Thirty-six Suffolk ewes were randomly divided into two equal groups. One group received a selenium (Se) deficient diet, while the other diet contained sodium selenite at a level of 0.2 ppm Se in the total diet. All ewes were sampled for plasma glutathione peroxidase (GPx) and whole blood Se at forty-day intervals for 160 days. The Se and GPx levels of lambs born to these ewes were determined at three and six weeks of age. Plasma creatine kinase (PCK) levels of lambs were determined weekly to monitor development of nutritional muscular dystrophy (NMD). Surgical biopsies of semitendinosus muscle were taken from alternate legs of lambs at two-week intervals, commencing at two weeks of age. Muscle specimens were prepared for histologic, histochemical and electron microscopic examination.

None of the Se supplemented lambs developed NMD, while 13 of 14 Se deficient lambs were affected. The earliest evidence of NMD was an elevated PCK, which preceded ultrastructural changes by two weeks, and histological and histochemical changes by four weeks. Early ultrastructural changes were characterized by swelling or condensation of mitochondria accompanied by swelling and fragmentation of sarcoplasmic reticulum. Subsequent ultrastructural changes included collapse of
the sarcoplasmic reticulum, rupture of mitochondria, loss of myofibrillar structure, and disruption of sarcomeres. Histochemical changes included reduced adenosine triphosphatase, reduced or enhanced nicotinamide adenine dinucleotide tetrazolium reductase, and heterogenous periodic acid schiffs, stain reaction, as well as foci of enhanced acid phosphatase reaction. The histochemical and ultrastructural results indicated a mitochondrial defect as a basis for Se deficiency-induced NMD.

Spontaneous recovery from NMD occurred in most lambs with complete remission of histologic, histochemical and ultrastructural abnormalities six weeks after peak PCK, except that some mitochondria remained swollen through the ten-week period following peak PCK. Two lambs with NMD were injected with sodium selenite. The nature of the recovery process in the muscles of these lambs paralleled non-injected lambs but was slightly accelerated.
Histological, Histochemical and Ultrastructural Changes
Associated with Nutritional Muscular Dystrophy
in Selenium Deficient Lambs

by

Eric Bruce May

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Doctor of Philosophy

Completed October 12, 1982

Commencement June, 1983
APPROVED:

Redacted for Privacy

Professor of Veterinary Medicine in charge of major

Redacted for Privacy

Chairman of Department of General Science

Redacted for Privacy

Dean of Graduate School

Date thesis is presented October 12, 1982

Typed by Karen I. Goulding for Eric Bruce May
This paper is dedicated to the following individuals
whose contributions far exceeded my expectations,
and without whom my professional career
would not have been the same:

Mr. George Muss
Dr. Ivan Pratt
Dr. Martha Tripp
Dr. Jack Schmitz
Dr. E. Edward Wedman
ACKNOWLEDGEMENTS

I gratefully acknowledge the help of the following individuals whose help and support throughout this research greatly contributed to its completion. Ms. Charlene Karr-May, Ms. Ann Birkholtz-Lambrect, Mr. Marcus Day, and Mr. Al Soeldner for their technical contributions and advice; Ms. Sharon Nelson, Ms. Karen Goulding, and Ms. Jo Kifer for manuscript preparation; Ms. Marilyn Slizeski for her diligent efforts in proofreading the manuscript; Ms. Peggy Harris for her kind assistance; Dr. Lloyd Guth and Dr. Tai Oh for reviewing the manuscript; Dr. David Willis for advice and support during the development and course of my doctoral program; and Ms. Rosemary Riggs for the support she gave and the patience she exhibited during the research and manuscript production.
# TABLE OF CONTENTS

I. Introduction and Review of Literature ............................................ 1

II. Materials and Methods ................................................................. 10
   Animals and Experimental Diets ................................................. 10
   Surgical Muscle Biopsy Procedures ........................................... 11
   Ultrastructural, Histochemical, and Histologic Procedures ............. 12
   Blood Sampling Procedures ..................................................... 14
   Staging for Comparison among Lambs of Histologic, Histochemical, and Ultrastructural Results ........................................... 15

III. Results ......................................................................................... 17
   Lamb Birth Rate, Survival, Clinical Characteristics, and Necropsy Findings in Selenium Supplemented and Selenium Deficient Groups ........................................... 17
   Histologic, Histochemical, and Ultrastructural Characteristics of Muscle from Selenium Supplemented Lambs ................................................... 26
   Histologic, Histochemical, and Ultrastructural Characteristics of Muscle from Selenium Deficient Lambs ................................................... 33
   Histologic, Histochemical, and Ultrastructural Characteristics of Muscle from Selenium Deficient Lambs Which Were Injected With Sodium Selenite at the Time of Acute Myopathy ................................................... 52

IV. Discussion .................................................................................... 60

V. Literature Cited ................................................................................ 78

VI. Appendix ....................................................................................... 83
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Photomicrographs of transverse sections of paraffin and plastic embedded muscle from a four-week-old selenium sufficient lamb.</td>
<td>27</td>
</tr>
<tr>
<td>2.</td>
<td>Photomicrographs of transverse serial sections of frozen muscle from a four-week-old selenium sufficient lamb.</td>
<td>30</td>
</tr>
<tr>
<td>3.</td>
<td>Electron micrographs of muscle from a four-week-old selenium sufficient lamb.</td>
<td>32</td>
</tr>
<tr>
<td>4.</td>
<td>Electron micrographs of muscle from a selenium deficient lamb four weeks prior to peak plasma creatine kinase level.</td>
<td>35</td>
</tr>
<tr>
<td>5.</td>
<td>Electron micrographs of muscle from a selenium deficient lamb four weeks prior to peak plasma creatine kinase level.</td>
<td>36</td>
</tr>
<tr>
<td>6.</td>
<td>Photomicrographs of transverse section of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb two weeks prior to peak plasma creatine kinase level.</td>
<td>37</td>
</tr>
<tr>
<td>7.</td>
<td>Electron micrographs of muscle from a selenium deficient lamb two weeks prior to peak plasma creatine kinase level.</td>
<td>40</td>
</tr>
<tr>
<td>8.</td>
<td>Electron micrographs of muscle from a selenium deficient lamb at peak plasma creatine level.</td>
<td>41</td>
</tr>
<tr>
<td>9.</td>
<td>Photomicrographs of transverse section of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb two weeks after peak plasma creatine kinase level.</td>
<td>44</td>
</tr>
<tr>
<td>10.</td>
<td>Electron micrograph of muscle taken from a lamb two weeks after peak plasma creatine kinase level.</td>
<td>45</td>
</tr>
<tr>
<td>11.</td>
<td>Photomicrographs of transverse sections of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb four weeks after peak plasma creatine kinase level.</td>
<td>46</td>
</tr>
<tr>
<td>12.</td>
<td>Electron micrographs of muscle from a selenium deficient lamb four weeks after peak plasma creatine kinase level.</td>
<td>48</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>13.</td>
<td>Photomicrographs of transverse sections of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb six weeks after peak plasma creatine kinase level.</td>
<td>49</td>
</tr>
<tr>
<td>14.</td>
<td>Electron micrograph of muscle taken from a selenium deficient lamb eight weeks after peak plasma creatine kinase level.</td>
<td>51</td>
</tr>
<tr>
<td>15.</td>
<td>Photomicrographs of transverse sections of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb with acute nutritional muscular dystrophy immediately prior to receiving a selenium injection.</td>
<td>53</td>
</tr>
<tr>
<td>16.</td>
<td>Electron micrographs of muscle from a selenium deficient lamb with acute nutritional muscular dystrophy immediately prior to receiving a selenium injection.</td>
<td>54</td>
</tr>
<tr>
<td>17.</td>
<td>Electron micrographs of muscle taken from a selenium deficient lamb three weeks after receiving a selenium injection at acute stages of nutritional muscular dystrophy.</td>
<td>57</td>
</tr>
<tr>
<td>18.</td>
<td>Electron micrograph of muscle from a selenium deficient lamb taken six weeks after receiving selenium injection at acute stage of nutritional muscular dystrophy.</td>
<td>59</td>
</tr>
<tr>
<td>19.</td>
<td>Proposed pathogenesis of selenium deficiency-induced nutritional muscular dystrophy in sheep.</td>
<td>73</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>1. Mean Whole Blood Selenium Values of Selenium Deficient and Supplemented Ewes After 40, 80, 120, and 160 Days on Experimental Diets.</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>2. Mean Whole Blood Glutathione Peroxidase Values of Selenium Deficient and Supplemented Ewes after 40, 80, 120, and 160 Days on Experimental Diets.</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>3. Mean Whole Blood Selenium (Se), Plasma Glutathione Peroxidase (GPx), and Creatine Kinase (CK) Values of Selenium Deficient and Supplemented Lambs at Three and Six Weeks of Age.</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>4. Creatine Kinase Levels in Plasma of Selenium Supplemented Lambs at Each Biopsy Period.</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>5. Creatine Kinase Levels in Plasma of Selenium Deficient Lambs at Each Biopsy Period.</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>6. Creatine Kinase Levels in Plasma of Selenium Deficient Lambs Which Were Injected With Sodium Selenite After Onset of Severe Nutritional Muscular Dystrophy.</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>7. Histochemical Staining Characteristics of Muscle Fiber Types in Semitendinosus Muscle from Selenium Supplemented Lambs.</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>
Histological, Histochemical and Ultrastructural Changes Associated with Nutritional Muscular Dystrophy in Selenium Deficient Lambs

INTRODUCTION AND REVIEW OF LITERATURE

White muscle disease is a chronic wasting condition that affects the young of both cattle and sheep (Jubb and Kennedy, 1970). This syndrome was originally characterized in the 1920's for both lambs and calves without knowledge of a specific etiology. Original work by Muth (1956) demonstrated a significant relationship between white muscle disease and the lack of Vitamin E; however, this relationship could not be clearly defined. Subsequent work by Muth et al. (1958) implicated selenium (Se) deficiency as a coincident factor along with a Vitamin E deficiency in producing the clinical signs and histopathological alterations characteristic of the disease. Myopathies associated with Se and Vitamin E deficiencies have also been recognized in a number of mammalian and avian species including chickens, rabbits, horses, cattle, and sheep. The syndrome has become known as nutritional muscular dystrophy (NMD).

There is little agreement in the literature regarding the comparative significance of Se and Vitamin E deficiencies as contributing factors in the development or treatment of NMD's. This is probably due to incomplete understanding of the exact biologic role of Se. Many studies exist that discuss the specific role of Vitamin E as a nutritional requirement (Muth, 1970; Harsch, 1977; Van Vleet, 1980; Trapp et al., 1970; Sylven and Glavind, 1977), while no such studies on the specific role of Se are available. It is known that injection
of Se alone can prevent development of NMD in sheep. This is probably because the diet of sheep usually contains adequate Vitamin E due to the high proportion of hay in most sheep diets. In actual management practice, studies have shown that Se deficiency alone can cause NMD in lambs (Muth et al., 1959, 1961; Muth, 1970; Whanger et al., 1978). Muth and Allaway (1963) demonstrated that the incidence of NMD parallels the distribution of Se deficient soils in the United States. The common druga widely used by sheep and cattle producers to prevent NMD contains both Se and Vitamin E.

To clinically assess the Se status in lambs and ewes, either whole blood or plasma glutathione peroxidase (GPx) levels are utilized. Selenium exists bound to amino acids either as selenomethionine or selenocysteine (Burk, 1975), more specifically as selenocysteine at a ratio of four moles selenocysteine to one mole GPx (Ladenstein et al., 1979; Ladenstein and Wendel, 1970). In field studies, the level of GPx has been shown to be directly proportional to the level of Se in either whole blood or plasma (Scholz and Hutchinson, 1979), and this relationship forms the basis of a field test to determine the Se status of cattle (Backall and Scholz, 1979). The combined use of GPx, creatine kinase (CK), and Se determinations on blood serves as a standard for assessing the Se status and severity of NMD in lambs (Whanger et al., 1976; 1977a,b; 1978).

Lambs from ewes deficient in Se or Vitamin E will often be still-born or will survive only for short periods (Gabbedy et al., 1977). Lambs born alive often are weak, unable to stand, and may die of cardiac arrest (Smith et al., 1972). Ewes marginally sufficient in

aBiotec; Omaha, Nebraska.
both Se and Vitamin E may bear live lambs; however, if those lambs are subjected to Se or Vitamin E deprivation, NMD will often ensue (Smith et al., 1972). Such lambs become thin and may exhibit signs of progressive muscular weakness. Occasionally individuals will appear asymptomatic; however, when exercised, will suddenly collapse and die due to heart failure (Smith et al., 1972). Severe myopathy and cardiomyopathy characterized by acute necrosis of myofibers is the hallmark of this disease. The muscles and heart may become grossly pale and sometimes have chalky white areas due to dystrophic calcification of necrotic muscle, thus accounting for the colloquial name of white muscle disease. A usual consequence of muscle damage is the release of CK into intercellular spaces; this release is related clinically to elevated plasma CK (PCK) (Sodeman and Sodeman, 1974). An elevated PCK is always the first clinical sign of NMD (Van Vleet and Ferrans, 1977; Van Vleet et al., 1977a), but the basis for this initial sign of NMD is not known. At this stage of NMD, lambs are still asymptomatic (Whanger et al., 1977a,b) and as the condition progresses, PCK activity rises to levels in excess of 2000 nanomoles per minute per milliliter (nm/min/ml) (Whanger et al., 1977a,b; 1978). Plasma CK has been a key enzyme for clinical evaluation of muscular dystrophies in humans (Dubowitz and Brooke, 1973) and swine (Van Vleet et al., 1977a,b).

