage to the muscle had occurred. Since the appearance of the lysosmal enzymes precluded or occurred at the same time as other indicators of the disease, the authors concluded that the lysosmal enzymes are most likely involved in the early stages of the disease and are not acting after muscle damage has occurred. The authors went on to speculate that one of the first consequences of E deficiency may be free radical damage to the the lipoprotein membranes of the cell and its organelles. This provides the inital damage to the cells and this triggers the cell to release lysosomal enzymes into the surrounding tissue. The proteolytic activity of the lysosomal enzymes

then take affect and muscle proteins are degraded. These conclusions were supported by the findings of Zalkin and Tappel (1960) who reported high levels of lipid peroxidation in the tissues of the E deficient rabbit. Similar results were obtained by Carpenter et al., (1959) in their work with E deficient rat and rabbit liver and muscle. They observed increased peroxide formation in vivo and in vitro in E deficient tissues. Pollack and Bird (1968) reported increased amounts of the lysosomal enzymes cathepsin D, arylsulfatase, acid ribonuclease, B-glucuronidase, and acid phosphatase in the atrophying degenerated leg muscles of the rat. These researchers also saw increased lysosomal enzyme concentrations when the tissue was exposed to osmotic stress. This is another situation where the release of lysosmal enzymes can be seen to play a role in tissue degradation after an initital triggering factor, in this case in vitro osmotic shock, caused their release. Other researchers (Neely et al., 1974; Neely et al., 1977) have demonstrated that when the osmotic sensitivity of lysosomes are increased so is the level of proteolysis observed in the tissue. This linked the lysosomal system to the proteolysis of muscle and the authors went on to conclude that the lysosmal system may play a role in the overall protein balance of animals. Support for the involvement of lysosomes in protein balance was

provided by the observation of reduced osmotic shock in lysosomes when agents known to reduce osmotic sensitivity, amino acids and insulin, were administered and decreased proteolysis observed. More direct evidence for the involvement of lysosomes in proteolytic processes was supplied by Ward et al. (1977) when they reported that lysosomes do increase their uptake of cellular protein under proteolytic conditions. The authors thought this increased lysosomal protein content was due to increased activity of the autophagic processes of these organelles and that the proteolytic conditions of low insulin and/or amino acid concentrations could trigger the initiation of this process. From the above discussion one can conclude that lysosomes are indeed involved in proteolytic processes and those conditions that are known to cause proteolysis may cause lysosomes to engulf and degrade proteins.

Wildenthal and Muller (1974) examined the role of the lysosomal enzyme cathepsin D in thyrotoxic rat cardiac tissue. They observed that as the heart underwent hypertrophy due to thyroxine adminstration, cathepsin D activity decreased by 15%. When hormone administration was stopped, the heart began a rapid regression of hypertrophy and a 40% increase in cathepsin D activity was observed. The authors concluded that the reduction in size was due to the proteolytic action

of cathepsin D. This regression in organ size and increase in lysosomal enzyme activity has been observed by other researchers. Wossner (1969) found that the regression in uterine smooth muscles after pregnancy was associated with increased activity of cathepsin D. In another experiment that examined cardiac muscle in perfused rat hearts, cathepsin D activity was found to be higher in hearts tht had been deprived of insulin (Wildenthal, 1973). The authors concluded that the effect of insulin was to increase the total synthesis or specific activity of cathepsin D and this resulted in higher rates of proteolysis. Further evidence of the involvement of lysosomal enzymes in muscle protein degradation has been supplied by Bird et al. (1980) when they demonstrated that cathepsins B and D definitely degrade myofibrillar proteins. Other researchers have demonstrated that cathepsins H and L also degrade muscle proteins and that while B and D only have limited proteolytic activity, L can degrade myosin to small peptides and amino acids (Schwartz and Bird, 1977; Bird et al., 1978). Rannels et al. (1973) measured the effect of insulin on protein turnover in rat hearts. Insulin, as expected, reduced the amount of protein degradation and as the degradation rates decreased, so did the activity of the lysosomal enzymes cathepsin D and B-acetylglucosaminidase. Mortimore and

Schworer (1977) reported increased activity of the lysosomal-vacuolar system in perfused rat livers when they were deprived of amino acids. This could indicate that while insulin may have some direct effect on cathepsin D specific activity or synthesis, other mechanisms may also exist that cause increased lysosomal enzyme when just the effect of insulin deprivation is occurring, i.e. lack of amino acids and not actual insulin deprivation (Wildenthal, 1975).

The lysosomal system has been shown conclusively to be involved in protein degradation of many tissues, one of the most important of these being skeletal muscle. Undoubtedly, any mechanisms which affect the rate of proteolysis of skeletal muscle will have some effect on the net turnover rate of the whole body. Schimke (1970) proposed tht the lysosomal system is probably involved in gross changes in the rates of protein degradation under such conditions as starvation, tissue involution, and muscle disease. Degradation that occurs in normal steady state conditions probably does not involve lysosomal enzymes since the degradation under "normal" conditions requires the selective and specific removal of certain proteins. The lysosomal system does not appear to be that selective but is rather an indiscriminate degrader of cellular proteins. In conditions such as starvation, the massive degradation of protein would be advanta-

geous but under steady state conditions this would not be the case (Schimke and Doyle, 1970; Bird, 1975). Nevertheless, the contribution of the lysosomal system to the protein turnover of the whole body has yet to be elucidated.

2.4 PROTEIN TURNOVER IN RELATION TO MUSCLE DEGRADATION

The above discussion concentrated on the involvement of the lysosomal system and the proteolytic processes that resulted from disease states such as E deficiency or insulin and amino acid depravation. One can not ignore however, the importance of the importance of the role that the lysosomal system plays in the regulation of normal whole body protein turnover.

A review of the basic mechanisms regulating protein synthesis and degradation is appropriate at this point. The equation Ks-Kg=Kd where Ks is the fractional synthesis rate, Kg the fractional degradation rate and Kd the fractional rate of protein deposition is a useful way to describe the relationship of synthesis and degradation to protein deposition (Millward et al., 1976; Scornik and Botbol, 1976). In his review of muscle protein metabolism, Young (1970) noted that skeletal muscle, because of its mass in relation to other body tissues, is the major tissue involved in whole body protein metabolism.

Turn over rates vary depending on the individual muscle or species being examined. Early researchers concluded that muscle proteins turn over at slower rates than those of the liver (Velick, 1956; McManus and Muller, 1966) Waterlow and Stephen (1968) reported that turnover rates can be influenced by sex with higher rates reported for male rats over female rats. In another study, Waterlow and Stephen (1967) found that whole body protein turnover slows with age and that in both young and old animals, rat muscle protein turnover as a percentage of whole body turnover was less than that of liver. The reduction in protein turnover with age is at least in part related to lower DNA populations or the reduced ability of DNA to code for RNA since a reduction in RNA content parallels the decrease in turnover rate with increasing age (Breuer and Florini, 1965; Srivastava and Chaudhary, 1969). A similar relationship exists when one compares the turnover rates of muscle and liver with RNA content (Erdos and Bessada, 1966; Gerber et al., 1960).

Increased turnover rates would require that cells have increased rates of protein synthesis and degradation. In order for protein to be synthesized and degraded, DNA and RNA must be present to code for the production of proteins that are responsable for protein synthesis and degradation. This has been shown to be the case in that the population of

DNA and RNA reflect a tissue's ability to turnover protein. (Munro and Gray, 1969). Goldberg et al. (1974) reported that in vitro rates of protein degradation are regulated by mechanisms which control RNA synthesis and that the overall balance between protein synthesis and degradation is affected by many physiological factors such as nutrient availability and hormones. The authors concluded that these physiological factors probably regulate protein turnover by influencing the cell's degradative mechanisms.

Millward and Waterlow (1978) published a review in which they related the rates of protein synthesis and breakdown to the amount of DNA in muscle. As was reported previously (Waterlow and Stephen, 1968), as rats grew older the fractional synthesis rates decreased. When two strains of rats were compared, one a fast growing strain, and the other a slow growing strain, other differences began to emerge. In the faster growing strain, the amount of tissue that was controlled by one unit of DNA increased as fractional synthesis rate decreased. DNA per unit of tissue decreased in the slow growing strain thus resulting in an overall reduced rate of synthesis (Stewart et al., 1975; Payne et al., 1972). Millward and Waterlow (1978) also reviewed work which related the amount of protein breakdown to the amount of DNA. These and other authors have demonstrated that frac-

tional breakdown rates have been highly correlated with fractional growth rates (Millward et al, 1975). Millward et al. (1975) concluded that protein breakdown is a necessary component of growth. This conclusion was supported by several studies which found both increased rates of protein synthesis and breakdown during rapid muscle growth (Waterlow et al, 1978; Turner and Garlick, 1974; Sola et al, 1973; Sola and Martin, 1968). Millward and Waterlow (1978) suggested that increased rates of degradation accompany higher rates of growth. These same researchers measured synthesis rates in vivo and discovered that rates of protein synthesis were always higher than necessary to achieve the observed rates of growth. From this they concluded that degradation rates had to also be increased. When protein or energy deficient diets were fed, fractional synthesis rates fell but fractional degradation rates did not (Garlick et al., 1975; Millward et al., 1975). One can conclude from these studies that protein degradation appeared to change in a direction that did not enhance either whole body synthesis or degradation but rather increased to facilitate the faster turnover associated with growth (an anabolic state) and slowed during what was considered to be a catabolic state. Thus, the overall net changes in protein deposition or breakdown were believed to be a result of changes in fractional synthesis

rate rather than a change in fractional degradation rate. The rates of protein breakdown are thought to rise only after a prolonged period of stress such as starvation (Millward and Waterlow, 1978).

Muscle protein breakdown is the major event associated with nutritional muscular dystrophy. Kruh et al. (1960) in an experiment which utilized injections of ^{14}C-glycine found that the muscle protein myosin turned over more rapidly in dystrophic as compared to normal mice. Whether this was due to increased rates of synthesis, degradation or both was not addressed by the authors, but examination of the data indicated that both processes were accelerated with an increased rate of amino acid incorporation by myosin and a more rapid decrease in radioactivity in muscles of dystrophic mice when compared to normal mice. Increased rates of protein metabolism in dystrophic mice have also been demonstrated by Simon et al. (1958). They observed faster rates of incorporation and disappearance of ^{14}C-labeled leucine in dystrophic mice.