Post-mortem examination of lambs with NMD reveals a variety of lesions; however, certain lesions are consistent. The musculature is usually pale, especially the semitendinosus muscle and, to a lesser extent, the epaxial muscles. The heart, if involved, will have pale to white streaks in the right ventricular myocardium and interven-
tricular myocardial septum. If the cardiac involvement is severe, both left and right ventricles are involved (Smith et al., 1972). The abdominal cavity may contain varying amounts of a clear, watery, ascitic fluid. All abdominal organs appear normal except the liver, which, if there is involvement of the heart, will be congested and slightly enlarged. The thoracic cavity may also contain varying amounts of a clear, watery fluid and edematous lungs.

Histologic examination of the striated muscles, especially semi-tendinosus and cardiac, reveals extensive interstitial (endomysial) inflammation composed largely of mononuclear cells and lymphocytes. Some myofibers are swollen and have deeply eosinophilic homogeneous cytoplasm (so-called Zenker's necrosis), while other myofibers have central nuclei but appear otherwise normal. Some of the more severely affected myofibers are pale with a patchy loss of cytoplasm, and others are infiltrated with or replaced by macrophages (Whanger et al., 1976).

Histochemistry has been widely used in the study of muscular diseases of animals and man, but the only such study on NMD has been done on pigs deprived of both Se and Vitamin E (Ruth and Van Vleet, 1974). In that study, animals were held until elevated PCK and lactic acid dehydrogenase (LDH) levels occurred, indicating the presence of myopathy. Animals were then sequentially killed at specific time intervals and frozen biopsies of cardiac and semitendinosus muscle taken. The histochemical studies revealed selective atrophy and necrosis of Type I myofibers with loss of mitochondrial nicotinamide dinucleotide tetrazolium reductase (NADH-TR) and adenosine triphosphatase (ATPase) activity. The studies by Ruth and Van Vleet (1974)
were conducted on cardiac and skeletal muscle only after myodegenerative changes were well advanced; thus, the early changes leading to the frank lesions and clinical signs were not determined.

Since only ATPase and NADH-TR were used by Ruth and Van Vleet (1974), selection of appropriate enzymatic reactions for this study was based on histochemical studies of other muscular diseases, primarily neuromuscular and inflammatory myopathies. Some neuromuscular disorders result in impaired myofiber utilization of glycogen (Dubowitz and Brooke, 1973). It is possible to determine if there is change in the myofiber's capacity to utilize glycogen by employing the periodic acid Schiff's reaction (PAS), as suggested by Cardinet and Holliday (1979), following the protocol of Pearse (1968, 1972) and Barka and Anderson (1963). This method detects only polysaccharides such as glycogen and not monosaccharides such as glucose (Pearse, 1968, 1972). It is an indirect measure of glycogen utilization and cannot be used to identify any specific site of glycogen metabolism dysfunction.

Muscle fiber typing is accomplished by use of the ATPase reaction and was used in this study to determine the muscle fiber type affected by selenium deficiency. The basis of the ATPase reaction is a calcium mediated reaction shown to be specific for calcium activated ATPases, particularly myofibrillar ATPase (Pearse, 1968, 1972). The ATPase reaction provides information regarding the pattern of Type I and II fibers as classified by Dubowitz and Brooke (1973). This method has been applied with great success by Cardinet and Holliday (1979) on domestic and small animals and has been used to determine the types of myofibers affected in myopathies (Cardinet and Holliday, 1979; Ruth
and Van Vleet, 1974). Intermediate fibers that show both Type I and Type II characteristics do occur (Ham and McCormack, 1979), but all intermediate types are classed as Type II (Dubowitz and Brooke, 1973).

The alkaline phosphatase (ALP) reaction is employed to test for active membrane transport (Pearse, 1968, 1972). Such active transport might be associated with the export of CK from myocytes prior to frank destruction of cytoplasmic membranes. A negative ALP reaction would suggest that the loss of CK from myocytes is passive due to loss of ATP or ATPase, or due to inapparent membrane damage.

A significant feature of NMD is infiltration of mononuclear leukocytes into affected muscles (Smith et al., 1972), but only in the more severely affected muscle fibers does the invasion of macrophages occur (Whanger et al., 1977a). Such macrophages have lysosomes containing hydrolytic enzymes which might contribute to the myodegeneration if released into the endomysium. The acid phosphatase (ACP) reaction was employed to determine if extracellular or intracellular myocytic hydrolases were significantly associated with myofiber degeneration. If myocyte organelle damage is a feature of early stages of NMD, then an increase of hydrolytic enzymes from autophagosomes or ruptured lysosomes may be a consequence.

Work by Calvin and Cooper (1979), as well as Wu et al. (1979) has shown a close association of Se with the mitochondria. The nicotinamide adenine dinucleotide reductase reaction (NAD-TR, NADH-TR) will demonstrate alterations in mitochondrial function (Dubowitz and Brooke, 1973). In addition, it has been shown that elements of the cytochrome chain are altered in cells depleted of Se (Diplock, 1974a,b). Any disruption of the cytochrome chain will result in increased
amounts of $H^+$ ions available to form the insoluble tetrazolium salt produced by the NADH-TR staining procedure (Dubowitz and Brooke, 1973). Thus, alterations of the mitochondrial membranes or mitochondrial viability will be reflected by changes in the NADH-TR reaction (Pearse, 1968, 1972).

In some human dystrophies, lipid accumulations due to dysfunction of lipid metabolizing systems have been demonstrated in myocytes (Dubowitz and Brooke, 1973). The oil red 0 stain in concert with osmium tetraoxide fixation is an accurate technique for localizing lipids in cells (Armed Forces Institute of Pathology, 1968) and was used herein to identify possible lipid accumulations in NMD.

Ultrastructural changes described for NMD in chicks (Van Vleet and Ferrans, 1976), in swine (Van Vleet and Ferrans, 1977; Van Vleet et al., 1977a), and in rabbits (Van Vleet et al., 1978) are consistent for late-stage muscle necrosis as described by Dubowitz and Brooke (1973) and Cardinet and Holliday (1979). At this stage, mitochondrial fragmentation and high amplitude swelling (Cheville, 1976) are observed, as well as swelling and fragmentation of the sarcoplasmic reticulum (Dubowitz and Brooke, 1973). Dissolution of myofilament bundles and irregular Z-line conformation are also typical characteristics in advanced stages of myofiber necrosis (Dubowitz and Brooke, 1973). Ultrastructural changes in swine exhibiting NMD were consistent with muscle undergoing necrosis, although myofilament bundle dissolution was not a common feature at earlier stages of the disease (Van Vleet et al., 1977a). Ultrastructural examinations of muscle from sheep with NMD have not been done, and it has been assumed that such changes at acute stages of NMD are consistent with those
seen in other animals. Studies of the ultrastructure of muscle prior to onset of NMD and during the recovery stages of the disease have not been done in any species.

Light microscopy will reveal tissue and cellular changes but will not detect the subcellular or organelle changes that lead to cellular and tissue pathologic changes. Ultrastructural analysis was thus employed to determine the organelle changes in the muscle biopsies taken from lambs prior to the clinical signs or histologic evidence of NMD. In reviewing models of human muscular dystrophies, Mendell et al. (1979) noted that mitochondrial abnormalities are consistent and appear to be a feature of the dystrophic process. There appear to be consistent changes in the mitochondria and sarcoplasmic reticulum of animals which have either nutritional myopathies or genetic muscular dystrophies, suggesting similar sites of organelle damage. Such changes are seen in hereditary muscular dystrophies of the hamster (Jasmin and Eu, 1979), mouse (Banker et al., 1979; Mrak and Baskin, 1978), and chicken (Wilson et al., 1979). Mitochondrial and sarcoplasmic reticular changes were also consistent with changes found in NMD of swine, chickens and rabbits (Van Vleet et al., 1978; Van Vleet and Ferrans, 1976; Van Vleet et al., 1976). Thus, a clear understanding of subcellular changes at pre-clinical as well as during acute and convalescent stages, would help in understanding NMD of sheep and other animals and possibly also muscular dystrophies of humans.

A characteristic feature of NMD in lambs and calves is the spontaneous remission of myodegeneration in some affected animals and the inability to induce NMD once they have reached a certain age (Smith et
Little information is available on histological characteristics of muscle following acute stages of NMD in any animal species, and no such information has been reported regarding the ultrastructural and histochemical features. This study provides this information by following the lambs through the recovery stages of NMD both during spontaneous recovery and during recovery promoted by injection of lambs with Se during the acute stage of NMD.

The purpose of this study was to examine the histochemical and ultrastructural changes in the skeletal muscle of Se deficient lambs prior to and during the acute phases of NMD as well as during the recovery stage. These changes were correlated with the histologic features of the muscle during the various stages of the disease. Sequential surgical biopsies were obtained from the lambs at weekly intervals starting at two weeks of age and extending to 16 weeks of age, thus encompassing the entire course of the disease. Light and electron microscopic examinations were conducted on the muscle specimens, and a battery of histochemical tests examining the glycolytic, hydrolytic, mitochondrial, and myofibrillar enzyme systems were done on serial sections of the muscle biopsies. The histochemical and ultrastructural changes were correlated with the histologic features and PCK levels to provide a greater understanding of the temporal characteristics of NMD as well as its overall pathogenesis.
MATERIALS AND METHODS

Animals and Experimental Diets:

Thirty-six Suffolk ewes were held on grass pasture from May, 1979 to November, 1979 and supplemented with mineralized salt containing Se at a level of approximately 0.2 ppm of the total diet during June and July. These ewes were bred three months prior to initiating the experiment. In November, the ewes were randomly divided into two equal groups and identified with ear tags. Ewes in both groups were fed four pounds chopped alfalfa hay, containing less than 0.015 ppm Se, and 150 gms rolled oats daily. Unsupplemented ewes (Se deficient) received oats without added Se, while supplemented ewes (Se supplemented) had 0.5 ppm Se added to the oats to equal approximately 0.2 ppm of the total diet. The ewes were fed two times daily in troughs. The sheep were housed in a building that was open on one side, and the pens extended to the outside. The pens were bedded with wood shavings.

During the lambing season, ewes were checked three times daily so that lambs were found immediately after birth. The lambs were ear tagged and their numbers were recorded with the dam's number. The lamb's umbilical cord was severed and the umbilical stub and the ear tag site were treated with Merthiolate™. Each lamb was also prompted to nurse to insure early ingestion of colostrum. The lambs remained with the ewes on their respective diets throughout the experiment.

In the Se supplemented group, four thrifty lambs were selected at two weeks of age for surgical muscle biopsy procedures. To insure that these four lambs were Se sufficient, they were injected intramuscularly in the dorsal cervical area with 1.0 mg of sodium selenite in 1.0 ml of sterile physiological saline solution (PSS). In the Se
deficient group, 14 lambs were randomly selected at two weeks of age for surgical muscle biopsy procedures. It was hoped that at least four of these would develop NMD but would survive through the early and acute stages of the disease and eventually recover. Four additional Se deficient lambs were monitored for PCK levels and when levels reached 20,000 nm/min/ml, indicative of severe myopathy, two lambs were selected for surgical muscle biopsies. After taking the initial muscle biopsies, both lambs were injected with 1.0 mg of sodium selenite as had been done previously with the Se supplemented lambs. These two lambs were designated as Se deficient and subsequently Se injected (Se Dprvd/Inj).

Surgical Muscle Biopsy Procedures:

Biopsy dates were established in advance for each lamb selected. The dates were set such that each animal to be biopsied (except lambs designated Se Dprvd/Inj) would have the first surgery performed on the left semitendinosus muscle (L), then, after two weeks, on the right semitendinosus muscle (R), returning again to the left semitendinosus muscle after a period of four weeks. The initial biopsy was not taken until each lamb was at least two weeks of age. Animals in the Se Dprvd/Inj group were biopsied weekly on alternate legs beginning with the left semitendinosus muscle. The first biopsies from the Se Dprvd/Inj lambs were taken immediately prior to Se injections. The lambs from the Se supplemented and Se deficient ewes were biopsied eight times ending with the fourth biopsy from the right semitendinosus muscle. Those animals in the Se Deprvd/Inj group were biopsied 10 times, ending when the right semitendinosus was biopsied for the
fifth time.

On the day of surgery, the lambs to be biopsied were moved to a separate pen, weighed, and blood samples drawn for PCK analysis. Each animal was given an intramuscular injection of a 2% solution of Xylazine-HCL\(^b\) in PSS at a rate of 0.3 mg/kg of body weight. The skin over the semitendinosus muscle was shaved and prepared for surgery by washing with surgical scrub\(^c\) and rinsing with 70% ethanol. A 3 cm longitudinal incision was made in the skin overlying the semitendinosus muscle; subsequent incisions were made immediately caudal to the initial incision. A 1.0 mm diameter sample of muscle was isolated, and a 2.0 cm length of muscle was isolated by clamping both ends with a pair of hemostats welded together in parallel. This sample was then removed and placed in fixative for subsequent electron microscopy. Another 1.0 mm by 2.0 cm sample, taken from the same area, was lightly stretched and pinned lengthwise on a sheet of dental wax. This was then immediately wrapped in PSS-soaked gauze and placed in a sealed jar for histochemistry and light microscopy. The surgical incision was closed by suturing the fascia and skin with "0" chromic catgut, and the area was sprayed with a nitrofurazone spray. All lambs recovered uneventfully from the general anesthetic within 1 hour and were returned to their dams within 2 hours.

Ultrastructural, Histochemical, and Histologic Procedures:

Ultrastructural Procedures. Samples of semitendinosus measuring 1.0 mm in diameter and 5 mm in length were prepared for electron

\(^b\)Rompun; Haver-Lockhart; Shawnee, KA.

\(^c\)Betadine; Med Tech; Elwood, KA.
microscopy by immediate immersion in 10 ml of an ice-chilled solution of 9.2 g sodium chloride, 0.142 g potassium chloride, 0.124 g hydrated calcium chloride, 0.15 g sodium bicarbonate, 0.1 g sucrose, and 10 g sodium nitrite dissolved in 1000 ml double distilled water and held there for three minutes to dilate vascular beds. Without rinsing, the tissue was transferred to 20 ml of chilled 2% glutaraldehyde which was prepared by adding 60 mls of 25% aqueous glutaraldehyde to 440 mls of 0.1 M cacodylate buffer containing 2 mM calcium chloride. After 10 minutes, the tissues were removed, cut into 1 mm cubes and returned to the solution for three hours. The specimens were then washed in cacodylate buffer and post-fixed in 1% osmium tetroxide solution in 0.1 M Malonigs phosphate buffer (pH 7.2) for one hour at 4°C. The samples were then rinsed in Malonig's phosphate buffer and embedded in diglycidylether of polypropylene glycol (DER)\textsuperscript{d} in accordance to the manufacturer's recommended procedures.

Sections for transmission electron microscopy were cut at 400 Å utilizing a Sorval MT-2 ultramicrotome, floated on distilled water, and adhered to 300 mesh copper grids that had been precleaned with acetic acid and acetone. The sections were examined with a Phillips EM 300 electron microscope.

Histochemical Procedures. Within an hour of surgery, samples of semitendinosus muscle for histochemistry were cut into 5 mm lengths, placed on cork squares, oriented to produce transverse sections, and immediately immersed in a vessel containing liquid Freon 500 which had been chilled to \(-150^\circ\text{C}\) with liquid nitrogen. The freon was kept fluid by constant agitation. The specimens were quenched in the freon for

\textsuperscript{d}Tousimis Chemical Co.; Rockville, MD.
30 seconds, then placed in plastic bags which were immediately heat sealed, and stored in an ultracold freezer at -80°C until sectioned. Sections were cut utilizing an Ames Cryostat Tek II held at -20°C. All sections were serially cut at 6 μm. The cut sections were then adhered to 1.0 grade thickness 22 mm square cover glass, air dried and stained with Hematoxylin and Eosin (HE), modified Gomori's Trichrome (Tric), Periodic Acid Schiffs (PAS), and Oil Red O (ORO), and stained for Adenosine Triphosphatase (ATPase), Nicotinamide Adenine Dinucleotide-Tetrazolium Reductase (NADH-TR), Acid Phosphatase (AcP), Alkaline Phosphatase (AlP), and Esterase (Es), as described in the Appendix.

Histologic Methods. Specimens for histological examinations were fixed in neutral buffered formalin (pH 7.0) and parallel specimens were embedded in both paraffin and glycol methacrylate plastic and processed appropriately for embedding in paraffin and glycol methacrylate plastic (Armed Forces Institute of Pathology, 1968). Paraffin embedded specimens were sectioned at 6 μm and stained by HE (Appendix). Plastic embedded muscle was sectioned at 3 μm and stained utilizing a triple strength Harris' Hematoxylin (Appendix).

Blood Sampling Procedures:

Samples were collected for whole blood Se and GPx assays from all ewes at 40, 80, 120, and 160 days after the start of experimental feeding. Blood was collected in heparinized Vacutainer® tubes for whole blood Se and whole blood GPx, placed on ice, and submitted to the laboratories of Dr. P. D. Whanger (Department of Agricultural Chemistry, Oregon State University) for analysis. Samples were

®Vacutainer; Becton, Dickinson, and Co.; Rutherford, NJ.
analyzed for Se following procedures adapted from those of Olson (1969) and for GPx following procedures of Paglia and Valentine (1967).

All lambs in both the Se supplemented and Se deficient groups were tested at three and six weeks of age for PCK and GPx, and whole blood Se. Plasma CK levels were also determined in lambs at the time of each biopsy. Blood for CK, GPx and Se were collected in ethylene diamine tetraacetic acid (EDTA)-treated Vacutainer tubes. Analysis for CK was done by the methods of Rotruck et al. (1973) and Whanger et al. (1976, 1977a,b). The values of CK and GPx were expressed as nanomoles per minute per milliliter (nm/min/ml), and Se was expressed as parts per million (ppm).

The blood Se and GPx levels of the ewes and lambs were measured to confirm that the experimental diets had the desired effect, i.e., the lambs of one group were deficient in Se while the lambs of the other group had adequate Se. The PCK levels were used in biopsied lambs to monitor the development of NMD. Levels of PCK above 2000 nm/min/ml are considered to indicate the presence of NMD (Whanger et al., 1976, 1977a).

Staging for Comparison Among Lambs of Histologic, Histochemical, and Ultrastructural Results:

Most Se deficient lambs will develop NMD between three and six weeks of age; however, the time of onset is not adequately uniform to allow comparisons between affected lambs merely on the basis of age. Therefore, a point of more definite reference was needed. This reference point was established as the biopsy period when the PCK level of
the lamb reached its highest level (peak PCK). Comparisons were then made among lambs in relation to the number of weeks before or after peak PCK.
RESULTS
Lamb Birth Rate, Survival, Clinical Characteristics and Necropsy Findings in Selenium Supplemented and Selenium Deficient Groups:

The average period of gestation for sheep is approximately 147 days with establishment of the body form and muscle masses complete by 40 days post-conception (Evans and Sack, 1973). All fetuses were between 44 and 52 days gestation at the time the ewes were placed on the experimental diets. The ewes of both groups had adequate blood Se levels at the outset of the experiment (Table 1). Thus, the Se deprivation of the ewe and fetus did not occur during the histogenesis stage of skeletal muscle development, but rather during the period of muscle growth (Campion et al., 1978).

Twenty-nine lambs were born to the Se supplemented ewes, two of which died shortly after birth. The exact cause of death in these two lambs was not determined; however, there was no evidence, either on gross or histopathologic examinations to suggest a diagnosis of NMD. All remaining lambs from the Se supplemented ewes survived to the end of the study. Twenty-eight lambs were born to the Se deprived ewes, 14 of which died. Three of the 14 mortalities occurred at birth and in these individuals there was no evidence of NMD. The remaining 11 lambs died between three and 14 days of age and had clinical, macroscopic and microscopic evidence of NMD. These lambs gained weight slowly and appeared inactive. When exercised, they stumbled, and on occasion collapsed and could not rise. On necropsy, all lambs had pale semitendinosus muscles, pale or white streaks in the myocardium (most obvious in the right ventricle), and ascitic fluid. Some lambs had congested livers and edematous lungs.
Table 1. Mean Whole Blood Selenium Values of Selenium Deficient and Supplemented Ewes After 40, 80, 120, and 160 Days on Experimental Diets.

<table>
<thead>
<tr>
<th>Days on Diet</th>
<th>Selenium (PPM)</th>
<th>Deficient (N = 18)</th>
<th>Supplemented (N = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>.102 ± .024 b</td>
<td>.102 ± .024</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>.052 ± .017</td>
<td>.085 ± .028</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>.031 ± .012</td>
<td>.144 ± .063</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>.026 ± .017</td>
<td>.119 ± .039</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>.015 ± .003</td>
<td>.131 ± .038</td>
<td></td>
</tr>
</tbody>
</table>

a Value represents all ewes (N = 36) prior to random distribution to experimental groups.

b Standard deviation.

Table 2. Mean Whole Blood Glutathione Peroxidase Values of Selenium Deficient and Supplemented Ewes After 40, 80, 120, and 160 Days on Experimental Diets.

<table>
<thead>
<tr>
<th>Days on Diet</th>
<th>Glutathione Peroxidase (nm/min/ml)</th>
<th>Deficient (N = 18)</th>
<th>Supplemented (N = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>62 ± 15 b</td>
<td>62 ± 15</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>67 ± 23</td>
<td>41 ± 17</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>34 ± 16</td>
<td>140 ± 32</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>34 ± 18</td>
<td>150 ± 38</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>18 ± 4</td>
<td>143 ± 37</td>
<td></td>
</tr>
</tbody>
</table>

a Value represents all ewes (N = 36) prior to random distribution to experimental groups.

b Standard deviation.
Selenium deficient lambs which did not die often developed clinical signs similar to those in lambs that eventually died of NMD. All lambs, as peak levels of CK were achieved, appeared either listless or fatigued easily when exercised. Three of the lambs selected for surgical biopsies died at or before eight weeks of age (Lambs #306, 309, and 346) and were not included in the study. These three lambs had lesions similar to those previously reported for NMD. Two lambs (#307 and 345) died at eight and nine weeks of age, respectively, after undergoing four surgical biopsies and were included in the study. Lamb #348 failed to show any elevation in PCK and could not be utilized in this study.

Blood Selenium and Glutathione Peroxidase and Plasma Creatine Kinase Changes in Selenium Supplemented and Selenium Deficient Ewes and Lambs:

The average whole blood Se level of the ewes at the time they were put on experimental diets was 0.102 ppm, and the average whole blood GPx levels was 62 nm/min/ml. After 40 days on the experimental diets, the Se and GPx levels of the Se deficient ewes were markedly lower than the Se supplemented group (Tables 1 and 2). This pattern persisted throughout the remainder of the experimental period. Ewes on the Se deficient diet had marked reductions in whole blood Se with mean values decreasing from 0.052 ppm at 40 days to 0.015 ppm after 160 days on the experimental diet. Ewes on the Se supplemented diet showed slight fluctuations in the blood Se with values ranging from a low of 0.085 ppm at 40 days to a high of 0.144 ppm at 80 days after introduction of experimental diets (Table 1).
Whole blood GPx levels of the ewes corresponded positively to changes in whole blood Se. In the Se deficient group, there was a steady decline in GPx after 40 days on the diet from a mean of 67 nm/min/ml at 40 days to only 18 nm/min/ml at 160 days. This contrasted with mean GPx levels in the supplemented ewes which rose from 41 nm/min/ml at 40 days to 140 nm/min/ml at 80 days and were 150 and 143 nm/min/ml after 120 and 160 days, respectively, on the experimental diet (Table 2).

Whole blood Se levels of lambs at three and six weeks of age demonstrated nearly 10-fold higher levels in lambs from Se supplemented dams. Mean Se values were 0.015 and 0.014 ppm at three and six weeks of age, respectively, in Se deficient lambs and were 0.112 and 0.111 ppm at three and six weeks of age in Se supplemented lambs (Table 3). Mean plasma GPx levels in Se deficient lambs were 21 and 16 nm/min/ml at three and six weeks of age, respectively, compared to means of 161 and 133 nm/min/ml at those same ages in Se supplemented lambs (Table 3).

Plasma CK levels increased in both groups between the ages of three and six weeks of age (Table 3). At three weeks of age, PCK values were similar in both groups, but at six weeks of age, the mean PCK level of the Se deficient lambs was approximately triple that of the Se supplemented lambs at 1785 and 583 nm/min/ml, respectively.

PCK values of lambs at the time of each biopsy are in Tables 4 and 5. Selenium supplemented lambs maintained low PCK levels ranging up to 238 nm/min/ml during the experimental period, except Lamb #321, whose PCK rose to 2186 nm/min/ml at the time of the eighth biopsy at 16 weeks of age (Table 4). Selenium deficient lambs achieved peak
Table 3. Mean Whole Blood Selenium (Se), Plasma Glutathione Peroxidase (GPx) and Creatine Kinase (CK) Values of Selenium Deficient and Supplemented Lambs at Three and Six Weeks of Age.