2.5 HISTOCHEMICAL CHANGES IN NUTRITIONAL MUSCULAR DYSTROPHY

The increases in proteolytic ativity during muscular dystrophy result in ultrastructural changes that characterize this condition. van Breeman (1960) was one of the first to examine dystrophic muscle with the electron microscope. He

described a vacuolation of the sarcoplasmic reticulum as one of the first events to occur. These vacuoles appeared singly or in small groups between the myofibrils. As the severity of the dystrophy progressed the sarcoplasmic reticulum became more vacuolated until it was completely disintegrated. Mitochondria became lobulated and eventually disintegrated. Myofibrils were found in a state of contraction and were presumed to be excessively shortened and apparently nonfunctional. These histopathological observations were correlated with an amount of gross muscle contracture found in the patients at time of biopsy. As the disease progressed, the striations in the muscle fibers became irregular and diffuse with only remnants of the contractile bands being present. Other features were a progressive loss of substance from the interfibrillar spaces with a wide scattering of mitochondria in the central mass of the fibers and a concentration of mitochondria around the periphery of the myofibrils. Although this paper appeared in the early period of electron microscope observation of muscular dystrophy, and not much information was available about what was actually causing these changes, the authors felt that the initial damage to the sarcocplasmic reticulum could be correlated to increased activity of the adenylic acid system.

Sweeny et al. (1972) published a comparative study of lambs and chickens suffering from nutritional muscular dystrophy. The earliest lesions observed by this group involved changes in the vascular bed, connective tissues and neuromuscular junctions. In areas that appeared histologically normal, capillary changes were characterized by swelling of the endothelium, with the capillary endothelial cells being generally enlarged, containing degenerating mitochondria, and sometimes devoid of all classic organelles. Collagen fibrils were difficult to find in the extracellular compartments. In sections that did contain lesions, occluded vessels were observed with endothelial cells showing phagocytotic characteristics. Degenerate mitohondria were seen in these endothelial cells. Along with the early changes in vascular beds, neuromuscular junctions were observed to have withdrawn from the muscle cells and undergone vacuolation rendering them nonfunctional. In muscle cells that had obvious muscle degeneration, no neuromuscular junctions were observed. In addition, degenerate fibers were found to contain a large number of satellite cells.

From their observations these authors felt that the important differences in muscle tissue would be related to factors that alter the differentiation of developing muscle cells. This hypothesis is supported by the fact the nutri-

tional deficiency seemed to more severely affect muscles when they were in a state of growth and differentiation (Cosmos, 1970; Sweeny and Brown, 1972). Sweeny and Brown (1972) also observed that one of the first changes in the cardiac tissue of E and Se deprived piglets was that of fibroblast degeneration and that abmormal fibrillogenesis. The authors concluded that the earliest lesion in muscular dystrophy is an alteration in the differentiation of the connective tissue compartment of the muscle. It is interesting to note here that while muscular dystrophy is considered to be a disease affecting muscular tissue, changes in muscle structure do not appear to be the first events to occur in the onset of the disease. Brown et al. (1967) had previously reported that E deficiency in rats increased the amounts of soluable collagen in the skin and decreased fibril formation and fibril stability. The authors suggested that this may implicate the involvement of E in fibril formation. Vitamin C has long been known to be involved in connective tissue maturation and the observations associated with E deficiency closely resemble the changes observed in scurvy (Friederici et al., 1960; Robertson, 1961).

Al-Tekrity and Telfer (1984) examined the ultrastructural changes in lamb tissue during E and Se depletion and found changes similar to those described previously. Generally,

they observed atrophy of the myofibrils and an increase in the intramyofibril space. This change persisted for a long period of time and appeared long before the animals were considered to be even marginally deficient. As the disease progressed, there was a loss of Z lines and I bands along with complete myofibrillar necrosis. Disintegration of mitochondria was also observed as well as vascular degeneration.

Pellegrino and Franzini (1963) were some of the earliest researchers to observe lysosomes in degenerating muscle fibers of the rat. Up to this point no lysosomes had been detected by electron microscopy in muscle. The lysosomes observed here appeared larger and more heavily loaded with material than those of normal muscle. There was a clear correlation between the appearance of lysosomes and the reduction of muscle cell constituents and it was apparent that the lysosomes were involved in muscle degeneration. Spanier (1977) also observed a higher population of lysosomes in his study of E deficient guinea pig tissue. He went on to describe that the lysosomes observed fell into two categories: 1) those found to be associated with the Golgi apparatus in the perinuclear region of the muscle and 2) those lysosomes found in the interior of the muscle tissue. He also described a structure known as a primary lysosome or "prelyso-

some" that was associated closely with the sarcoplasmic reticulum. In addition to the changes observed with lysosomes, the characteristic Z-line destruction and decrease in the diameter of individual myofibrils was also noted. When muscle tissue was incubated in the presence of purified cathepsin B or D or both, the muscle showed all the chacteristics of of muscle from a dystrophic animal (Z-line spreading, organelle disruption, myofibril thinning, and myosin and actin disappearance). The authors concluded that this was conclusive evidence of the involvement of the lysosomal system in muscle degeneration. They supported this theory by the cytochemical localization of cathepsin B and D in the lysosomes and the increased concentration of these enzymes as the dystrophy progressed.

Chapter III

OBJECTIVES

The objectives of this study were to evaluate the in vitro synthesis and degradation capabilities of Vit E-Selenium deficient lamb muscle and to explore the involvement of the lysosomal enzyme system in muscle degradation.

Chapter IV

MATERIALS AND METHODS

4.1 DESIGN AND STATISTICAL ANALYSIS

Data were obtained on 21 Suffolk x (Dorset x Coopworth) lambs were blocked according to weight and assigned to one of three treatments in a randomized block design.

1. The Se-E deficient basal diet (-Se-E).
2. The Se-E deficient basal diet with a added protected fat supplement (-Se-E+fat).
3. The basal diet supplemented with Se and Vit E (+Se+E).

The data were statistically analyzed using single variable least squares analysis of variance.

4.2 FEED PREPARATION AND HOUSING

4.2.1 Protected Fat Supplement

In an effort to supply polyunsaturated fat (PUFA) to the animals and allow it to escape hydrogenation in the rumen, a protected fat supplement was prepared using the methods of Machlin (1961) and Astrup and Krekling (1979) (table 1).. Linseed oil to comprise 17.6% of the final mixture was heated in a 19 liter Hobart mixer to 35 to 40°C and Lauryl Peroxide to become 2% of the final mixture w/v (Alperox-C, Lu-

cidiol Division, Buffalo, New York) was added. The lauryl peroxide-oil mixture was stirred until dissolved and then heated to 115°C and maintained at that temperature for 20 minutes. At the end of 20 minutes the oil was allowed to cool and .01% w/v Tenox-6 antioxidant (BHA/BHT antioxidant mixture, Eastman Kodak) was added and mixed. Sodium caseinate to make up 17.6% of the final mixture was slowly added to the oil with water, mixing, and heat until a dough-like consistancy was achieved. $CaCl_2$ at .7% w/v , NaOH at .7% w/v and a 40% formaldehyde solution at 1.7% v/v was added and mixed into the doughy mass and mixing was continued with heat for 30 to 40 minutes until formation of granular rubbery particles. This protected oil supplement was then stored at 4°C until fed at a level of $3g \cdot kg^{-1}$ body weight during the first part of the experiment or $6g \cdot kg^{-1}$ of body weight during the latter part of the experiment.

4.2.2 Basal Corn-Hay Diet

A mixture of 85% whole, air dried, corn grain was combined with 15% orchardgrass hay (table 2) in a vertical mixer after being ground through a 2.54 cm screen. The mixed feed was then dumped into a mix wagon where a solution of NaOH to supply 3.5g of $NaOH \cdot kg^{-1}$ of feed was sprayed on the feed (McMurry et al., 1980) and allowed to mix thoroughly.

TABLE 1

COMPOSITION OF PROTECTED FAT SUPPLEMENT

	%
Sodium Caseinate	17.60
Linseed Oil	17.60
CaCL	.70
$CaOH^2$	.70
40% Formalin	1.70
Texox-6	.01
Lauryl Peroxide	2.00
H_2O	58.99
TOTAL	100.00

The feed was transferred to a grain bin where air was forced through the mixture for 48 hours to facilitate drying. As soon as feed was dry enough to prevent spoilage the feed was bagged and stored until use.

4.2.3 Vitamin-Mineral Supplement

A vitamin-mineral premix was prepared using the relative amounts depicted in table 3. All treatment groups received this mixture as .5% of the total diet with the +Se+E treatment group being further supplemented to recieve .2 ppm Se and 330 $IU \cdot kg^{-1}$ of feed of E.

4.3 FEEDING AND MANAGEMENT

Prior to the start of the experiment the lambs were fed the experimental basal diet for 30 d during which time the lambs were ear tagged and wormed. During the trial feeding was done twice daily with the mineral and fat supplements being added to the feed during the PM feeding. Water was provided ad libitum. Initially, all three treatment groups were fed .6 kg of $feed \cdot pen^{-1} \cdot day^{-1}$. After the start of the trial the amount fed was adjusted to match the consumption of the lambs. The -Se-E treatment consumed .3 $kg \cdot pen^{-1} \cdot day^{-1}$, and the lambs on the -Se-E+fat and +Se+E treatments recieved .5 $kg \cdot pen^{-1} \cdot day^{-1}$. The lambs were weighed every 14 days.

TABLE 2

COMPOSITION OF BASAL DIET

Corn Grain (IFN#: 4-02-935)	84.25%
Orchardgrass Hay (IFN#: 1-03-438)	14.25%
Mineral Mix	.5%

Composition:

DM (%)	CP (%)	CF (%)	Ca (%)	P (%)
88.5	8.5	5.8	.25	.17

TABLE 3

COMPOSITION OF VITAMIN-MINERAL PREMIX

		kg	%
Macro:	$KHCO_3$	23.74	20.66
	$NaCl$	9.10	7.92
	$MgSO_4 \cdot 7H_2O$	21.36	18.58
	$CaHP_4 \cdot 5H_2O$	51.03	44.40
	$CaCO_3$	8.45	7.35
			%
Micro:	$FeSO_4 \cdot 7H_2O$	.469	0.40
	$CuSO_4 5H_2O$	.090	0.08
	$ZnSO_4 \cdot 7H_2O$	.280	0.24
	$MnSO_4 \cdot 4H_2O$	.397	0.35
	$CoCl_2 \cdot 6H_2O$	.010	0.01
	KIO_3	.004	0.01
	TOTAL	114.93	100.00

Vit A 21,112 IU|kg of Mineral Mixture
Vit D 2,111 IU|kg of Mineral Mixture
For a portion of this mixture Se was added to provide .2 ppm in the feed and E was added to 330 IU|kg of feed.

4.4 HOUSING

During the trial lambs were housed in three seperate 2.4 x 2.4 m pens on expanded metal floors for approximately 110 days under continual light. The pens were located in the large animal quarters of the animal science building with the tempreature of the room maintained at 18 to 20°C.

4.5 SAMPLING

During the course of the trial blood was obtained weekly via veinipuncture. Blood was also collected the day of slaughter. Lambs were slaughtered in groups of three that were comprised of the original weight blocks. The semitendinosus and triceps brachi muscles of the lambs were exposed immediately after exsanguination and muscle clamps (one inch alligator clamps) were put in place to provide continual tension on a portion of the muscle and to allow sampling for in vitro synthesis and degradation. Within 3 to 5 min after slaughter the clamped muscle subsample was taken and the remainder of the muscle removed along with the heart, kidney, and a portion of the liver. The tissues were then weighed, frozen in liquid nitrogen and stored at -80°C until time of assay.