<table>
<thead>
<tr>
<th>Age</th>
<th>Selenium Deficient</th>
<th>Selenium Supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 Weeks</td>
<td>6 Weeks</td>
</tr>
<tr>
<td>N =</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Se (PPM)</td>
<td>.015 ± .008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.014 ± .007</td>
</tr>
<tr>
<td>GPx (nm/min/ml)</td>
<td>21 ± 9</td>
<td>16 ± 9</td>
</tr>
<tr>
<td>CK (nm/min/ml)</td>
<td>76 ± 51</td>
<td>1785 ± 2964</td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard deviation.
Table 4. Creatine Kinase Levels in Plasma of Selenium Supplemented Lambs at Each Biopsy Period.\(^a\)

<table>
<thead>
<tr>
<th>Lamb No.</th>
<th>Biopsy Period (Age In Weeks)</th>
<th>CK (nm/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(L_1(2)^b)</td>
<td>(R_1(4)^c)</td>
</tr>
<tr>
<td>321</td>
<td>51</td>
<td>58</td>
</tr>
<tr>
<td>322</td>
<td>84</td>
<td>55</td>
</tr>
<tr>
<td>361</td>
<td>30</td>
<td>48</td>
</tr>
<tr>
<td>363</td>
<td>32</td>
<td>47</td>
</tr>
</tbody>
</table>

\(^a\) Blood samples were collected prior to surgery.

\(^b\) \(L_1\) = muscle sample from left semitendinosus, subscript denotes the sample number i.e. 1 = first, 2 = second. First biopsy was taken at 2 weeks of age.

\(^c\) \(R_1\) = muscle sample from right semitendinosus, subscript denotes the sample number i.e. 1 = first, 2 = second.
Table 5. Creatine Kinase Levels in Plasma of Selenium Deficient Lambs At Each Biopsy Period.

<table>
<thead>
<tr>
<th>Lamb No.</th>
<th>Biopsy Period (Age In Weeks)</th>
<th>L₁(2)</th>
<th>R₁(4)</th>
<th>L₂(6)</th>
<th>R₂(8)</th>
<th>L₃(10)</th>
<th>R₃(12)</th>
<th>L₄(14)</th>
<th>R₄(16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK (nm/min/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>302</td>
<td>154</td>
<td>63</td>
<td>2572</td>
<td>7878</td>
<td>1865</td>
<td>5595</td>
<td>563</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td>306</td>
<td>1865</td>
<td>1043</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td></td>
</tr>
<tr>
<td>307</td>
<td>4630</td>
<td>7335</td>
<td>7460</td>
<td>12058</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td></td>
</tr>
<tr>
<td>309</td>
<td>3730</td>
<td>10450</td>
<td>3213</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td></td>
</tr>
<tr>
<td>341</td>
<td>418</td>
<td>498</td>
<td>2512</td>
<td>2830</td>
<td>1607</td>
<td>547</td>
<td>167</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td>342</td>
<td>42</td>
<td>193</td>
<td>3601</td>
<td>1286</td>
<td>450</td>
<td>125</td>
<td>244</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td>343</td>
<td>37</td>
<td>116</td>
<td>3160</td>
<td>1250</td>
<td>480</td>
<td>160</td>
<td>255</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>344</td>
<td>26</td>
<td>257</td>
<td>58</td>
<td>1580</td>
<td>2485</td>
<td>286</td>
<td>472</td>
<td>418</td>
<td></td>
</tr>
<tr>
<td>345</td>
<td>48</td>
<td>174</td>
<td>193</td>
<td>12540</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td></td>
</tr>
<tr>
<td>346</td>
<td>47</td>
<td>6431</td>
<td>3858</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td>--d</td>
<td></td>
</tr>
<tr>
<td>348</td>
<td>34</td>
<td>93</td>
<td>68</td>
<td>86</td>
<td>109</td>
<td>251</td>
<td>154</td>
<td>193</td>
<td></td>
</tr>
<tr>
<td>349</td>
<td>26</td>
<td>296</td>
<td>772</td>
<td>2443</td>
<td>3215</td>
<td>313</td>
<td>174</td>
<td>514</td>
<td></td>
</tr>
<tr>
<td>351</td>
<td>93</td>
<td>1672</td>
<td>434</td>
<td>1543</td>
<td>109</td>
<td>154</td>
<td>67</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>353</td>
<td>29</td>
<td>26</td>
<td>48</td>
<td>1607</td>
<td>135</td>
<td>321</td>
<td>61</td>
<td>206</td>
<td></td>
</tr>
</tbody>
</table>

*a* Blood samples were collected prior to surgery.

*b* L₁ = muscle sample from left semitendinosus, subscript denotes the sample number i.e. 1 = first, 2 = second. First biopsy was taken at two weeks of age.

*c* R₁ = muscle sample from right semitendinosus, subscript denotes the sample number i.e. 1 = first, 2 = second.

*d* Lamb died, no biopsy or CK determination.
PCK values up to 12,540 nm/min/ml between biopsy periods R₁ and R₂ which corresponded with four and eight weeks of age, respectively. In eight of the 14 lambs, the PCK levels steadily decreased following the peak value until the end of the experiment. Lamb #348 never had any significant PCK elevation, and values remained similar to those of controls. Lamb #351 had two PCK peaks, the first at 1672 nm/min/ml at biopsy period R₁ and the second at 1543 nm/min/ml at biopsy period R₂. These peaks occurred at four and six weeks of age, respectively. Likewise, Lamb #302 had a second PCK peak, the first of 7878 nm/min/ml at biopsy period R₂ (8 weeks old) and the second at 5595 at biopsy period R₃ (12 weeks old). Lambs #306, 307, 309, 345, and 346 died soon after peak PCK levels (Table 5).

Two of the four Se deprived lambs which were tested weekly for PCK but not biopsied developed PCK levels that exceeded 20,000 nm/min/ml on the same day. These two lambs were selected to receive Se injections and have muscle biopsies during the recovery phase (Se Dprvd/Inj lambs). The PCK of Lamb #311 was 21,543 nm/min/ml two days prior to surgery but by the day of surgery and Se injection the PCK had decreased to 2379 units. These values steadily decreased to 1994, 707, and 233 nm/min/ml, respectively on the first, second, and third days after Se injection (Table 6). The other Se Dprvd/Inj lamb, #312, had PCK levels of 25,723 nm/min/ml two days prior to surgery and Se injection, 29,904 units on the day of surgery and Se injection, and 54,019 units the day after surgery and Se injection. Thereafter, the PCK values of each lamb decreased daily to 80 and 186 nm/min/ml on the seventh post-injection day (Table 6). Both lambs had a second elevated PCK peak phase with values ranging from 530 to 6366 nm/min/ml.
Table 6. Creatine Kinase Levels in Plasma of Selenium Deficient Lambs Which Were Injected With Sodium Selenite\(^a\) After Onset of Severe Nutritional Muscular Dystrophy.

<table>
<thead>
<tr>
<th>Days Relative to Time of Injection</th>
<th>Biopsy Period (Age in Weeks)</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>311</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK (nm/min/ml)</td>
</tr>
<tr>
<td>-15</td>
<td></td>
<td>61</td>
</tr>
<tr>
<td>-8</td>
<td></td>
<td>2122</td>
</tr>
<tr>
<td>-2</td>
<td></td>
<td>21543</td>
</tr>
<tr>
<td>0</td>
<td>(L_1) (3)</td>
<td>2379</td>
</tr>
<tr>
<td>+1</td>
<td></td>
<td>1994</td>
</tr>
<tr>
<td>+2</td>
<td></td>
<td>707</td>
</tr>
<tr>
<td>+3</td>
<td></td>
<td>233</td>
</tr>
<tr>
<td>+4</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>+5</td>
<td>(R_1)</td>
<td>96</td>
</tr>
<tr>
<td>+6</td>
<td></td>
<td>106</td>
</tr>
<tr>
<td>+12</td>
<td>(L_2) (5)</td>
<td>80</td>
</tr>
<tr>
<td>+19</td>
<td>(R_2) (6)</td>
<td>353</td>
</tr>
<tr>
<td>+26</td>
<td>(L_3) (7)</td>
<td>530</td>
</tr>
<tr>
<td>+33</td>
<td>(R_3) (8)</td>
<td>530</td>
</tr>
<tr>
<td>+40</td>
<td>(L_4) (9)</td>
<td>212</td>
</tr>
<tr>
<td>+47</td>
<td>(R_4) (10)</td>
<td>199</td>
</tr>
<tr>
<td>+54</td>
<td>(L_5) (11)</td>
<td>141</td>
</tr>
</tbody>
</table>

\(^a\) Dose equals 1 mg sodium selenite injected subcutaneously in dorsal cervical region.
starting at 26 days post-injection and continuing until 33 days post-injection (Table 6).

Histologic, Histochemical, and Ultrastructural Characteristics of Muscle from Selenium Supplemented Lambs:

Examination of sections from plastic and paraffin embedded muscle biopsies taken from Se supplemented lambs revealed normal muscle histology. The architecture did not appreciably vary between individuals, nor between muscle samples taken from the same individual at different biopsy periods. Paraffin embedded, HE stained muscle sections were characterized by round to polygonal fibers having one or two peripheral nuclei. The myofiber cytoplasm was characterized as granular, with the appearance of Conheim's fields typical of skeletal muscle. Endomysial connective tissue was scant with some degree of separation between fibers typical of paraffin embedded samples (Figure 1A). Sections from plastic embedded muscle had similar features, although the thinner sections provided greater cellular clarity and detail (Figure 1B).

There were very minimal histopathological changes associated with surgical sites. These consisted of few densely eosinophilic myofibers surrounded by fibrous tissues bordering the site of previous surgery. Such fibers appeared rounded and contained swollen central nuclei. Occasional fibers contained multiple nuclei, and some fibers appeared small and angular, indicating atrophy. Macrophages were at the junctions between zones of fibroplasia and normal fibers with some extension of macrophages into adjacent endomysial spaces. The inflammatory and degenerative changes did not extend beyond a zone of three or four
Figure 1. Photomicrographs of transverse sections of paraffin and plastic embedded muscle from a four-week-old selenium sufficient lamb, HE, X80: A) Characteristic appearance of paraffin embedded muscle with angular myofibers (mf), peripheral nuclei of the myofibers (n) which are indistinguishable from fibroblast nuclei of the endomysium. Artifactual separation (s) is seen between muscle bundles; B) Characteristic appearance of plastic embedded muscle with angular myofibers (mf) more clearly seen and artifactual separation (s); C) Section from plastic embedded muscle taken from a site of previous surgery showing normal myofibers (no) next to a zone of fibrosis (F) occurring as a result of surgery. Macrophage (m) infiltrates are seen near myofibers and between hypereosinophilic necrotic fibers (en).
myofiber thickness (Figure 1C).

Frozen sections stained with HE revealed characteristics comparable to HE-stained paraffin and plastic sections (Figure 2A). The histochemical characteristics of the myofibers conformed with those of other animal species and were consistent between lambs in this group. Table 7 lists the histochemical staining characteristics of Type I and II muscle fibers. No abnormalities were detected in the histochemistry of frozen muscle sections from the Se supplemented lambs. The histochemical reactions further failed to reveal any significant alterations as a result of the surgical procedure. Frozen sections stained by the trichrome method revealed little intermyofiber connective tissue consistent with results of both the HE-stained paraffin and plastic embedded samples. The AcP reactions on muscle samples having evidence of fibrosis and macrophagic activity due to previous biopsies demonstrated intense positive reactions within macrophages and in zones adjacent to areas of fibroplasia. As with the histologic findings, this reaction extended only to a depth of three or four myofibers thickness.

The ATPase stain on muscle biopsies from lambs at two and four weeks of age revealed that there were generally only two or three Type I fibers per muscle bundle (Figure 2B). In serial sections from the same samples, these Type I fibers stained slightly darker for glycogen by the PAS reaction (Figure 2C) and more intensely with NADH-TR reactions (Figure 2D) than did the Type II fibers. No differential staining between fiber types was demonstrated with the AcP reaction (Figure 2E). In muscle biopsies taken at 10 to 16 weeks of age, the numbers of Type I fibers had increased to approximately 5 or

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Fiber Type</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase pH 9.4</td>
<td>Light</td>
<td>Dark</td>
<td></td>
</tr>
<tr>
<td>pH 4.5</td>
<td>Light</td>
<td>Light-Dark</td>
<td></td>
</tr>
<tr>
<td>pH 4.2</td>
<td>Dark</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>NADH-TR</td>
<td>Dark</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>PAS</td>
<td>Dark</td>
<td>Light-Dark</td>
<td></td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>Light</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>Light</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>Esterase</td>
<td>Light</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>Oil Red O</td>
<td>Light</td>
<td>Light</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Photomicrographs of transverse serial sections of frozen muscle from a four-week-old selenium sufficient lamb, X80: A) HE stained section. Type I myofibers (I) are located by comparison; B) ATPase stained section with light staining Type I myofibers (I) and dark staining Type II which comprise the remainder of the muscle bundle; C) PAS stained section with dark Type I myofibers (I) and dark or light staining Type II myofibers; D) NADH-TR stained section with dark staining Type I myofibers (I) and dark or light staining Type II myofibers; E) AcP stained frozen section showing no reaction in either Type I (I) or Type II myofibers.
Figure 2.
more per muscle bundle. The staining characteristics of the histo-
chemical reactions remained consistent in muscle biopsies taken from 
lambs at two weeks through 16 weeks of age except for some hetero-
gegeneity of the PAS reaction observed in muscle sections from lambs at 
2 weeks of age when compared to those biopsies taken at ages of 12 and 
16 weeks. The PAS staining characteristics were consistent in muscle 
biopsies from lambs of comparable age, however.