4.6 IN VITRO SYNTHESIS AND DEGRADATION

The in vitro synthesis and degradation capabilities of the two skeletal muscles were estimated by the methods of Hentges et al. (1983). As described previously in the sampling section, the muscles were exposed and 250 to 350 mg samples were removed with the use of two alligator type clamps connected by a stiff wire to maintain tension on the samples and keep them in their natural state of contraction for the duration of the incubation. Upon removal, the muscle samples were immediately placed in preincubation media which consisted of oxygenated, cold Krebs Ringer Buffer (KRB), pH 7.4, containing five times the highest levels of amino acids found in sheep plasma (Bergen, 1979; Wolfrom and Asplund, 1979; Moore et al., 1980; Young et al., 1981). After the muscle samples were removed and preincubated at 37°C in KRB for 30 min they were placed in to their respective incubation media and kept at 37°C for 2.5 h for synthesis estimation and 3 h for degradation and inulin estimation. In addition to the amino acids, the synthesis incubation media contained .3uCi•ml^{-1} of ^{14}C-tyrosine and 2 mg of tyrosine•100 ml^{-1} of KRB. Degradation incubation media contained .5 mM cyclohexamide to block synthesis and inulin incubation media contained .03 uCi•ml^{-1} of ^{14}C-Inulin. The preincubation and incubation media for synthesis, degra-

dation and inulin estimates was supplemented with .3 $mg \cdot liter^{-1}$ of choramphenicol, 1.0 mM glucose, and .5 $IU \cdot ml^{-1}$ of insulin. Muscle samples used as blank values were homogenized immediately after the preincubation period and prepared for assay.

Upon completion of incubation, all of the muscle samples were removed from their incubation vials, weighed and placed in a test tube containing 2.0 ml of phosphate buffer and .5 ml cold 50% TCA and homogenized using a Tekmar Tissuemizer (Tekmar Products, Cincinnati, Ohio) in two or three 5 to 10 sec bursts at high speed. The tip of the homogenizer was washed with 1.0 ml of 10% TCA with the washings being allowed to run into the tube with the homogenized tissue. For all samples, 2.0 ml of the incubation media was added to .5 ml of cold 50% TCA, vortexed and centrifuged at 2300 x g, 4°C for 15 min. The resulting supernates from both the tissue and media samples were decanted, with the pellets from the tissue samples being washed twice with the 1.0 ml volumes of cold 5% TCA. The supernates from the two washes were combined with the original tissue supernates and the pellet resuspended in 1 ml of ether and centrifuged. The supernate was discarded and the pellet was allowed to dry at room temperature for 12 to 18 h. The pellets and supernates from both the tissue samples and the media samples were frozen and stored at -80° until time of assay.

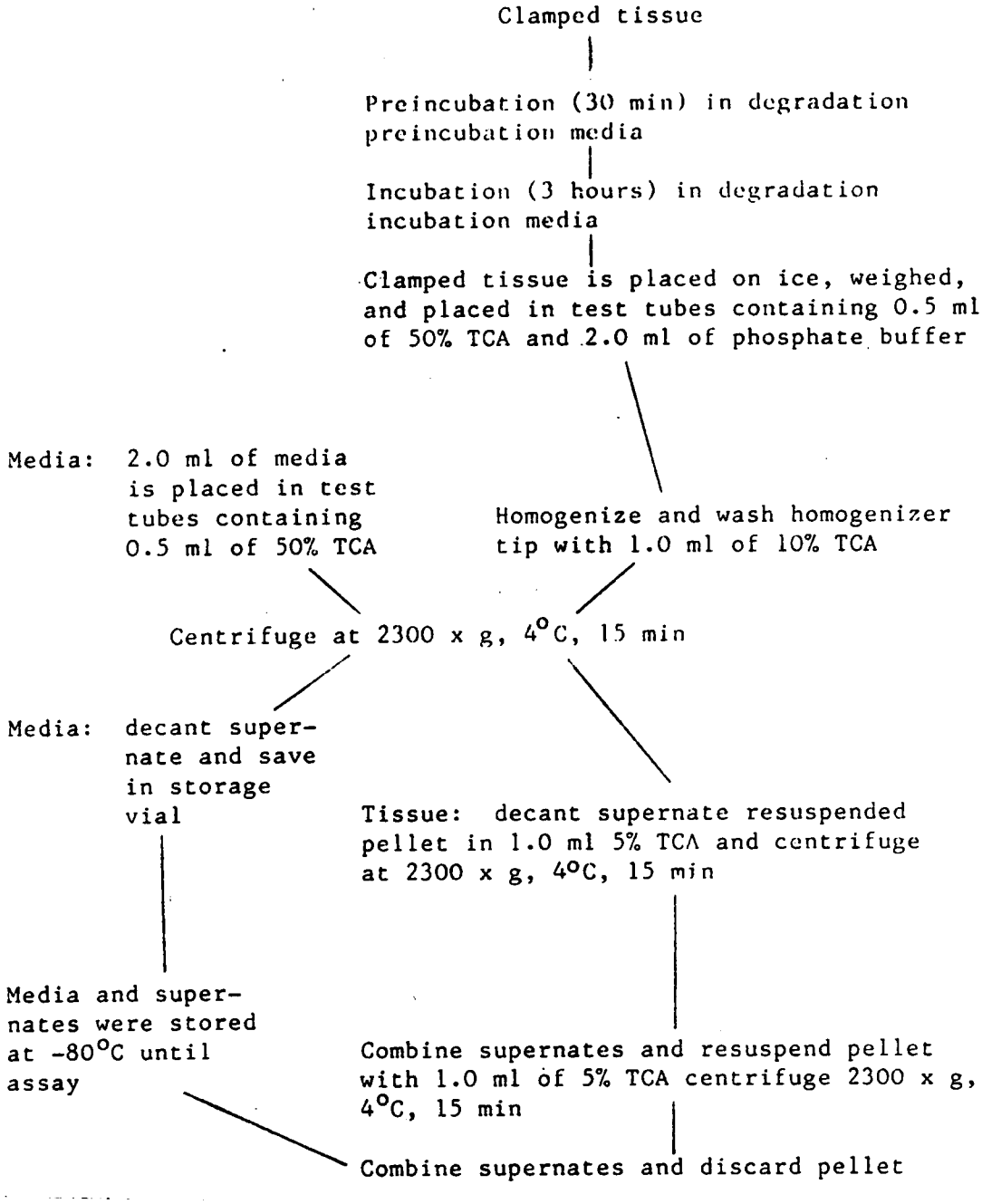

Figure 1: SCHEMATIC OF DEGRADATION PROCEDURE

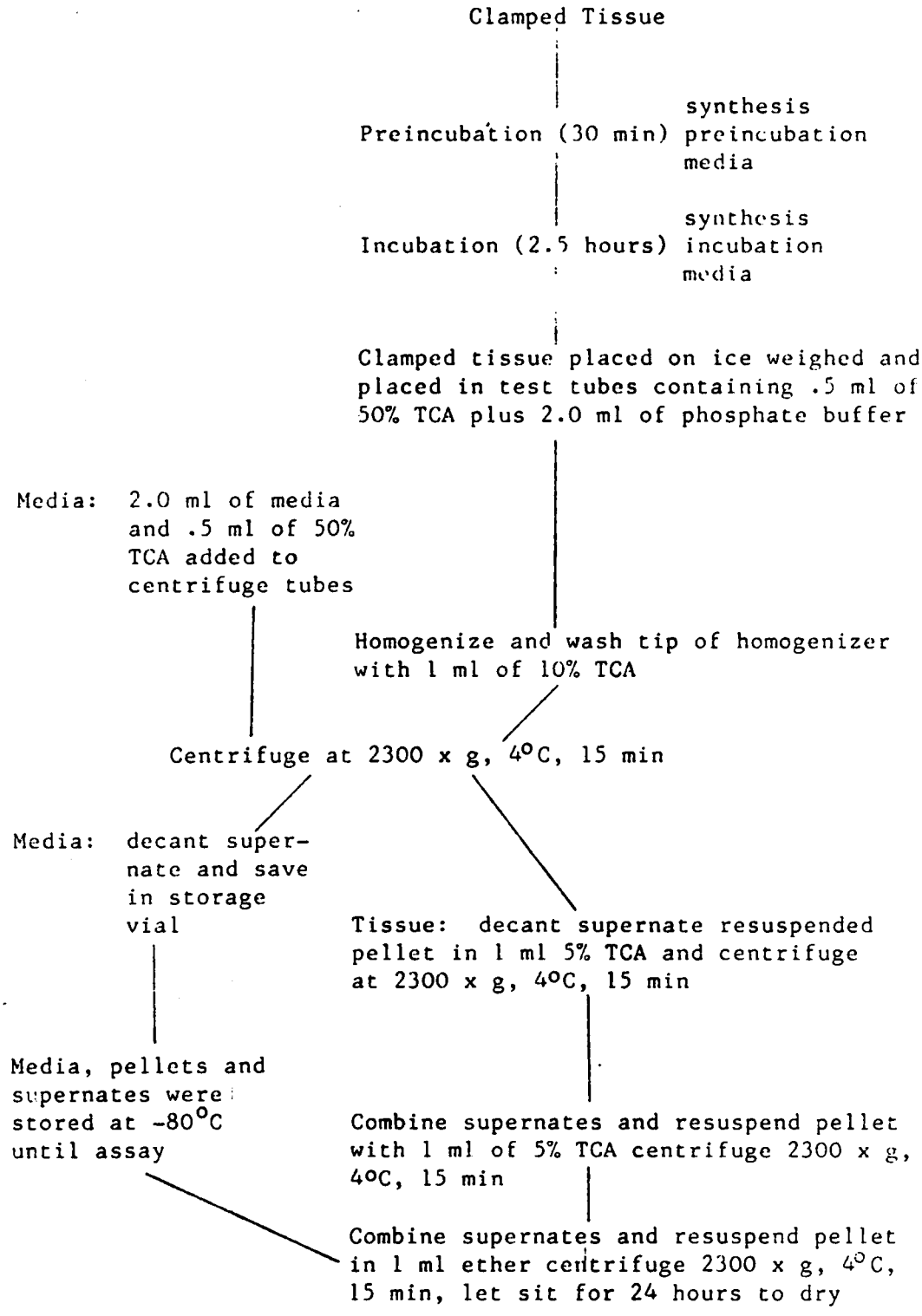

Figure 2: SCHEMATIC OF INULIN AND SYNTHESIS PROCEDURE

4.7 ESTIMATION OF SYNTHESIS RATE AND INULIN SPACE

Muscle protein synthesis was estimated by measuring the incorporation of ^{14}C-tyrosine into muscle protein and interstitial space was estimated by incubating muscle in the presence of ^{14}C-inulin. The synthesis and inulin pellets were prepared for liquid scintillation counting by digestion in 1 ml of Solulene 100 tissue solubilizer (Packard Instrument Co.) for 12 h at 50°C. One ml aliquots of solublized tissue was counted in 10 ml of scintillation solution comprised of tolulene containing 4 g of a 98:2 PPO•bis-MSB mixture•liter^{-1}. Before counting, .15 ml of water and .05 ml of glacial acetic acid were added to each scintillation vial and mixed with the scintillation fluid. For the acid soluble supernates from the synthesis and inulin samples, 1.0 ml of the supernate was combined with 10 ml of scintillation fluid that contained toluene-trition X-100 (2:1) mixed with 6 g of a 98:2 PPO•bis-MSB mixture•liter^{-1}. The samples were counted in a Beckman LS 7500 Scintillation Counter with quench corrections made by H-number.

Intracellular specific activity was calculated by subtracting the amount of tyrosine in the inulin space from the ^{14}C-tyrosine detected in the synthesis samples and dividing by the total tyrosine in the muscle pools minus the amount of tyrosine in the inulin space. Inulin space was calculat-

ed by adding the inulin detected in the tissue to the inulin in the supernates and combined washes and dividing that figure by the amount of inulin $\cdot ml^{-1}$ of media after incubation. Inulin space was found to be .242 $ul \cdot mg^{-1}$ of tissue. Synthesis was calculated by dividing the activity of the incubated tissue by the intracellular specific activity. Mathematical formulas for the calculation of synthesis rates and inulin space may be seen in the appendix.

4.8 ESTIMATION OF DEGRADATION

The degradation of muscle protein was estimated by measuring the release of tyrosine from the muscle tissue into the incubation media. Tyrosine was assayed for by the method of Ambrose (1974) with the following reagents being used.

1. 2.5 M nitric acid: 156 ml of 16 M nitric acid was diluted to 1 liter with H_2O that had been filtered through a .45 um millipore filter. Storage was at room temperature in a glass container.
2. Phosphoric acid reagent: 533 ml of 15 M phosphoric acid were diluted to 1 liter with millipore-filtered H_2O to result in an 8 M solution. 100 g of $Na_2P_2O_7$ $10 \cdot H_2O$ (sodium pyrophsophate) was dissolved in 500 ml of the 8 M phosphoric acid solution and diluted to 1 liter with millipore- filtered water. The solution

was then filtered once more with .45 um filters and stored at room tempreature in a glass container.

3. Ethanol: Absolute ethanol was mixed with water to contain 5 ml of water per deciliter and filtered through .45 um millipore filters and stored at room temperature in a glass bottle.

4. 1-Nitroso-2-Napthol-Sodium Nitrate Reagent: This reagent was prepared immediately before use. 1-nitroso-2-napthol (50 mg) and 345 mg of $NaNO_2$ were placed in a 50 ml beaker and 40 ml of 50 mM NaOH were added. The mixture was stirred with a glass rod until dissolved and transferred quantitatively to a 100 ml volumetric flask. The glass rod and beaker were washed several times with 50 mM NaOH with the washings added to the volumetric. The volume was adjusted to 100 ml with 50 mM NaOH. The solution was then shaken vigorously and filtered through a .45 um millipore filter and stored in the dark in a glass container covered with aluminum foil.

5. Nitric Acid Reagent: This reagent was prepared in glass immediately before use. One volume of nitric acid (2.5 M) was added to two volumes of the nitroso-naphthol-sodium nitrite reagent. The mixture was stirred with a glass rod, covered, and allowed to

stand for 20 min. After 20 min, 1.5 volumes of the phosphoric acid reagent were added and the mixture stirred again with a glass rod.

6. Trichloroacetic Acid (.6 M): 98.10 g of TCA was diluted in 1 liter of H_2O to make a .6 M solution. The solution was then filtered and stored at 4°C in glass.

7. Trichloroacetic Acid (60 mM): 100 ml of .6 M TCA were transferred to a 1 liter volumetric flask and diluted to volume with H_2O. This was then filtered with .45 um millipore filters and stored at 4°C in glass.

8. Primary stock standard and working standard: A 50 $mg \cdot dl^{-1}$ stock solution of L-tyrosine was prepared by placing 50 mg of L-tyrosine in a 50 ml beaker and adding 10 ml of filtered .6 M TCA plus 20 ml of filtered water. The tyrosine was then dissolved by stirring with a glass rod and transferred quantitatively to a 100 ml volumetric flask. The beaker and glass rod were rinsed several times with millipore-filtered water with the washings added to the volumetric flask. The solution was then diluted to 100 ml with millipore-filtered water. This solution was stored at 4°C in the glass volumetric flask for up to

30 d. A 1 mg/dl working standard was prepared fresh each week by transferring 1 ml of the stock standard to a 50 ml volumetric flask and diluting to volume with .6 M TCA. This solution was stored at 4 C in the glass volumetric flask.

4.8.1 Tyrosine Assay Procedure

Reagent blanks were prepared by placing .5 ml of .06 M TCA into clean screw cap culture tubes in triplicate. A .5 ml aliquot of sample was also pippetted into screw cap culture tubes with a standard curve being constructed by putting .1 ml to .5 ml of L-tyrosine working standard into the tubes and adjusting the volume to .5 ml with .06 M TCA. After the blanks, standard curve, and samples were dispensed into culture tubes, 1.0 ml of the nitric acid reagent was added, the tubes capped, vortexed and then heated in an 85°C water bath for exactly 6 min then cooled in a 33°C water bath for 10 min. After cooling, the tubes were removed from the water bath and 5 ml of ethanol added to each tube. The tubes were then inverted to mix the contents and returned to the 33°C water bath for 30 min. At the completion of incubation the samples were removed from the water bath and read in a Turner Model 111 Fluorometer equipped with a constant tempreature door at 33 C. (G. K. Turner Associates, Palo

Alto, Ca.) The fluorometer setting was a IIX aperture with a blue lamp, T-5 envelope (Turner No. 110-853) and a primary filter combination of 2A + 47B to result in a 436 nm activation wavelength. A No. 16 secondary filter (>535 nm emission wavelength) in combination with a 10% neutral density filter was used on the secondary side. Before reading, all cuvets were wiped with laboratory tissue to remove any lint or dust particles that could contribute flourescence. The fluorometer was zeroed with a black dummy cuvet. The readings were then taken with the average blank value being subtracted from each standard and sample reading. Degradation was calculated by adding the tyrosine content of the acid soluble homogenate to the tyrosine content of the acid soluble media (nmoles/mg muscle) and subtracting from that the tyrosine content of the acid soluble homogenate of the blank.

4.9 CATHEPSIN D DETERMINATION

The following solutions were used in the assay of cathepsin D.

1. 2.5% hemoglobin solution: A 5.0% (w/v) solution of acid denatured bovine hemoglobin (Sigma Chemical Company) was dialyzed against double distilled H_2O for 48 hours and diluted to 2.5% with doubled distilled

H_2O. Just prior to use, 25.0 ml of acetate buffer were added for each 100 ml of 2.5% hemoglobin.

2. 1.35 M acetate buffer, pH 3.8.
3. 5% (w/v) Trichloroacetic acid
4. 0.5 N NaOH
5. 0.667 N Folin-Ciocalteau Phenol reagent.

4.9.1 Preparation of Muscle Samples

Frozen tissue samples (stored at -80°C as described in the vitro synthesis and degradation section) were ground to a powder with the use of a Bel-art tissue mill (Bel-art Products, Pequannock, NJ) at -20°C with the addition of dry ice to the grinding head to insure against heat resulting from the friction of grinding. The samples were then returned to -80°C storage until homogenization.

4.9.2 Homogenization

Tissues were prepared for fractionation using the method of Spanier (1977). Homogenization media was prepared to contain the following.

1. 0.25 M sucrose
2. 0.02 M KCl
3. 10.00 mM Tris-HCl

The pH was adjusted to 7.2 with .5 N NaOH and stored at 4°C until use.

A 2.65 g sample of powdered muscle was added to 20 ml of cold homogenization media, homogenized throughly with a Tekmar Tissuemizer, filtered through four layers of cheesecloth and placed on ice. The homogenized tissue was then further homogenized by two strokes of a Wheaton Potter Elvehjem teflon homogenizer in the cold. The tissue was returned to the ice, the pH adjusted to 7.2 with .05 N NaOH and, the volume adjusted to 35 ml with homogenization media to yield a 7.5% tissue homogenate.

4.9.3 Fractionation

The lysosome rich fraction was isolated by the method of Spanier (1977). Thirty ml of total 7.5% tissue homogenate was centrifuged at 2000 x g for 10 minutes, 4°C. The supernate was decanted, saved, and kept on ice. The pellet was resuspended with homogenization media to one half the original volume and centrifuged at 1200 x g for 10 min at 4°C. The resulting supernate was combined with the supernate from the first centrifugation and the pellet discarded. A 30 ml aliquot of the combined supernate was put into a clean centrifuge tube and centrifuged at 23,000 x g for 15 min at 4°C. The resulting supernate was discarded and the pellet

resuspended with homogenization media to one half original volume and centrifuged again at 23,000 x g for 15 min at 4°C. The supernate was discarded and the pellet was washed as in the previous step. The resulting pellet is the lysosome rich fraction.