The ultrastructural characteristics of the muscle of the Se 
supplemented lambs varied little between lambs, and there was little 
age-related change. Mitochondria appeared round or ovoid with usual 
length and width dimensions of 0.07 microns and 0.05 microns, respect-
tively. Occasionally, the length of normal mitochondria increased 
approximately two to three times but their width remained approxi-
mately constant. Most mitochondria were restricted to interfibrillar 
spaces (Figure 3 A and B) and were partially sequestered by both the 
sarcoplasmic reticulum and myofilament bundles; however, some mito-
chondria were observed which traversed myofilament bundles at the zone 
between the Z line and the beginning of the M line (Figure 3B).

Little variation was seen in the sarcoplasmic reticulum of 
control lamb muscle throughout the experimental trial period. Myo-
filament bundles on cross-section varied in cross-sectional dimensions 
and the densities of myofilaments in adjacent muscle fibers also 
varied as a result of different cutting planes (Figure 3C). No 
significant variations were evident in the myofiber nuclei.

Even though Lamb #321 developed elevated PCK levels indicative of 
myopathy (466 at L3, 1067 at L4 and 2186 at R4), there was no evidence 
of myopathy by light microscopic, histochemical, or electron micro-
Figure 3. Electron micrographs of muscle from a four-week-old selenium sufficient lamb: A) Longitudinal section showing the position of mitochondria (m) relative to myofibrils (my) as well as to I(i) and H(h) band positions, X10,000; B) Longitudinal section showing Z lines (z). A sarcomere unit (sarcomere) lies between two Z bands. The I band (i) is always nearest the Z line and the H band (h) in the center of the sarcomere. Mitochondria (m) are present with one mitochondrion traversing the sarcomere at the I band (lower right), X18,000; C) Transverse section showing mitochondria (m) and triads (t), one of which lies in the center of the myofibril (my). Note that the myofibril is surrounded by the sarcoplasmic reticulum (sr) which maintains myofibrillar integrity. Note the stippling effect created by the myofilaments which make up the myofibril, X18,000.
Figure 3.
scopic studies.

Histologic, Histochemical, and Ultrastructural Characteristics of Muscle from Selenium Deficient Lambs:

**Eight Weeks Prior to Peak PCK** - Two lambs, #344 and 349, were biopsied at this period. Both were two weeks of age and both had PCK levels of 26 nm/min/ml. Histologic examination of HE-stained frozen, plastic, and paraffin sections of muscle biopsies revealed no differences from those taken from Se supplemented lambs of equivalent ages. Histochemical and ultrastructural examination of muscle samples revealed no differences from Se supplemented lambs of the same age.

**Six Weeks Prior to Peak PCK** - Eight lambs were biopsied at this period. Lambs #302, 307, 341, 345, 351, and 353 were two weeks old and thus were undergoing their first biopsies. Lambs #344 and 349 were four weeks old and were on their second biopsy. The mean PCK level was 199.3 nm/min/ml. Histologic, histochemical and ultrastructural examination of muscle samples revealed no differences from Se supplemented lambs of the same ages.

**Four Weeks Prior to Peak PCK** - Ten lambs were biopsied at this period. Lambs #342 and 343 were two weeks old (first biopsies). Lambs #302, 307, 341, 345, 351, and 353 were four weeks old (second biopsies). Lambs #344 and 349 were six weeks old (third biopsies). The mean PCK level was 209.8 nm/min/ml. Histologic and histochemical examinations revealed no differences from Se supplemented lambs.

Ultrastructural changes were seen at this stage in all lambs except Lamb #342, which appeared normal. The ultrastructural changes were characterized by swelling of triad sarcoplasmic cisternae,
presence of sarcoplasmic reticular vesicles, and presence of dilated sarcoplasmic reticular cisternae (Figure 4). Increased numbers of electron dense particles, typical of glycogen, were at the periphery of myocytes and between myofilaments (Figure 5A). The mitochondria were swollen with internal circular structures formed by cristal membranes (Figure 5B). Condensed mitochondria characterized by increased density and reduced size were present. These changes were uniformly present in all myofibers.

Two Weeks Prior to Peak PCK - Ten lambs were biopsied at this period. Lambs #342 and 343 were four weeks old (second biopsies). Lambs #302, 307, 341, 345, 351, and 353 were six weeks old (third biopsies). Lambs #344 and 349 were eight weeks old (fourth biopsies). The mean PCK level was 1168.5 nm/min/ml. Histologic examination revealed that in all biopsy specimens a mild mononuclear cell infiltrate was present in the interstitial tissue of the perimysium and endomysium (Figure 6 A and B). The majority of myofibers appeared unaffected during this period but there were many myofibers that were necrotic, as evidenced by loss of internal structure and hypereosinophilia (Figure 6B). There were some degenerative fibers with central nuclei (Figure 6A). There were also low numbers of hypertrophic fibers, many of which were slightly hypereosinophilic and moderate numbers of atrophic fibers which demonstrated normal eosinophilia. There appeared to be some increase in the number of macrophages associated with the capillaries and arterioles, but the vessels were not altered.

Histochemical reactions demonstrated changes in the ATPase, NADH-TR, PAS and AcP staining reactions of low to moderate numbers of
Figure 4. Electron micrographs of muscle from a selenium deficient lamb four weeks prior to peak plasma creatine kinase levels. Longitudinal sections, X18,000: A) Section showing dilated terminal cisternae (tc) of the triad (t) and sarcoplasmic reticular (sr) membranes which are dilated and are formed into vesicles (v) and irregular membranes; B) Section showing slightly dilated terminal cisternae (tc) and sarcoplasmic reticulum (sr) as dilated structures and vesicle formation.
Figure 5. Electron micrographs of muscle from a selenium deficient lamb four weeks prior to peak plasma creatine kinase level, X28,000: A) Longitudinal section showing swollen mitochondrion (ma) with cristae in irregular array and numerous glycogen bodies (arrows). B) Transverse section showing swollen mitochondrion (ma) which contain circular membranous structures. A normal mitochondrion (mn) is present. The sarcoplasmic reticulum (sr) can be seen as small vesicles and dilated cisternae.
Figure 5.
Figure 6. Photomicrographs of transverse section of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb two weeks prior to peak plasma creatine kinase level, X80: A) HE stained plastic section showing hypertrophied myofibers (mn) and mononuclear cell (m) infiltrates into the endomysium. Many myofibers appear rounded (mr) as compared to normal myofibers (mf). Occasional myofibers with central nuclei are present; B) HE stained frozen section showing mononuclear cell (m) infiltrate. Necrotic myofibers (mfn), hypertrophic myofibers (mfh), and normal myofibers (mf) are present. In comparison with Figure 2B, a greater variation in myofiber size is noted; C) ATPase stained frozen section showing normal Type I myofibers (I) and normal Type II myofibers (II). Abnormal Type II myofibers are hypertropic and intense-staining (IIi), atrophic and weak-staining (IIa), or necrotic with irregular staining (IIin); D) PAS stained frozen section showing PAS positive foci in hypertrophic Type II myofibers that stained intensely for ATPase (IIi), and irregular PAS staining in atrophic Type II myofibers that stained weakly for ATPase (IIa); E) NADH-TR stained frozen section showing pale foci in hypertrophic Type II myofibers that stained intensely for ATPase (IIi). Atrophic Type II myofibers that stained weakly for ATPase were intensely stained by the NADH-TR reaction (IIa); F) AcP stained frozen section showing intense staining where macrophages (m) and phagocytized myofibers (mfp) were seen.
myofibers. The majority of the myofibers appeared histochemically normal when compared to Se sufficient lambs of the same age. Frank myofiber necrosis or swelling and nuclear changes were confined to Type II myofibers where the ATPase reaction occurred as a non-uniform accumulation of the reaction product within the necrotic fibers (Figure 6C). With the PAS stain, some necrotic myofibers appeared entirely pale or contained pale foci, while others exhibited intense peripheral staining and pale centers (Figure 6D). The NADH-TR reaction revealed pale or non-staining areas in the necrotic myofibers denoting abnormal mitochondria or abnormal distribution of mitochondria within these myofibers (Figure 6E).

The ATPase stain demonstrated that the hypertrophic and atrophic fibers were all Type II myofibers. Hypertrophic myofibers and some Type II myofibers of normal size stained intensely for ATPase (Figure 6C). The hypertrophic myofibers showed PAS positive foci (Figure 6D), and pale foci when with the NADH-TR reaction (Figure 6E). The ATPase intense but normal sized myofibers were normal in their PAS and NADH-TR reactions. Atrophic myofibers, which were quite inconspicuous on HE sections, were quite prominent by histochemical stains where they exhibited diminished ATPase staining (Figure 6C), diminished but irregular staining with PAS (Figure 6D), and an intense NADH-TR reaction (Figure 6E). Intense staining with the NADH-TR reaction was present in many otherwise normal Type II myofibers, except that these myofibers had intense AcP reactions in both intra-fiber and interstitial zones (Figure 6). No abnormalities were detected in Type I myofibers.

Ultrastructural changes were similar to those at four weeks prior
to peak PCK, the major change being an increased severity of organelle damage. Marked increase in the size of the mitochondria and early signs of mitochondrial rupture were present at this stage (Figure 7A). There was also a paucity of mitochondrial cristae with irregularity in the size and shape of remaining cristae (Figure 7A). The spaces between myofibrils were increased in size and contained membrane fragments which appeared to be from disrupted organelles (Figure 7A and B). Although the myofibrils were not altered structurally, some irregularity of the Z lines suggested early damage of sarcomeres (Figure 7A and B).

Peak PCK Period - Ten lambs were biopsied at this period. Lambs #342 and 343 were six weeks old (third biopsy). Lambs #302, 307, 341, 345, 351, and 353 were eight weeks old (fourth biopsy). Lambs #344 and 349 were ten weeks old (fifth biopsy). The mean PCK of these lambs was 4731.1 nm/min/ml.

The histologic appearance of the muscle at this stage remained unchanged from biopsies taken two weeks prior to peak PCK except for greater numbers of necrotic myofibers. This was accompanied by intense inflammatory changes in perimysial or endomysial spaces that included large numbers of macrophages and mononuclear cells. Marked edema was also present and caused increased interfiber distance. Necrotic myofibers were either very pale (lytic) or deeply eosinophilic with a homogeneous, amorphous character. These are features of Zenker's type necrosis.

Lamb #351 four weeks prior to this period experienced a slightly higher PCK level; however, no histological or histochemical evidence of NMD could be observed. Histologic, histochemical and ultrastruc-
Figure 7. Electron micrographs of muscle from a selenium deficient lamb two weeks prior to peak plasma creatine kinase level. Longitudinal sections, X18,000: A) Section showing normal (m) and disrupted mitochondria (md) which are likewise condensed (mc). The Z lines are greatly distorted as compared with those at four weeks prior to peak PCK (Figure 4). The myofibrils (MYF) have irregular margins, and the intermyofibrillar distance is greatly increased over that seen at four weeks prior to peak PCK (Figure 4). Membrane fragments (mf) are seen in abundance; B) Section showing glycogen (arrows) in the intermyofibrillar zone as well as membrane fragments (mf). The myofilaments (mfil) are no longer tightly organized as in Figure 4.
Figure 7.
Figure 8. Electron micrographs of muscle from a selenium deficient lamb at peak plasma creatine kinase levels. Transverse section: A) Section through macrophage (M) adjacent to severely affected myofiber (upper right). Loss of myofibril (myf) integrity is seen as compared to myofibrils (myf) of myofiber at lower left. Condensed mitochondria (mc) are obvious in this section, X10,000; B) Section showing increased myofibril (myf) dissociation when compared to myofibers at four weeks prior to peak PCK (Figure 5). Condensed mitochondria are obvious (mc) as well as mitochondria showing loss of internal structure (mi).
tural changes paralleled the second peak PCK occurring at eight weeks of age. For this reason, the second peak was considered as more indicative of the course of the disease for this lamb.

The ATPase, NADH-TR, and AcP histochemical reactions on frozen muscle biopsies taken from lambs at this peak were unchanged from the previous period but more swollen, rounded myofibers which were hyper-eosinophilic with the HE stain and darkly stained by the ATPase reaction were seen. The AcP reaction also demonstrated acid phosphatase in these myofibers and decreased acid phosphatase in the endomy-sium around these myofibers.