4.9.4 Assay Procedure for Cathepsin D

The pellet obtained in the fractionation procedure was resuspended to 2.0 ml with cold homogenization media and placed on ice until assay. A 1.0 ml aliquot of the hemoglobin-acetate buffer solution was pipeted into individual test tubes and incubated for 4.0 min at 37°C. A .5 ml aliquot of the tissue fraction was dispensed into the tubes containing the hemoglobin-acetate buffer substrate, vortexed, covered, and incubated at 37°C for 5 h. To stop the reaction, 3.0 ml of the 5% TCA solution was added to each tube. The samples were then filtered with number 42 Whatman paper. A 1.0 ml aliquot of the filtrate was combined with 2.0 ml of .5 N NaOH in a clean tube and vortexed. With timing 0.6 ml of the 0.667 N phenol reagent was added and vortexed vigorously. The samples were then read exactly 5 minutes after the addition of the phenol reagent at 660 nm on a Perkin-Elmer Lamda 75 Spectrophotometer. Blanks were prepared by incubation of the hemoglobin substrate without enzyme (sample)

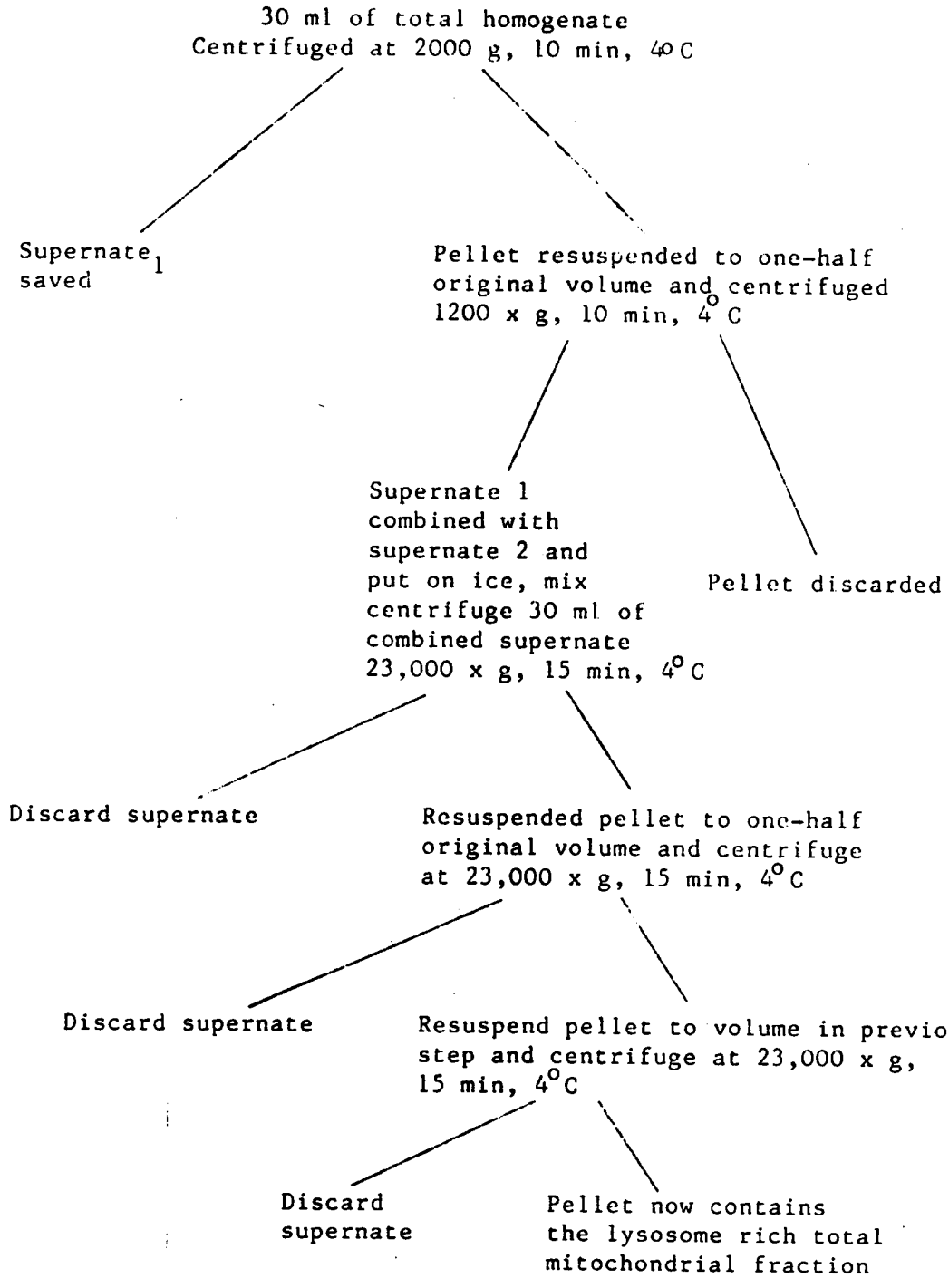

Figure 3: ISOLATION OF THE LYSOSOME RICH FRACTION OF MUSCLE TISSUE

followed by addition of the enzyme to the TCA precipitated substrate just before filtration.

Chapter V

JOURNAL ARTICLE

In Vitro Protein Synthesis and Degradation and Cathepsin D Activity in the Muscle Tissue of Selenium and Vitamin E-Deficient Lambs

5.1 SUMMARY

The lysosomal enzyme cathepsin D and the in vitro synthesis and degradation capabilities of lamb muscle were evaluated in 21 Selenium-Vit E (Se-E) deficient lambs. Suffolk x (Dorset-Coopworth) lambs were blocked by weight and assigned to one of three treatments: 1)a Se-Vit E deficient diet (-Se-E), 2) a Se-E deficient diet plus a mixture of sodium caseinate and linseed oil to supply a high level of polyunsaturated fats to the lambs (-Se-E+fat),and 3)a Se-E supplemented diet (+Se+E). All lambs received a basal diet of 85% shelled corn and 15% hay which had been treated with 3.5 g NaOH/kg of feed. Liver, heart, kidney, and skeletal tissues were analyzed for selenium content and differences ($P<.05$) were observed for all tissues when the two deficient diets were compared to the supplemented diet. Blood Se and E levels were also significantly different ($P<.05$) for the lambs

fed the deficient diets as compared to the controls. Means revealed that the protected fat supplemented diet resulted in lambs with higher blood Se levels and lower blood E levels than lambs fed the treated basal diet only. The Se-E supplemented lambs were heavier at slaughter and had higher semitendinosus, triceps brachi and cardiac muscle weights than either of the two unsupplemented treatments ($P<.05$). In vitro degradation rates were highest for the -Se-E treatment and lowest for the -Se-E+fat treatment ($P<.05$). In vitro synthesis estimates revealed that skeletal muscles from lambs fed unsupplemented diets tended to have higher rates of synthesis than muscles of Se-E supplemented lambs.

Cathepsin D activity tended to be higher in the -Se-E treatment than in the other two treatments. Cardiac cathepsin D activity was higher than either the triceps brachi or the semitendinosus muscles ($p<.05$). The observations made in this study indicate that a higher rate of turnover may be occurring in Se-E deficient muscle and that cathepsin D may be partly responsible for the observed increase in proteolysis.

5.2 INTRODUCTION

Selenium (Se) and vitamin E (E) responsive diseases have long been recognized in animal production. Nutritional muscular dystrophy, also known as white muscle disease in cattle and sheep, is one such disease. While the addition of Se and E to the diets of animals has enabled producers to effectively prevent and treat deficiency conditions, the losses resulting from E and/or Se deficiency can be very subtle. Decreased rates of gain, poor reproductive performance, and depressed levels of overall health, have been observed in animals that are only marginally deficient and not exhibiting clinical signs of white muscle disease (Robertson and During, 1961).

Se-E deficiency is a condition that can be induced in the laboratory and provides researchers with a unique opportunity to study the mechanisms of muscle protein breakdown and synthesis under catabolic conditions. In theory, increased rates of muscle protein turnover should result from increased rates of synthesis and degradation (Millward et al., 1976; Scornick and Botbol, 1976). In addition, the relationship of synthesis rates and degradation rates as they affect net protein deposition have come under extensive investigation over recent years with no definitive conclusions (Millward and Waterlow, 1978; Goldberg, 1974).

The lysosomal enzymes are involved in the degeneration of muscle and are thought to be one of the main factors involved in nutritionally induced muscular dystrophy (Weinstock et al., 1955; Tappel et al., 1962). The role of the lysosomal enzymes in the overall net protein metabolism of the animal has yet to be elucidated but it could be of considerable importance when one considers the amount of protein represented by the muscles of the animal (Young 1970).

The use of nutritionally induced muscular dystrophy to study muscle metabolism may prove to be a useful tool in that it can provide insights into the etiology of general degenerative muscle disease, and at the same time also provide a unique opportunity to relate how the degradative and synthetic processes of muscle tissue contribute to the net deposition of muscle protein in the meat animal. The following study was undertaken to investigate the in vitro protein synthesis and degradation capabilities of dystrophic and healthy lamb muscle using Se-E deficiency as a model. In addition, the role of the lysosomal enzyme cathepsin D as it relates to muscle protein degradation was also investigated.

5.3 MATERIALS AND METHODS

Twenty one lambs of Suffolk x (Dorset-Coopworth) breeding were blocked by weight and assigined to three treatments. Lambs were housed on expanded metal floors for approximately 110 days during which they received a basal diet of 85% shelled corn and 15% chopped hay. In order to lower the E content of the feed, the basal diet was treated with 3.5g $NaOH \cdot kg^{-1}$ (McMurry et. al., 1983). A high level of polyunsaturated fats (PUFA) was included one of the treatment groups, via a protected fat supplement prepared by stripping linseed oil of its E content with lauryl peroxide by the method of Machlin (1961) and then protectiing the oil from hydrogenation in the rumen by encapsulating it in sodium caseinate by the method of Astrup and Krekling (1979). One of three different supplements (treatments) were added to the basal diet: 1) a Se-E deficient mineral supplement, 2) the same mineral supplement that was fed in group 1 with 3g•kg body $weight^{-1} \cdot day^{-1}$ of casein encapsulated linseed oil (protected fat) added to the diet, and 3) a Se-E supplemented mineral mixture. Water was provided ad libitum. Throughout the trial the lambs were fed their respective supplemented basal diets at maintainance levels or ad libitum when intake was not up to maintaince (NRC, 1968).

Upon completion of the trial, the lambs were killed by exsangunation and the triceps brachi and semitendinosus muscles exposed. Muscle subsamples (150-350 mg) for in vitro protein synthesis and degradation estimates were taken using two 2.54 cm alligator clamps connected by a stiff wire to maintain the muscle samples in their natural state of contraction throughout the incubation period. As soon as the subsamples were taken, the remainder of the of the triceps brachi and the semitendinosus were removed as was the heart, kidney and a portion of the liver. The samples were weighed, frozen in liquid nitrogen and stored at -80 °C until analysis.

In vitro muscle synthesis and degradation rates were estimated using modifications of the methods described by Hentges et al. (1983) and Fulks et al. (1975) in which synthesis was estimated by measuring the incorporation of ^{14}C-tyrosine in the muscle and degradation was estimated by measuring the release of tyrosine into the incubation medium. Interstitial space was estimated by incubating muscle tissue in the presence of ^{14}C-inulin (Hentges et al.,1983; and Fulks et al., 1975).

Immediately after the muscle clamps were applied to the muscle the muscle subsamples (250 to 350 mg) were preincubated for 30 min in oxygenated cold Krebs Ringer buffer

(KRB), pH 7.4, to contain 5 times the levels of amino acids found in sheep blood (Bergen, 1979; Wolfrom and Asplund, 1979; Moore et al., 1980; Young et al., 1981). At the completion of preincubation, the muscle samples were placed in their individual incubation media for a period of 2.5 h for the estimation of synthesis and 3.0 h for the estimation of degradation and interstitial space. Synthesis incubation media consisted of oxgenated amino acid supplemented KRB plus .3 $uCi \cdot ml^{-1}$ of ^{14}C-tyrosine. Degradation incubation media contained .5 mM cyclohexamide in oxygenated, amino acid supplemented KRB. Inulin incubation media contained .03 $uCi \cdot ml^{-1}$ of ^{14}C-Inulin in oxygenated, amino acid supplemented KRB. All preincubation and incubation media were supplemented with .3 $mg \cdot l^{-1}$ of choramphenicol, .1 mM l-tyrosine, 1.0 mM glucose, and .5 $IU \cdot ml^{-1}$ of insulin. Muscle samples taken to be used as blanks were homogenized immediately after the preincubation period and prepared for assay.

Intracellular specific activity was calculated by subtracting the amount of tyrosine in the inulin space from the ^{14}C-tyrosine detected in the synthesis samples and dividing that value by the total amount of tyrosine in the muscle pools minus the tyrosine in the inulin space. Interstital space was calculated by adding the amount of inulin detected in the tissue to the amount of inulin in the combined super-

nates and dividing that figure by the amount of inulin/ml of incubation media after incubation. Synthesis was calculated by dividing the total activity of the incubated tissue by the intracellular specific activity.

For degradation estimates, tyrosine was determined fluorometrically using a Turner 111 Fluorometer by the method of Ambrose (1974). Degradation was calculated by adding the tyrosine content of the acid soluble homogenate to the tyrosine content of the acid soluble media (nmoles•mg^{-1} muscle) and subtracting the tyrosine content of the acid soluble homogenate of the blank.

Cathepsin D activity was assayed for by the method of Barrett (1977) in the lysosomal-rich fraction of the muscle as isolated by the method of Spainer (1977). Using this method, the proteolytic activity of Cathepsin D is estimated by measuring the concentration of peptides released after the enzyme has been incubated with a hemaglobin substrate. Peptide concentration was determined by the Folin-Lowery reaction (Lowery, 1951).

Vit E levels were determined fluorometrically (Storer, 1974) and Se was assayed using gas chromotography by the method of McCarthy (1971).

5.4 RESULTS AND DISSCUSSION

5.4.1 Blood Selenium and Vitamin E Levels

Blood selenium levels, reported in Table 4, were not different between treatment groups at the start of the feeding period (initial whole blood Se), but were different at the end of the trial ($p<.05$). The initial Se levels for this experiment coencided with the lower end of the range of values reported by Maas et al. (1984) of 41 to 89 ppb for normal lambs. Jenkins et al. (1974) reported a wider range of values, 33 to 151 ppb in normal lambs, with Lunde and Odegaard (1972) reporting values of 70 to 150 ppb for normal lambs. Paulson et al. (1968) however, reported slightly higher values in Se supplemented lambs with blood levels ranging from 180 to 290 ppb. Therefore, according to the values reported previously, the lambs used in this experiment could be considered to be marginally deficient at the beginning of the trial.

Blood Se levels at termination of the trial (slaughter whole blood Se) for the two deficient treatments were 47.3 ppb for -Se-E treatment and 77.0 ppb for the -Se-E+fat treatment, slightly higher than at the beginning of the trial. These values were lower than those measured in the supplemented control lambs (231.5 ppb) ($p<.05$). The control lambs had values falling into what is considered the normal

TABLE 4

BLOOD SELENIUM AND VITAMIN E LEVELS

	Diets					
	-Se-E	S.E.	-Se-E+Fat	S.E.	+Se+E	S.E.
Initial whole blood Se (ppb)	45.7	7.9	68.4	9.0	52.5	7.9
Slaughter whole blood Se (ppb)	49.3[a]	16.0	77.0[a]	18.3	231.5[b]	16.0
Slaughter plasma vitamin E (ppm)	2.0[a]	.2	1.6[a]	.2	6.3[b]	.2

[a,b]Means within a row with different subscripts differ ($P<.05$).

range for supplemented lambs (Lunde and Odegaard, 1972; Jenkins et al., 1974; Paulson, 1968) with the other two treatments having values that coencided with the levels of 29-250 ppb observed in deficient lambs (Jenkins et al., 1974; Lunde and Odegaard 1972; Paulson et al., 1968). It is evident from the values previously reported in the literature that a certain amount of overlap exists in the literature when one compares the the blood Se levels of normal lambs to deficient lambs. Although not statistically different, the mean Se concentration was higher for the fat supplemented group than for the -Se-E group possibily due to the fact that the fat supplement did contribute some Se to the diet. This difference could also be a reflection of the slight difference in blood Se levels observed at the beginning of the trial. The Se levels obtained in this experiment could only be considered borderline deficient since no clinical signs of NMD were observed, but the values were close to the values considered to be diagnostic of Se deficiency by Whanger et al., (1977) and Baxter (1981) of 40 ppb. Allen et al. (1974) suggested a slightly higher value of 50 ppb to be diagnostic of Se deficiency.

Plasma levels of Vit E at the end of the trial were significantly different only for the supplemented group which had a concentration of 6.32 ppm ($p<.05$). The -Se-Vit E

treatment lambs had mean a-tocopherol blood levels of 2.0 ppm with the -Se-Vit+fat group having a slightly lower concentration at 1.6 ppm. The non-supplemented groups had E values considered deficient by Maas et al. (1984) who reported values of 2 ppm as diagnostic of E deficiency. In lambs that have been diagnosed as having nutritional muscular dystrophy, E values of .81 to 2.67 ppm were reported (Culica, 1951; Hidiroglou et al., 1972; Whanger et al., 1977). Maas et al. (1984) reported values of 2 to 3.6 ppm in sheep that were considered normal. In a study by Hidiroglou et al. (1972) values of 1.89 ppm to 7.49 ppm were reported for normal lambs that had been supplemented with Vit E. Therefore, as with the blood Se levels, there is some overlap in the literature for E levels when on compares normal lambs to dystrophic lambs. Although not statistically different, the lower concentrations for the -Se-E+fat group could be explained in part by the inclusion of the fat supplement. The higher concentration of polyunsaturated fat in the diet could cause increased oxidation of E by increasing activity of the mechanisms which protect the cell from peroxidative damage (Dam, 1962; Hoekstra, 1974; McMurry et al., 1983). The fat supplement was included in the diet in an effort to increase the peroxidative damage that could be caused by this pathway but since no clinical signs of the

disease were observed in this group, it can not be determined if the reduction in E levels resulted in any physiological changes. Due to the higher plane of nutrition, the lambs which received the fat supplemented diet had heavier body weights and seemed to be in overall better health than the -Se-E group. They were also more active and less likely to refuse feed than the -Se-E group. The energy and protein that was provided through the fat supplement could have enabled that group to withstand their low Se and E status better than the -Se-E group.

5.4.2 Tissue Selenium Content

Tissue levels of Se are reported in Table 5. Liver Se levels were different for the +Se+E group ($p<.05$) with a mean concentration of 487.6 ppb. Jenkins et al. (1974) reported similar values of 375 to 393 ppb when a diet containing 50 ppb of Se was fed. Paulson et al. (1968) reported values ranging from 920 to 2540 ppb for normal lambs receiving 160 to 520 ppb Se in the diet. The values observed for the deficient treatments coencide with the values observed by Hartley (1967) of 80 ppb in deficient lambs fed a diet containing 8 ppb. Paulson et al. (1968) reported values of 190 ppb in the livers of deficient lambs. The higher value obtained for the -Se-E+fat treatment could be due to the

fact that the fat supplement was found to contain some Se, probably from the sodium caseinate used to protect the fat from hydrogenation in the rumen.

The mean kidney Se concentration of 563 ppb for the -Se-E group was different ($p<.05$) from the -Se-E+fat group value of 859 ppm and the +Se+E group value of 1088 ppb. Lambs from selenium deficient areas in Canada have been reported to have kidney Se levels of 2600 ppb (Hoffman et al., 1973), consistant with the values obtained for Se deficient animals by Jenkins and Hidiroglou (1972) for lambs raised in the United States and New Zealand. Paulson et al. (1968) reported kidney Se values of 3870-4500 ppb for lambs that had received no Se supplementation and consumed a hay-corn diet similar to the one used in this experiment. The values obtained for the supplemented group are not as high as have been reported in other studies that have supplemented lambs with Se (Hoffman,1973).

When the dietary intake of selenium is low, the kidney of the lamb has been observed to contain more selenium than does the liver, whereas when dietary selenium is adequate, the reverse has been observed with the liver having higher concentrations than the kidney. (Ewan et al., 1968; Oh et al., 1976). In the present experiment, there were higher Se concentrations in the kidney than in the liver on the defi-

TABLE 5

TISSUE SELENIUM LEVELS

	Diets					
	-Se-E	S.E.	-Se-E+Fat	S.E.	+Se+E	S.E.
Liver	168[b]	35	191[b]	39	487[c]	35
Kidney	563[b]	82	859[c]	93	1088[c]	82
Heart	85[b]	12	126[c]	14	237[d]	12
Skeletal	28[b]	4	31[b]	5	64[c]	4

[a]All values expressed on a wet basis as ppb.

[b,c,d]Means within a row with different superscripts differ ($P<.05$).

cient diets but this situation did not reverse when selenium was supplemented, which could be due to the lower amount of supplementation in this study than in the reports cited previously.

Cardiac tissue Se concentrations exhibited the same pattern as did liver Se concentrations, with the the highest values of 237 ppb observed in the +Se+E group. The -Se-E group had a mean cardiac Se concentration of 85 ppb and the -Se-E+fat group was observed to have a cardiac Se concentration of 126 ppb. The Se concentrations observed in cardiac muscle were higher than those observed in skeletal muscle. This is in agreement with patterns reported by Ullrey et al. (1983).

Skeletal muscle Se concentrations were significantly higher ($p<.05$) in the +Se+E group which had a mean concentration of 64.83 ppb. The means for the two deficient treatments were not different from each other. Selenium concentrations in the skeletal muscle of healthy lambs have been reported to range from 53 to 330 ppb (Jenkins et al., 1974; Paulson et al., 1968; Hartley, 1967). Those same researchers observed values of 20 to 140 ppb in lambs that were considered deficient in Se.

TABLE 6

SELENIUM CONTENT OF THE DIET

	ppm
Feed, Fat and Unsupplemented Salt	.086
Feed, Se Supplemented Salt	.658
Feed With Plain Salt	.076
Feed With No Supplementation	.028

5.4.2 Body, Muscle and Organ Weights

Body weight changes were also recorded with the final ending weights for the -Se-E+fat group being the greatest at 42.15 kg ($p<.05$) (table 7). The body weights at slaughter for the -Se-E diet were lowest at 31.45 kg with the +Se+E diet being intermediate at 35.81 kg. The higher weights for the +Se-E+fat group were probably due to the increased amount of dietary protein and energy provided by the fat supplement. The +Se+E lambs were heavier than the -Se-E lambs, indicating a beneficial effect of the Se and E supplementation. This positive effect of supplementation on body weight gain has been previously reported by several investigators (Paulson et al., 1968; Ewan et al., 1968; Rotruck et al., 1968). Although not statistically different, the semitendinosus (ST), triceps brachi (TB), and heart weights were also higher for the -Se-E+fat group in comparison to the -Se-E and +Se+E treatments. Kidney weights were highest for the -Se-E treatment, differing from the pattern displayed by the muscles and overall body weights but were not significantly different from the weights observed for the other two treatments.