Ultrastructural changes seen at four and two weeks prior to peak PCK were still present at this period but there were increased numbers of the condensed mitochondria (Figure 8B). Occasional macrophages had phagosomes which contained structures resembling remnants of mitochondrial or sarcoplasmic reticular membranes (Figure 8A). At this stage, the myofibrils appeared to have lost much of their internal organization, with moderate to severe dissociation of myofilaments in which myofibrils appeared spread apart on transverse section (Figure 8B).

Two Weeks After Peak PCK - Eight lambs were biopsied at this period. Lambs #342 and 343 were eight weeks old (fourth biopsy). Lambs #302, 341, 351, and 353 were ten weeks old (fifth biopsy). Lambs #344 and 349 were 12 weeks old (sixth biopsy). Lambs #307 and 345 died at eight and nine weeks of age, respectively. Both lambs had gross and histopathological changes characteristic of NMD. The mean PCK level of the lambs biopsied at this period was 928.2 nm/min/ml. Histologic changes were consistent with those in muscle at the period two weeks prior to peak; however, greater numbers of macrophages were
present infiltrating the endomysium and lytic myofibers and there were increased numbers of hypertrophic myofibers. Histochemical changes remained consistent with those seen at two weeks prior to peak PCK (Figure 9). The number of unaffected myocytes remained in the majority.

The ultrastructural characteristics at this period were very similar to those at the peak PCK period; however, lysis of the sarcomeres and irregularity of Z lines had become much more pronounced. In addition, myelin figures, characterized as bilipid membranes arranged in concentric lamellae, were detected for the first time (Figure 10).

**Four Weeks After Peak PCK** - Eight lambs were biopsied at this period. Lambs #342 and 343 were ten weeks old; Lambs #302, 341, 351, and 353 were 12 weeks old; Lambs #344 and 349 were 14 weeks old. At this period, the plasma PCK values ranged from 154 to 5,595 nm/min/ml with a mean of 1024.8 nm/min/ml. The mean PCK value of the lambs at this period was greater than the previous period because Lamb #302 had a second PCK elevation of 5,595 nm/min/ml at this time.

Histologic examination revealed a markedly diminished inflammatory response in all samples with fewer mononuclear cells and macrophages present. Correspondingly, fewer myofibers undergoing phagocytosis were evident, but the number of hypertrophic hypereosinophilic myofibers remained essentially unchanged (Figure 11 A and B).

Histochemical stains revealed that the number of abnormal Type II myofibers demonstrated by the ATPase and NADH-TR reactions remained unchanged from the two weeks prior to PCK peak period (Figure 11C); however, intense foci of NADH-TR staining were seen in the endomysium at sites where myofibers meet (Figure 11D). There was little extra-
Figure 9. Photomicrographs of transverse section of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb two weeks after peak plasma creatine kinase level, X80: A) HE stained plastic section showing hypertrophic myofibers (mh), atrophic fibers (ma) and mononuclear cell infiltrates (m), similar to changes seen at two weeks prior to peak PCK (Figure 6A); B) HE stained frozen section showing hypertrophic myofibers (mh), only occasional atrophic myofibers (ma), and a mononuclear cell infiltrate (m); C) ATPase stained frozen section showing intensely stained hypertrophic myofibers (III) and weakly staining atrophic myofibers (IIa). The results were not appreciably different than those seen at two weeks prior to peak PCK. Atrophic myofibers are more obvious than in the HE stained frozen section (micrograph B of this figure); D) PAS stained frozen section showing focal accumulations of stain (s) at sites of both hypertrophic and atrophic myofibers; E) NADH-TR stained frozen sections showing irregular deposition of stain throughout as compared to Figure 6E. Myofibers that stained weakly with ATPase (IIa) stain darker for NADH-TR than surrounding fibers; F) AcP stained frozen section showing less endomysial reaction (er) and more intrafiber reaction (ir) as compared with sections taken at two weeks prior to peak PCK (Figure 6F).
Figure 9.
Figure 10. Electron micrograph of muscle taken from a lamb two weeks after peak plasma creatine kinase level, X18,000: Section showing more severe myofibrillar damage when compared with sections taken at two weeks prior to peak PCK (Figure 7). Though the Z lines are present and less distorted than at two weeks prior to peak PCK, they are lysed (Zd) and there is segmental loss of sarcomeres (Sd). Myelin figures (mf) are present and the mitochondria is disrupted (Md).
Figure 10.
Figure 11. Photomicrographs of transverse sections of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb four weeks after peak plasma creatine kinase level, X80: A) HE stained plastic section showing fewer hypertrophic myofibers (mh) and diminished mononuclear cell infiltrate as compared with sections taken at two weeks after peak PCK (Figure 9A); B) HE stained frozen section showing few hypertrophic myofibers and few mononuclear cells. The degree of change is much reduced over that of two weeks prior to peak PCK (Figure 6B) and slightly reduced over that of two weeks after peak PCK (Figure 9B); C) ATPase stained frozen section showing similar reactions to that seen at two weeks after peak PCK (Figure 9C) with hypertrophic intense staining (IIi) and atrophic weak staining (IIa) myofibers. Normal Type I and II fibers are easily seen (I and II respectively); D) NADH-TR stained frozen section showing atrophic intense staining myofibers (IIa) and fewer hypertrophic weak staining myofibers (IIi) when compared to two weeks after peak PCK (Figure 9E). Intense foci of NADH-TR staining (if) present between myofibers is a new feature; E) PAS stained frozen section showing few intense staining foci (arrows) as compared with the previous period (Figure 9D).
fiber AcP reaction, but the intrafiber accumulation of AcP was diminished relative to the previous two weeks prior to peak PCK period (Figure 11F).

The most prominent ultrastructural changes involved the mitochondria which were either markedly swollen (Figure 12A) or condensed (Figure 12B). A few normal mitochondria were seen at this period. Many myofibers contained increased accumulations of glycogen over that seen at four weeks prior to PCK peak. The myofibrils were beginning to recover normal conformation. There was less dissociation of the sarcomeres with the Z lines only slightly altered while I and H bands appeared normal.

**Six Weeks After Peak PCK** - Eight lambs were biopsied at this period. Lambs #342 and 343 were 12 weeks old, Lambs #302, 341, 351, and 353 were 14 weeks old, Lambs #344 and 349 were 16 weeks old and were biopsied for the last time ($R_4$). The mean PCK value was 268.8 nm/min/ml. Histologic examination revealed little change in myofibers when compared to the previous period. Few inflammatory cells remained in endomysial spaces and most myofibers appeared normal, but occasional atrophic and hypertrophic myofibers were present (Figures 13 A and B). The ATPase reaction revealed fewer light-staining atrophic myofibers than at four weeks post peak PCK. At this period, moderate numbers of intense-staining NADH-TR foci appeared in endomysial spaces between normal staining fibers (Figure 13D). These foci did not react with the other histochemical stains and were not noted in the histologic examinations. The PAS reaction demonstrated a few positive intrafiber foci and a general decrease in the number of pale fibers (Figure 13E).
Figure 12. Electron micrographs of muscle from a selenium deficient lamb four weeks after peak plasma creatine kinase level. Longitudinal sections, X18,000: A) Section showing dissolved sarcomeres (sd) but also near-normal sarcomeres (s). Mitochondria are greatly swollen (Ns) but cristae (c) are present that are normal (See C of this figure); B) Section showing condensed mitochondria (Mc) but near normal sarcomeres (s) and less intermyofibrillar distance than seen two weeks prior to peak PCK (Figure 7). The sarcoplasmic reticulum (sr) appears nearly normal; C) Section from Se sufficient lamb taken at 12 weeks of age for comparisons between A and B of this figure. Normal mitochondria are shown (m) as well as normal sarcomeres (s).
Figure 12.
Figure 13. Photomicrographs of transverse sections of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb six weeks after peak plasma creatine kinase level, X80: A) HE stained plastic embedded muscle showing normal myofibers with no mononuclear cells. This section is consistent with that of normal muscle (Figure 1B); B) HE stained frozen section showing only a mild mononuclear (m) infiltrate. Few hypertrophic myofibers can be observed as compared to the previous period (Figure 11B); C) ATPase stained frozen section showing few weakly stained (IIa) atrophic and intensely stained hypertrophic (IIi) myofibers. The number of these myocytes was slightly less than that of the previous period (Figure 11C); D) NADH-TR stained frozen section showing very few intense atrophic myofibers (IIa) and large numbers of intensely stained foci (if) which cannot be recognized as myofibers. The number of these foci was increased over the previous period (Figure 11D); E) PAS stained frozen section showing little intrafiber stain accumulation.
Ultrastructural changes evident at this stage consisted principally of swollen and condensed mitochondria as present in the previous periods (Figure 5B). There were also swollen sarcoplasmic reticular vesicles associated with triads, similar to those present in muscle biopsies beginning at four weeks prior to peak PCK (Figure 4A). Myelin figures were present. The myofilaments appeared architecturally intact on transverse and longitudinal section.

**Eight Weeks After Peak PCK** - Six lambs were biopsied at this period. Lambs #342 and 343 were 14 weeks old; Lambs #341, 351, and 353 were 16 weeks of age. The mean PCK value of the lambs biopsied at this time was 207.2 nm/min/ml.

Histologic examination revealed no changes at this stage. Histochemical reactions revealed that the hypertrophic fibers which had stained intensely for ATPase were no longer present. Few NADH-TR intense fibers remained and there were few NADH-TR intense foci relative to the previous period. Ultrastructurally, at this stage there were sarcoplasmic reticular vesicles as in the previous period. Normal mitochondria were present as well as swollen mitochondria. Some variation in the size of the myofilament bundles which was not seen previously was observed at this period (Figure 14 A, B and C).

The histochemical reactions involving AlP and Es, as well as the trichrome and oil red 0 stains on frozen muscle sections remained unchanged in the Se deficient lambs when compared to Se supplemented lambs of equivalent ages. They were thus normal in all biopsies from lambs at all ages. A summary of the histologic, histochemical and ultrastructural characteristics of the muscle from the Se deficient lambs throughout the experimental period is presented in Table 8.
Figure 14. Electron micrograph of muscle taken from a selenium deficient lamb eight weeks after peak plasma creatine kinase level, X18,000: A) Transverse section showing some irregularity of myofibrils (myf) as compared with normal myofibrils in C of this figure; B) Longitudinal section showing normal sarcomeres (s); however, there is some sarcomere disruption (d) and accumulation of membrane fragments. The damage is much less than that seen at peak PCK (Figure 10) and slightly less than that at four weeks after peak PCK (Figure 12A); C) Transverse section from Se sufficient lamb taken at 14 weeks of age showing normal mitochondria (m) and myofibrils (myf) for comparison.
Figure 14.
Histologic, Histochemical and Ultrastructural Characteristics of Muscle From Selenium Deficient Lambs Which Were Injected With Sodium Selenite at the Time of Acute Myopathy:

Lambs #311 and 312 both developed plasma CPK levels exceeding 20,000 nm/min/ml at three weeks of age. At this time, both lambs fatigued easily and both collapsed when forced to run. They were thus considered to have severe NMD and were selected to receive Se injections and to undergo the surgical biopsy procedures during the convalescent period.

Histologic examination of the muscle biopsy taken immediately prior to injection of sodium selenite revealed lesions more severe than in the other Se deficient lambs. There was extensive infiltration of macrophages and mononuclear cells into the endomysium (Figure 15 A and B). Most of the myofibers were lytic, hypertrophic or atrophic, but there were normal fibers present as well. Many fibers, both hypertrophic and atrophic, had single or multiple central nuclei.

Histochemical staining revealed changes in the PAS, ATPase, NADH-TR and AcP reactions consistent with those observed for non-injected Se deficient lambs at acute stages of NMD, though a greater number of myofibers were affected (Figure 15 C, D, and E). No change occurred with the Es or AlP reactions. Ultrastructural changes were likewise consistent with those of non-injected Se deficient lambs at the most severe stages of NMD (Figure 16).

Five Days After Selenite Injection - At this time, #311 had a PCK level of 96 nm/min/ml, while #312 had a PCK level of 545 nm/min/ml. Both lambs continued to show clinical evidence of NMD. Histologic
Figure 15. Photomicrographs of transverse sections of plastic embedded muscle and serial sections of frozen muscle from a selenium deficient lamb with acute nutritional muscular dystrophy immediately prior to receiving a selenium injection, X80: A) HE stained plastic section showing NMD which is more severe than acute NMD of previous lambs (Figure 6A). Hypertrophic (mh) and atrophic (ma) myofibers can be more easily seen than with the HE stained frozen section (Figure 15B); B) HE stained frozen section showing similar changes as those seen with previous Se deficient lambs at acute stages of NMD (Figure 6B); C) ATPase stained frozen section with changes similar to those seen with previous Se deficient lambs. Hypertrophic intensely staining (III), atrophic weakly staining (IIa), and normal Type II myofibers (II) are seen as well as Type I myofibers (I); D) PAS stained frozen section showing irregular PAS accumulations (s) and fibers with peripheral staining (ps); E) NADH-TR stained frozen section showing some intensely stained atrophic myofibers (IIa) and pale staining hypertrophic myofibers (III).
Figure 16. Electron micrograph of muscle from a selenium deficient lamb with acute nutritional muscular dystrophy immediately prior to receiving a selenium injection: A) Section showing altered mitochondria which are disrupted (md) and condensed (mc). Changes here are consistent with those seen with previous Se deficient lambs at acute stages of NMD (Figure 7); B) Section showing fusion of sarcomeres (s) and irregular Z lines (z). The sarcomeres are indistinct and I and H bands are absent.
Figure 16.
examination revealed a diminished mononuclear cell infiltrate in the interstitial tissue (both endomysial and perimysial) but the number of lytic, hypertrophic, and atrophic fibers remained the same as in the previous period. Histochemical observations were similar to those prior to Se injection except for a decrease in the extrafiber AcP reaction that paralleled the diminished mononuclear and macrophage infiltrates. There were intrafiber PAS positive foci. The ultrastructural characteristics of the muscle were comparable to the previous biopsy.

**Two Weeks After Selenite Injection** - At this period, PCK levels for Lambs #311 and 312 were 80 and 186 nm/min/ml, respectively. Both lambs were four weeks old and no longer exhibited clinical signs of NMD. Histopathologic examination revealed further diminishment of the number of hypertrophic fibers and a further decrease in the mononuclear cell infiltrates. The relative number of necrotic, atrophic, and normal myofibers appeared unchanged. Histochemical stains revealed further diminishment of interstitial AcP and an increase in intrafiber AcP. Fewer intense PAS-positive foci were seen at this period. The number of ATPase and NADH-TR intensely stained myofibers appeared to remain unchanged. Ultrastructural examination revealed that the myofibril bundles had distorted Z lines and occasional lysis of sarcomeres. The mitochondria at this time were swollen or condensed as in the Se deficient lambs at four weeks after peak PCK values.

**Three Weeks After Selenite Injection** - At this period, PCK levels for Lambs #311 and 312 were 353 and 836 nm/min/ml, respectively. These were increased from the previous period, but neither lamb
exhibited clinical signs of NMD. Histologic examination revealed a few hypertrophic or atrophic myofibers but most of the myofibers appeared normal, and there was no evidence of myolysis. There was only mild mononuclear infiltration. The histochemical reactions paralleled those of the previous period, but there were fewer atrophic fibers which reacted weakly for ATPase and stained intensely by NADH-TR and PAS reactions. Present at this stage were some AcP positive myofibers which also stained intensely by NADH-TR. Ultrastructural examination revealed swollen mitochondria with circular inner cristal structures (Figure 17 A and B). The sarcoplasmic reticular cisternae had alterations similar to those in the previous biopsy period but there was less degeneration of myofilament bundles resulting in overall improvement of the myofiber architecture (Figure 17B).

Four Weeks After Selenite Injection - At this period, PCK levels for Lambs #311 and 312 were 530 and 6,366 nm/min/ml, respectively. For both lambs, this was the second PCK peak; however, histologic and histochemical examinations revealed no changes from the previous week. Ultrastructural changes from the previous period included increased numbers of condensed mitochondria, fragmented sarcoplasmic reticulum and some myofibers with complete loss of myofibrillar architecture. Thus, there was increased organelle damage evident at this stage relative to the previous period.

Five Weeks After Selenite Injection - At this period, PCK levels for Lambs #311 and 312 were 530 and 1929 nm/min/ml, respectively. Examinations of muscle from Lamb #311 revealed normal histology and histochemistry as compared to Se supplemented lambs. Lamb #312,
Figure 17. Electron micrographs of muscle taken from a selenium deficient lamb three weeks after receiving a selenium injection at the acute stage of nutritional muscular dystrophy, X18,000: A) Longitudinal section showing normal sarcomeres (s) as compared to those of the deficient lambs prior to injection (Figure 16 A and B). The mitochondria are swollen (ms) and show inner circular membranes (cm) and loss of cristae. No disrupted mitochondria are seen as compared to pre-injection mitochondria (Figure 16 A and B); B) Transverse section showing normal myofibril (myf), condensed mitochondria (mc), and near-normal sarcoplasmic reticulum (sr) as compared to normal myofibers shown in Figure 3C.
Figure 17.
however, had histologic and histochemical changes similar to those at one week after sodium selenite injection. Ultrastructural examination revealed changes in both lambs similar to those of the previous week, except that fewer degenerating myofibril bundles were seen in Lamb #311 as compared with #312.

**Six Weeks After Selenite Injection** - Values of PCK six weeks after selenium injections were 212 and 160 nm/min/ml for Lambs #311 and #312, respectively. Histologic examination revealed that Lamb #311 was normal, but the muscle of Lamb #312 still had a slight mononuclear cell infiltrate. Histochemical ATPase characteristics of both lambs were similar to those at one and two weeks post-Se injection; thus, Lamb #311 appeared to have regressed slightly. Lamb #312 was still more severely affected at this period. Ultrastructural examination revealed no change from the previous week, except that in Lamb #311 the mitochondria were swollen to a greater degree than previously seen in any muscle biopsy from any lamb in the experiment (Figure 18).

**Seven Weeks After Selenite Injection** - At this period, PCK levels for Lambs #311 and 312 were 199 and 122 nm/min/ml, respectively. Histologic and histochemical examinations revealed normal ovine muscle as compared to Se supplemented lambs. The ultrastructural abnormality was swollen mitochondria comparable to those in the previous biopsy.

**Eight Weeks After Selenite Injection** - At this period, PCK levels for Lambs #311 and 312 were 141 and 112 nm/min/ml, respectively. Histologic, histochemical and ultrastructural examinations revealed normal ovine muscle when compared to Se supplemented lambs of the same age except that the mitochondria remained swollen in muscle samples from both lambs.
Figure 18. Electron micrograph of muscle from a selenium deficient lamb taken six weeks after receiving a selenium injection at acute stage of nutritional muscular dystrophy. Longitudinal section, X18,000: Severely swollen mitochondria (MS) are still present but cristae (c) are normal.
DISCUSSION

White muscle disease or nutritional muscular dystrophy (NMD) can occur in lambs receiving adequate Vitamin E but deficient in Se (Muth, 1956; Muth et al., 1961, 1967; Whanger et al., 1977a,b; 1978). In some species, e.g., pigs, a combined deficiency of Vitamin E and Se is necessary to produce NMD (Van Vleet et al., 1976, 1977a,b). Previous histochemical and ultrastructural studies on NMD have been conducted in swine and have involved combined Vitamin E and Se deficiencies (Ruth and Van Vleet, 1974; Van Vleet et al., 1976; Van Vleet and Ferrans, 1976). The study described herein utilized sheep and involved a Se deficient, Vitamin E adequate diet, a situation analogous to that in the naturally-occurring syndrome of sheep (Muth et al., 1967; Whanger et al., 1978).

A unique feature of this experiment was that histologic, ultrastructural, and histochemical analyses were performed on the same biopsy specimens. Because of the individual variation between animals in developing myopathy, utilization of the same muscle biopsy specimen for all three procedures improved the correlation of findings by the three methods. Another novel aspect of this study was the collection of muscle samples from the experimental animals prior to and during the developing stages of NMD. Muscle biopsies were also collected during the recovery stage of NMD, both in lambs spontaneously recovering and in lambs injected with Se during the acute phase of NMD. In previous experiments with sheep and swine, test animals were held on experimental diets until serum enzyme levels were significantly elevated or until they displayed clinical evidence of NMD, before sampling procedures were attempted. Both features occur as a result of
advanced myopathy. In such studies (Ruth and Van Vleet, 1974; Van Vleet et al., 1976; Van Vleet and Ferrans, 1976), only pigs that exhibited clinical signs of myopathy were selected, and from this group, individuals were euthanized at specified intervals after NMD onset for histopathological, histochemical, or ultrastructural evaluation. Thus, these studies only examined the acute stages of NMD in swine. Similar limitations existed for studies on other species (Nafstad, 1971; Van Vleet et al., 1976, 1977a, 1978; Van Vleet and Ferrans, 1976).

The survivability of lambs born to Se deficient ewes was a concern prior to project initiation. In similar previous trials, a high mortality of lambs born to Se deficient ewes was experienced. Since surgical procedures would represent an added stress on the lambs, it was feared that the Se deficient lambs might not survive through enough surgical biopsies to provide meaningful information. Thus, the ewes did receive a low level of Se in their salt (approximately equivalent to 0.02 ppm total diet) during June and July prior to being bred. The Se status of the ewes was therefore declining during the gestation period (Table 1), and the lambs born to Se deprived ewes were deficient in Se at birth, but their liveability was possibly enhanced.

In order to examine the pathogenesis of NMD in lambs, muscle biopsies were collected as early after birth as possible. Thus, the initial biopsies were taken at approximately two weeks of age, which in most lambs was six to eight weeks prior to peak PCK levels (Table 5). By utilizing PCK levels to monitor the severity of myopathy, it was possible to make comparisons among lambs by assigning each biopsy
a time designation relative to the lamb's peak PCK level. This was of considerable value in evaluating histochemical and ultrastructural changes, especially since the age at time of peak myopathy, as indicated by peak CK levels, ranged from 6 weeks to 12 weeks of age.

The biopsy procedures were initiated at two weeks of age for several reasons. First, at birth and during the first week of life, the lambs were probably too weak to withstand surgical procedures. Second, lambs generally begin developing NMD during the fourth and fifth week of life; thus, muscle biopsies taken at one week of age would probably yield little information.

The blood Se and GPx analyses on lambs taken at three and six weeks of age indicated that the experimental diets had the desired effects, i.e., produced Se deficiency which promoted NMD, or alternately, provided adequate Se and prevented NMD. The earliest indication of NMD onset was an elevated PCK which preceded ultrastructural changes by approximately two weeks. Thus, CK loss from the myocytes occurred prior to demonstrable morphologic changes in any organelles. Farber (1981) has suggested that enzyme loss from hypoxic cells is a result of early mitochondrial damage. Ultrastructural lesions were first detected four weeks prior to peak PCK.

The ultrastructural studies of the semitendinosus muscle from Se supplemented lambs revealed features parallel to those of striated muscle from other mammalian species (Ham and McCormack, 1979). Early ultrastructural muscular lesions observed in all Se deficient lambs occurred in the mitochondria, triads, and sarcoplasmic reticulum. The mitochondrial alterations included high amplitude swelling, as described by Hill and LaVia (1975) and Cheville (1976), which is indica-
tive of some alteration in the organelle's ability to regulate its internal osmolarity. Similar changes occur in mitochondria in organs deprived of oxygen (Hill and LaVia, 1975) and in organs to which has been added arsenic, an agent which blocks substrate level phosphorylation (Sodeman and Sodeman, 1974). In both situations, the ability of mitochondria to produce ATP is lost, and ATP is critical to mitochondrial integrity (Hill and LaVia, 1975). Alteration of mitochondrial cristae was also apparent at early stages of NMD and consisted largely of irregular circular structures and loss of cristae. These mitochondrial changes, occurring as one of the earliest morphologic changes in Se-deficiency-induced NMD, are consistent with the suggestion that Se may be an integral part of the mitochondrial structure (Calvin and Cooper, 1979).

In early stages of the myopathy, the sarcoplasmic reticulum developed into membrane-bound vesicles of varying size. Some authors suggest that this represents sarcoplasmic reticular fragmentation (Van Vleet and Ferrans, 1976; Van Vleet et al., 1976, 1977a); however, this change may reflect a collapse of the sarcoplasmic reticular structure such that transection would create the impression of fragmented vesicles. Such a collapse could develop as a consequence of diminished ATP production since the sarcoplasmic reticulum requires ATP for active transport of calcium back into its cisternae following muscle contraction, as well as to maintain existing calcium levels. A loss of ATP would result in calcium loss from the sarcoplasmic reticulum leading to a collapse of the sarcoplasmic reticular structure.

These mitochondrial and sarcoplasmic reticular changes were consistent at four weeks prior to peak PCK levels except in one lamb,
which did not have any ultrastructural aberrations until two weeks prior to its PCK peak. These ultrastructural changes were correlated with sharp rises in PCK levels (Figure 5); however, as noted earlier, PCK was already beginning to rise prior to development of ultrastructural changes. In nine of ten lambs, the ultrastructural lesions preceded histochemical and histologic changes by two weeks; in the remaining lamb, the ultrastructural changes were coincident with histochemical changes. This was consistent with the principle that biochemical lesions precede ultrastructural lesions with histologic changes occurring as a result of continued organelle dysfunction (Hill and LaVia, 1975).