TABLE 7

BODY, MUSCLE AND ORGAN WEIGHTS

	Diet					
	-Se-E	S.E.	-Se-E+Fat	S.E.	+Se+E	S.E.
Initial body wt. (kg)	31.82	1.82	32.36	1.98	32.42	1.82
Slaughter body wt. (kg)	31.46[a]	5.62	42.16[b]	6.59	35.82[ab]	5.62
Semitendinosus (g)	88.10	8.37	110.28	9.54	100.93	8.37
Triceps brachi (g)	158.49	10.58	203.42	12.07	180.94	10.58
Heart (g)	177.98	9.93	182.41	11.33	177.51	9.93
Kidney (g)	73.91	7.17	58.69	9.48	51.01	7.17

[a,b]Means within a row with different subscripts differ ($P<.05$).

5.4.3 In Vitro Synthesis and Degradation

The in vitro degradation rate of the skeletal muscle (table 8) was greatest for the -Se-E group which had a mean degradation rate of .0383 mg tyr•mg^{-1} muscle. Degradation rates for the -Se-E+fat group was .0298 mg tyr•mg^{-1} and the +Se+E group had a degradation value of .0338 mg tyr•mg^{-1}. When one compared the degradation rates of the ST to the TB, the ST had a higher rate, .0343 mg tyr•mg^{-1}, than the TB, .0291 mg tyr•mg^{-1} muscle ($p<.05$).

Although not significant, the highest value for in vitro synthesis was observed in the -Se-E+fat treatment which had a mean value of .0605 nmoles tyr•mg^{-1} muscle. The -Se-E and +Se+E treatments were intermediate with .0433 nmole tyr•mg^{-1} muscle and .0387 nmole tyr•mg^{-1} muscle being observed for each of the two treatments respectivly. When comparing the ST to the TB, the synthesis rates were essentially the same with .0412 nmole tyr•mg^{-1} being observed for TB and .0403 nmole tyr•mg^{-1} being observed for the ST.

The - Se-E+fat group had the lowest degradation rates and the highest synthesis rates. This would indicate that the possibility existed for a greater net deposition of protein to occur in that treatment. Although the protein contents of the individual muscles were not determined the muscle weights were higher in the -Se-E+fat treatment when compared

TABLE 8

CATHEPSIN D ACTVITY AND INVITRO SYNTHESIS AND DEGRADATION

Trt	Cathepsin D (% ABS)	STD ERR	Degradation ($mgTYR \cdot mg^{-1} \cdot 3HR^{-1}$)	STD ERR	Synthesis ($nmoles \cdot mg^{-1} \cdot 2.5HR^{-1}$)	STD ERR
-Se-E	.0658	.0059	.0383	.0035	.0433	.0090
-Se-E+Fat	.0525	.0065	.0298	.0038	.0605	.0127
+Se+E	.0640	.0059	.0338	.0035	.0387	.0090
Muscle						
TB	.0391[a]	.0056	.0344[a]	.0027	.0413	.0073
Card	.0953[b]	.0056	---	---	---	---
ST	.0365[a]	.0056	.0290[b]	.0027	.0403	.0073

[a,b]Means within a column with different subscripts differ ($P<.05$).

to the other two treatments. In all treatment groups, the higher the degradation rates were associated with the smaller the muscle weights. Conversly, the higher synthesis rates were not observed in all cases to be highest in the largest muscles. The highest synthesis rates were observed in the heaviest muscles (the -Se-E+E treatment) but the next highest rates were observed in the -Se-E treatment which had the smallest muscle weights. This could indicate that while synthesis and degradation rates do indeed play a role in muscle size, they may not necessarily result in net protein gain or muscle size increases. While other factors such as fat, water, and connective tissue content contribute to differences in muscle weight, the results obtained in this study seem to indicate that the in vitro estimates of protein synthesis and degradation can partially explain the differences in muscle weights observed. In all cases, the largest muscle weights occured in the lambs with the heaviest body weights. The large muscle weights and greater synthesis and lower degradation rates observed in the -Se-E+fat group are probably a function of increased dietary protein and energy rather than the Se or E content of the diet. Comparisons of in vitro synthesis and degradation rates to dietary Se and E levels in this case are more valid when one compares the +Se+E treatment to the -Se-E treatment because some con-

founding occured due to the increased protein and energy supplied by the protected fat supplement. The higher synthesis rates and higher degradation rates observed for the -Se-E lambs as compared to the +Se+E lambs indicate a higher rate of turnover. This observation has also been made by other researchers in animals under dystrophic conditions. (Kruh et al., 1960; Simon et al., 1958)

5.4.4 Cathepsin D Activity

The activity of the lysosomal enzyme, cathepsin D (Cat D) was also examined. Average Cat D activity (absorbance at 660 nm) in the TB and ST was highest for the -Se-E treatment, .0658, and lowest in the -Se-E+fat treatment at .0525. The increases in Cat D activity parallel the changes observed in degradation rates with the higher enzyme activities corresponding to the higher degradation rates. Cathepsin D activities for the individual muscles were higher for the TB than the ST. This corresponded to increased degradation rates for the TB as opposed to the ST. These results indicate that the lysosomal enzyme was involved in the degradative processes and that increased rates of degradation could be due in part to the proteolytic activity of the enzyme.

It is well established that Cat D is a proteolytic enzyme that degrades muscle proteins. (Schwartz and Birad, 1977; Bird et al., 1980; Hershko and Ciechanover, 1982) Also, other studies have shown that increased levels of lysosomal enzymes occur in animals which are suffering from genetic or nutritionally induced muscular dystrophy. (Tappel et al., 1962; Zalkin et al., 1962; Desi et al., 1964). Therefore, neither the occurance of increased enzyme activity nor increased degradation rates in -Se-E animals is an unexpected observation. However, it has not been previously reported that in vitro muscle degradation coincides with increased lysosmal enzyme activity. It is known that in vitro muscle degradation estimates reflect changes occuring in the muscle in vivo (Waterlow et al., 1978), thus our results suggest that cat D is responsible for some of the increased muscle degradation in dystrophic animals.

Chapter VI

SUMMARY AND CONCLUSIONS

A study was conducted with 21 Suffolk x (Dorset-Coopworth) lambs to investigate the in vitro synthesis and degradation capabilities and the involvement of lysosomal enzymes in Se-E deficient ovine skeletal muscle. A randomized block design was utilized in which the lambs were subjected to 3 treatments: 1) A basal NaOH treated 85% corn, 15% hay diet to which was added a Se-E deficient mineral mixture (-Se-E), 2) The basal diet and deficient mineral mixture as fed in treatment 1 with the addition of a casein encapulated fat supplement fed at $3g \cdot kg^{-1}$ body weight (-Se-E+fat), and 3) The basal diet plus a Se-E supplemented mineral mixture (+Se+E). Three lambs were killed at the start of the trial with the remainder of the lambs killed after being on the diets an average of 110 days. In vitro protein synthesis and degradation was measured on the semitendinosus and triceps brachi muscles. The activity of the lysosmal enzyme cathepsin D was estimated in skeletal and heart muscle. The skeletal, cardiac, kidney and liver tissues were analyzed for Se content. In addition, plasma E and whole blood Se levels were determined.

Blood concentrations of Se were lowest in the initial kill lambs and highest in the +Se+E lambs. The -Se-E and -Se-E+fat groups had blood Se levels significantly lower than those of the +Se+E groups ($P<.05$).

Plasma concentrations of Vit E were different only for the +Se+E group ($p<.05$) however the -Se-E+fat group had slightly lower E levels than the -Se-E group indicating a responce to the protected fat supplement probably causeing and increase in the oxidation of the E in the fat supplemented lambs. None of the lambs in this experiment were observed to have the classic clinical signs of WMD. At slaughter no gross muscle lesions were observed in any of the three treatments. The -Se-E group was observed to be slower to rise and less vigorous in general appearance than the other two treatment groups. The relationship of Se levels to overall health and vigor was not clear with some of the most deficient lambs appearing to be more healthy than lambs with higher Se levels. This could indicate that while general conclusions can be made as to how deficient the lambs as a group were, there seemed to be tremendous amount of individual animal variation in their ability to withstand low Se and E levels.

Tissue blood Se levels exhibited similar patterns between treatment groups. Again the lowest Se levels were in the

inital kill lambs and the highest in the supplemented lambs. Across treatments, kidney was observed to have the highest Se content with liver, heart, and muscle following in that order.

In vitro degradation rates were highest for the -Se-E group and lowest for the inital controls. Between muscles the semitendinosus muscle tended to have higher degradation rates than the triceps brachi. In vitro synthesis rates were highest for the -Se-E+fat treatment and the lowest in the initial kill lambs. The -Se-E group appeared to have a lower synthesis rate than the +Se+E group although none of the three treatment groups were statistically different. When degradation rates were compared to overall muscle weights, in each case the higher degradation rates were associated with smaller muscle weights. When synthesis rates were compared to muscle size, the highest synthesis rates were observed in the treatment which produced the largest muscles (the -Se-E+fat treatment). The next highest synthesis rate was observed in the -Se-E treatment which had the smallest muscle weights. The high synthesis and degradation rates of the -Se-E treatment could indicate a higher rate of turnover for those muscles. This higher rate of turnover could be explained by increased degradation due to the release of the lysosomal and other enzymes in response to the

Se-E deficiency and an increase in synthesis rates to offset the increased degradation. This increase in synthesis in response to increased degradation could be an effort to maintain muscle size and function but it could also be a means by which other parts of the body could be supplied with needed protein during the disease state. The body could be drawing on the protein stores of the muscles and the increased synthesis and degradation could be acting to replenish this store and/or provide more available protein. The scenerio depicted with synthesis and degradation processes during this experiment seem to be similar to those of another muscle wasting condition, that of starvation (Millward and Waterlow, 1978).

Cathepsin D activity was found to be highest for the -Se-E treatment and lowest for the initial kill lambs. Cardiac tissue was consistently higher in cathepsin D activity than the two skeletal muscles ($P<.05$). In all cases the higher cathepsin activities were associated with the higher degradation rates. This relationship indicates that the proteolytic enzyme action of cathepsin D may be in part responsible for the higher degradation rates of the skeletal muscles.

The deficiency of Se and E has long been known to cause increases in the degradative processes of skeletal muscles.

This study has demonstrated, via in vitro estimates of degradation and synthesis, that the skeletal muscles of sheep respond to Se and E deficiency with an increased turnover rate of muscle protein. The overall net result of increased degradation and synthesis rates remain cloudy at best and one could infer from the data presented here that a simple relationship between protein turnover rates and overall muscle size does not exist. They can at least in part explain the differences in muscle size in that the largest muscles indeed had the highest synthesis rates and the lowest degradtion rates. This relationship was different in the more deficient lambs who exhibited high synthesis and high degradation rates for smaller muscles. The increased muscle weights were paralleled by overall heavier live weights at slaughter. Therefore, the net result of changes in synthesis and degradation rates depends on both of these processes with either process being able to alter the affect of the other.

Chapter VII

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'APPENDIX'

TABLE 9

INDIVIDUAL LAMB DATA

Tag No.	Sex	Wt. kg 8/27/84	Birth Date
1	F	34.5	3/22/84
2	F	35.5	3/02/84
3	F	22.7	3/06/84
4	F	37.3	3/18/84
5	F	32.7	3/02/84
6	F	32.7	3/06/84
7	M	32.7	3/11/84
9	M	28.2	3/19/84
10	F	37.3	2/18/84
11	M	26.4	2/20/84
12	F	26.4	3/14/84
13	M	27.3	3/14/84
14	M	26.4	2/26/84
15	F	35.5	3/02/84
16	M	32.7	3/11/84
17	M	36.4	2/20/84
19	M	32.7	3/19/84
20	F	33.6	2/26/84
21	F	34.5	2/19/84
22	M	32.7	3/11/84
23	M	37.3	2/26/84

TABLE 10

SLAUGHTER MUSCLE, ORGAN, AND BODY WEIGHTS

Tag No.	ST (g)	TB (g)	Heart (g)	Kidney (g)	Bodyweight (kg)
1	119.70	193.36	156.66	50.93	--
2	136.76	222.74	223.69	56.25	47.3
3	74.48	121.86	156.56	62.32	28.2
4	113.02	207.48	191.73	66.36	40.9
5	94.15	166.51	178.90	43.16	--
6	76.97	146.29	158.98	--	--
7	95.37	192.94	171.49	70.60	34.5
10	71.94	130.51	141.75	44.95	25.0
11	62.84	139.99	133.73	--	--
12	118.06	179.23	178.06	46.46	36.4
13	69.55	156.65	130.56	38.48	--
14	67.16	170.44	165.51	--	37.3
15	88.64	192.49	217.00	65.16	37.3
16	70.97	143.37	173.28	123.59	31.8
17	105.59	156.83	199.05	70.07	30.9
19	84.07	166.46	180.67	56.29	31.8
20	98.10	169.50	186.80	60.55	--
21	113.50	223.08	174.46	61.21	43.2
22	87.81	175.58	147.76	59.25	34.1
23	144.99	241.30	201.56	47.05	46.4

TABLE 11

BLOOD SELENIUM AND VITAMIN E LEVELS

Tag No.	Initial Blood Se (ppb)	Slaughter Blood Se (ppb)	Slaughter Blood Vit E (ppm)
1	62	65	--
2	45	62	1.6
3	27	47	1.6
4	70	90	1.9
5	60	112	--
6	30	--	--
7	64	58	1.8
10	66	213	6.5
11	31	--	--
12	49	324	7.4
13	33	--	--
14	109	86	1.7
15	46	243	5.6
16	39	32	1.6
17	45	38	2.9
19	47	62	2.1
20	52	59	--
21	56	82	1.4
22	43	276	5.7
23	51	221	6.4

TABLE 12

TISSUE SELENIUM LEVELS

Tag No.	Slaughter Liver Se (ppb)	Slaughter Skeletal Muscle Se (ppb)	Slaughter Cardiac Se (ppb)	Slaughter Kidney Se (ppb)
1	144	28	80	749
2	166	33	116	885
3	141	32	88	645
4	180	30	114	766
5	245	36	132	1073
6	68	12	35	626
7	209	32	75	506
10	508	56	262	1082
11	56	17	31	634
12	389	62	242	1028
13	61	14	34	688
14	285	37	160	926
15	669	72	262	834
16	164	28	82	274
17	190	30	68	494
19	151	18	111	662
20	155	33	88	798
21	181	30	132	970
22	548	87	282	1035
23	567	76	242	1480

TABLE 13

CATHEPSIN D ACTIVITY AND IN VITRO SYNTHESIS AND DEGRADATION

Tag No.	Muscle	Cathepsin D Activity (% ABS)	Degradation (nmoles·mg^{-1}·3HR^{-1})	Synthesis (nmoles·mg^{-1}·2.5HR^{-1})
1	TB	0.023	0.026	0.016
	CARD	0.105	--	--
	ST	0.024	0.026	0.169
2	TB	0.039	0.002	--
	CARD	0.073	--	--
	ST	0.018	0.020	--
3	TB	0.050	0.092	0.036
	CARD	0.090	--	--
	ST	0.065	0.086	0.009
4	TB	0.067	0.038	0.033
	CARD	0.058	--	--
	ST	0.038	0.020	0.051
5	TB	0.017	0.034	0.020
	CARD	0.094	--	--
	ST	0.057	0.046	0.020
6	TB	0.034	0.012	0.014
	CARD	0.036	--	--
	ST	0.015	0.030	0.006
7	TB	0.049	0.026	0.048
	CARD	0.077	--	--
	ST	0.006	0.020	0.040
10	TB	0.043	0.008	0.068
	CARD	0.105	--	--
	ST	0.024	0.026	0.047
11	TB	0.034	0.014	0.040
	CARD	0.043	--	--
	ST	0.025	0.020	0.030
12	TB	0.055	0.006	0.050
	CARD	0.082	--	--
	ST	0.045	0.010	0.027

TABLE 13

Tag No.	Muscle	Cathepsin D Activity (% ABS)	Degradation (nmoles·mg^{-1}·3HR^{-1})	Synthesis (nmoles·mg^{-1}·2.5HR^{-1})
13	TB	0.036	0.016	0.014
	CARD	0.055	--	--
	ST	0.018	0.012	0.017
14	TB	0.029	0.059	--
	CARD	0.114	--	--
	ST	0.049	0.026	--
15	TB	0.051	0.052	0.041
	CARD	0.179	--	--
	ST	0.037	0.016	0.027
16	TB	0.053	0.040	0.012
	CARD	0.118	--	--
	ST	0.041	0.034	0.025
17	TB	0.049	0.056	0.053
	CARD	0.114	--	--
	ST	0.032	0.024	0.069
19	TB	0.040	0.028	0.105
	CARD	0.195	--	--
	ST	0.040	0.028	0.058
20	TB	0.044	0.012	0.017
	CARD	0.066	--	--
	ST	0.055	0.014	0.048
21	TB	0.023	0.040	0.072
	CARD	0.089	--	--
	ST	0.038	0.041	0.036
22	TB	0.024	0.088	0.036
	CARD	0.079	--	--
	ST	0.038	0.062	0.022
23	TB	0.023	0.038	0.068
	CARD	0.134	--	--
	ST	0.065	0.020	0.039

$$\frac{\text{Amount of Tyrosine in acid soluable supernate of the tissue } (\mu g/ml) + \text{Amount of Tyrosine in acid soluable supernate of the incubation media } (\mu g/ml)}{\text{mg tissue}} - \frac{\text{Amount of Tyrosine in acid soluable supernate of blank preincubation media } (\mu g/ml)}{\text{mg tissue}}$$

= µg Tyrosine released/mg tissue for the time of incubation

Inulin:

$$\frac{\text{DPM in inulin pellet + DPM of supernate and combined washes of inulin pellet}}{\text{DPM/ml in the media after incubation}} \div \text{mg tissue}$$

= µl/mg tissue space that is taken up by inulin

Figure 4: CALCULATION OF DEGRADATION RATE

$$\text{Intracellular Specific Activity (DPN/nmole)} = \frac{\overbrace{\text{Total TCA Soluable DPM/mg tissue}}^{A} - \overbrace{\text{Inulin space } \mu l/mg \times \text{DPM/}\mu l \text{ medium}}^{B}}{\underbrace{\text{Total TCA Soluable Tyrosine of the blank (nmole/mg)}}_{C} \quad \underbrace{\text{Inulin space } \mu l/mg \times \text{nmole Tyrosine/}\mu l \text{ medium}}_{D}}$$

A: Total TCA Soluable DPM of the Synthesis Supernate:

DPM/ml Supernate x Total Volume of Supernate = Total TCA Soluable DPM of the Synthesis Supernate

Total TCA Soluable DPM of the Synthesis Supernate/mg of Tissue Sample = Total TCA Soluable DPM/mg Tissue

B: Inulin Space of the Synthesis Media

Total DPM in the Synthesis Incubation Media After Incubation (DPM/ml)/1000 µl = DPM/of Synthesis Media

DPM/µl of Synthesis Media x .252 µl of Inulin Space = DPM that represents Inulin Space

Figure 5: CALULATION OF SYNTHESIS RATE

C: TCA Soluable Tyrosine in the Blank Tissue

µg Tyrosine/ml of Supernate of the Blank Tissue Pellet x Total Volume of Supernate = Total TCA Soluable Tyrosine for the Blank Tissue

Total TCA Soluable Tyrosine for the Blank Tissue/mg Tissue = TCA Soluable Tyrosine of the Blank/mg Tissue

TCA Soluable Tyrosine of the Blank/mg Tissue + .181 = nmole of tyrosine/ mg tissue

D: Inulin Space of the Soluable Blank

Total TCA Soluable Tyrosine in Degradation Incubation Media After Incubation + 6 = µg/ml Tyrosine Released Over 30 Minutes (the length of the Preincubation)

µg/ml Tyrosine Released in Preincubation + .181 µg/nmole = nmole/ml Tyrosine Released

nmole/ml Tyrosine Released + 1000 µl = nmole/µl Tyrosine Released

nmole/µl Tyrosine Released x .252 µl = Inulin Space of the Acid Soluable Blank

$$\text{Synthesis} = \frac{\text{DPM/mg of Tissue for the Synthesis Pellet}}{\text{Intracellular Specific Activity DPM/nmole}} = \text{nmole/mg of Tyrosine Incorporated Over the Incubation Period}$$

Figure 5:

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ABSTRACT

In Vitro Protein Synthesis and Degradation and Cathepsin D Activity in the Muscles of Selenium and Vitamin E Deficient Lambs

by

Mitchell T. Gore

Invitro synthesis and degradation capabilities and the cathepsin D activity of the skeletal muscles of experimentally induced Selenium-Vitamin E deficient lambs were investigated. Twenty one Dorset x Coopworth x Suffolk lambs were blocked by weight and assigned to one of the following treatments: 1)a basal diet of 85% whole shelled corn and 15% mixed grass hay plus a mineral supplement that was low in Se and E (-Se-E), 2)the basal diet and deficient mineral mixture as fed in treatment 1 with the addition of a casein encapsulated fat supplement (-Se-E+fat), 3)the basal diet plus a mineral mixture which contained supplemental Se and E (+Se+E). Upon completion of the feeding trial, lambs which had been recieving the -Se-E treatment had the greatest body and individual muscle weights. The -Se-E+fat treatment had the lowest degradation rates and the highest synthesis rates. Cathepsin D activity was found to be lowest in the

-Se-E+fat treatment. The -Se-E treatment was observed to have the highest degradation rate and the next highest synthesis rate indicating an increased rate of muscle turnover. In all cases the degradation rates were positively correlated with cathepsin D activity.