The ultrastructural lesions in the lamb muscle were consistent at the acute stage of NMD with those previously described for pigs (Van Vleet et al., 1977a), chickens (Nafstad, 1971; Van Vleet and Ferrans, 1977) and rabbits (Van Vleet et al., 1978). These earlier studies described mitochondrial and sarcoplasmic reticular changes, but such lesions were associated with extensive changes in other organelles, including myofibrillar lysis and fusion as evidenced by Z line distortion and loss of sarcomere integrity (Figure 10). This study demonstrated that the mitochondrial and sarcoplasmic reticular alterations preceded the myofibrillar and other ultrastructural changes previously reported with NMD.

Glycogen bodies were present in many myofibers of Se deficient lambs at four weeks before peak PCK and through the recovery stage. No mention of this change has been found in ultrastructural studies on NMD of swine, chickens, or rabbits. Accumulation of glycogen bodies has been found in some human genetic muscular dystrophies due to a
loss of the phosphorylase enzyme (Dubowitz and Brooke, 1973); however, mitochondria and sarcoplasmic reticular damage are not features of those dystrophies.

Histochemical studies on sheep skeletal muscle have not been previously reported. In this study, observations on muscle biopsies from normal lambs up to 16 weeks of age revealed no significant differences in the ATPase, NADH-TR, AcP, AlP, and Es reactions relative to those described in other animals (Cardinet and Holliday, 1979). The ATPase reaction showed that the lamb semitendinosus muscle is a mixed muscle composed largely of Type II fibers with only a few randomly distributed Type I fibers (Figure 2B). This suggests that the semitendinosus muscle of sheep is a fast twitch muscle (Guth and Samaha, 1969) and contains only a few slow twitch myofibers. With the NADH-TR reaction, the Type I myofibers were dark staining. The ATPase reaction did not differentiate Type II myofiber subtypes as described in human muscle by Guth and Samaha (1969), nor were the Type II myofiber subtypes described in humans by Barnard et al. (1971) clearly distinguishable by the NADH-TR reaction. The inability to clearly differentiate Type II fiber subtypes has been previously described in animals (Cardinet and Holliday, 1979). The pattern of Type I and Type II fibers in lambs is distinct from that of swine semitendinosus muscle. In swine, Type I fibers are numerous and confined to the central portion of muscle bundles (Ruth and Van Vleet, 1974).

Reactions with the PAS stain demonstrated heterogeneity among Type II myofibers as seen by varied intensity of the reaction in myofibers. This heterogeneous staining was more pronounced in the younger lambs and diminished with age. Such heterogeneity is normal.
in adult human muscle (Dubowitz and Brooke, 1973) and in adult animals
(Cardinet and Holliday, 1979). No studies are available on the histochemo-
cal development of muscle which would assist in interpreting these results.

The AcP reaction for hydrolases did not produce any positive staining reaction in myofibers of muscle biopsies from Se supplemented lambs. This was expected since a positive AcP reaction signifies lysosomal activity either in macrophages or in myofibers. Normal muscle fibers do not show any stainable acid phosphatases (Dubowitz and Brooke, 1973; Cardinet and Holliday, 1979). In some biopsies from Se supplemented lambs, small lesions caused by previous surgery were found, and these areas contained AcP-positive foci.

Esterases which are stainable in muscle are usually cholinester-
as (Dubowitz and Brooke, 1973), and thus motor end plates are the usual sites of intense reaction (Ham and McCormack, 1979). In neuro-
dystrophic muscle, group or bundle atrophy involving large numbers of myofibers is common (Dubowitz and Brooke, 1973). Such muscles appear to have excess acetylcholinesterase which blocks effective neurotrans-
mission. Such an excess is demonstrable by the Es procedure. No excess cholinesterase was demonstrated in muscle from either Se sup-
plemented or Se deficient lambs, although some motor end plates did react as would normally be expected (Dubowitz and Brooke, 1973;
Cardinet and Holliday, 1979).

Oil red O was used to detect excessive levels of fats within myofibers as occur if lipodystrophic-like lesions are present. No such lipid excess occurs in normal muscle (Dubowitz and Brooke, 1973) and none was detected in any of the muscle specimens in this study.
One of the earliest histochemical changes in muscle of Se deficient lambs was with the ATPase reaction which demonstrated atrophic fibers having decreased staining intensity. These fibers were more rounded and the cell outlines were more irregular than in myofibers with angular atrophy of neuromuscular dystrophy (Cardinet and Holliday, 1979). Lytic myofibers, which were greatly diminished in their ATPase staining, were present in the acute stages of NMD. The ATPase reaction is specific for myofibrillar ATPase; thus alterations in the ATPase stain reflect changes in the myosin molecule of which myofibrillar ATPase is an inherent component (Sodeman and Sodeman, 1974). Ultrastructurally, there was damage to the sarcomeres which reflects myofibrillar damage. Thus, the diminished ATPase staining was a consequence of myofibrillar damage. Some hypertrophic myofibers with intense ATPase staining were observed; however, no ultrastructural changes could be demonstrated that could provide a possible explanation for this observation.

As the NMD progressed into acute stages, the predominant myocytic change, in addition to myofiber lysis, was the appearance of atrophic myofibers having weak ATPase stain intensity. This would suggest that myofiber atrophy and lysis are major components of NMD. The numbers of hypertrophic myofibers having intense ATPase staining did increase during the course of the disease, but were never a preponderant feature in acute stages of NMD.

During the recovery stage of NMD, in both the Se-injected and non-injected lambs, there was a reduction in numbers of both intense ATPase-staining hypertrophic myofibers and lytic myofibers; however, the number of atrophic myofibers with faint ATPase staining remained
constant until six weeks after PCK peaks. Since lytic fibers are phagocytized by macrophages (Smith et al., 1972), their disappearance would be expected. Hypertrophy, on the other hand, is a reversible change which can lead to recovery if the initiating cause is removed (Hill and LaVía, 1975). The fate of the hypertrophic myofibers, prominently present at the acute stage of NMD, during the recovery stage could not be ascertained. Since the number of lytic fibers remained constant until late in the recovery stage, some of the hypertrophic fibers must have proceeded to the lytic stage, otherwise the number of lytic fibers should have decreased more rapidly.

This study has shown that Type II myofibers, based on the ATPase stain, were affected in the muscles of lambs with NMD, whereas in a study with pigs (Ruth and Van Vleet, 1974), there was selective destruction of Type I myofibers. The swine in that study were deficient in both Vitamin E and Se, whereas the sheep in this study were deficient only in selenium. Whether this difference had any effect on the type of fibers affected can only be speculated. Ruth and Van Vleet point out that the selective destruction of Type I fibers is a unique situation because in animal muscular dystrophies, the myopathy involves solely Type II fibers in some entities or both Type I and Type II fibers in other myopathies. Van Vleet et al. (1977b) has reported vascular damage in pigs with myopathy caused by Vitamin E and Se deficiency and suggests that some of the myopathy may be secondary to ischemia caused by vascular abnormalities. In this study, no evidence for vascular damage was detected.

Based on the results from the NADH-TR stain, damage to mitochondria can be suggested as a major feature of NMD. Those myofibers
which were lytic exhibited loss of NADH-TR staining visualized as foci within the myofibers devoid of stain product. This type of staining pattern is caused by a loss of mitochondria (Dubowitz and Brooke, 1973). Conversely, those myofibers which were atrophic exhibited intense staining by the NADH-TR stain. Any alteration of the cytochrome chain, which is present in the mitochondrial membranes, will cause increased NADH-TR staining. Thus, increased NADH-TR staining in the atrophic myofibers is indicative of mitochondrial damage (Pearse, 1968, 1972) or increased density of mitochondria within the confines of the myofiber. The ultrastructural examinations did indeed indicate the presence of damaged mitochondria.

As the lambs entered the acute period of NMD, the number of intense NADH-TR staining atrophic fibers increased, and with recovery, the number of such myofibers declined. However, as recovery progressed, indistinct foci, which stained intensely for NADH-TR, appeared in increasing numbers in endomysial spaces. Ultrastructural studies did demonstrate deposits of membrane fragments between myofibers. Thus, these foci probably represented atrophic fibers that had degenerated past the point of recognition but still contained membrane fragments that reacted to the staining procedure. Hypertrophic myofibers, which had stained intensely for ATPase, stained weakly for NADH-TR. This pattern of staining is most indicative of a reduced number of mitochondria in the hypertrophic myofibers or a dilution effect caused by a decreased density of mitochondria within the swollen myofibers.

The PAS technique utilized for this study will demonstrate carbohydrates, particularly glycogen, but simple sugars such as glucose do
not have the capacity to react with PAS (Pearse, 1968, 1972). In myofibers with reduced ATPase intensity and elevated or reduced NADH-TR intensity, irregular accumulations of PAS positive material were observed, indicative of glycogen accumulation in these myofibers. Conversely, those fibers showing increased ATPase intensity and lowered NADH-TR intensity showed a low PAS staining intensity. The more prevalent condition was that of glycogen accumulation seen histochemically and ultrastructurally. Glycogen utilization is not directly dependent on ATP availability (Sodeman and Sodeman, 1974); thus, myofiber accumulation of glycogen cannot be explained directly by the loss of ATP. Many enzyme-substrate interactions require cellular compartmentalization which places the enzyme in close proximity with its substrate. Ultrastructural examinations demonstrated a loss of sarcoplasmic reticulum integrity, and it is this organelle that serves to compartmentalize the myofibers (Ham and McCormack, 1979). Thus, glycogen accumulation may have resulted from the loss of the structural relationships by which glycogen and its catalytic enzyme are normally brought into functional contact.

The primary change associated with AcP was an intense reaction around some of the myofibers starting at two weeks prior to peak PCK. The AcP reaction is indicative of lysosomal activity and is usually associated with phagocytic activity; thus, the increased AcP was reflective of early infiltration of leukocytes or phagocytes around myofibers which were still intact. As the lambs began to recover, the interfiber AcP gradually decreased, but there was some increase in intrafiber AcP reaction, suggesting that autophagocytic activity was occurring (Dubowitz and Brooke, 1973). The presence of the AcP in
some hypertrophic fibers would suggest that irreversible cell injury had occurred in these fibers.

In this study, no significant changes were found in muscles of Se deficient lambs by the AlP, Es, or ORO stains. The lack of changes in AlP activity would suggest that CK is not actively exported since AlP activity usually increases during active membrane transport. This would be consistent with a failure of mitochondria to supply the ATP required for maintaining cellular membrane integrity.

Histologic changes seen in paraffin embedded muscle samples during this study were consistent with changes previously reported for acute NMD (Whanger et al., 1976, 1977a,b, 1978). The number of affected myofibers observed during acute phases of NMD in this study appeared to be lower than reported in the previous studies, but in those reports, the pathology was done primarily on lambs that died of NMD, while most of the lambs examined in this experiment recovered spontaneously. It is possible that the steps taken to promote lamb survival in this study resulted in less severe myopathy. Since neither the atrophic nor hypertrophic fibers appeared capable of recovery, the lamb's recovery from NMD would then depend on the survival of the histologically unaffected fibers. However, ultrastructural examinations revealed that all myofibers were affected; thus fibers which were unchanged histochemically and histologically would have been affected ultrastructurally. Thus, the ultrastructural changes were not irreversible and did not lead necessarily to necrosis of the myocyte.

The spontaneous recovery of the lambs from NMD in this study indicates that additional Se was not essential to the recovery of the
muscle. Indeed, the various examinations revealed parallel features in the muscle of lambs injected or not injected with Se. This may be due to a very low Se requirement by the organism to achieve satisfactory function. Perhaps lambs become more efficient in their recovery or metabolism of Se as they mature. Whatever the reason, the muscle from Se deficient lambs returned to normal, except for the presence of swollen mitochondria, by approximately ten weeks after the peak PCK period. The muscle of lambs injected with Se returned to normal in approximately nine weeks, slightly faster than the non-injected lambs, but the swollen mitochondria persisted in the Se injected lambs also. Apparently, the swollen mitochondria were functional since the myocyte could not otherwise survive and indeed the swollen mitochondria did have normal-appearing cristae at this stage.

The results of this study provide a basis for proposing the following pathogenesis of Se deficiency induced NMD in lambs. A summary of this hypothesized pathogenesis is provided in Figure 19. The effect of Se deficiency on myocytes may be analogous to the effect of hypoxia on cells. However, unlike other cell types, the myocyte is organized structurally and biochemically to control rapid movement of ions moving from one cell compartment to another, particularly sodium ($\text{Na}^+$), potassium ($\text{K}^+$) and calcium ($\text{Ca}^{+2}$) (Ham and McCormack, 1979). The control of these ionic fluxes in the myocyte is largely ATP dependent. The ultrastructural, histochemical, and histologic myocytic changes demonstrated in this study can be explained by diminished capacity of the myocyte to produce the ATP needed to maintain its normal function and structure.
Figure 19. Proposed Pathogenesis of Selenium Deficiency-Induced Nutritional Muscular Dystrophy in Sheep

LYSOSOMES | SARCOPLASM | MITOCHONDRIA | CELL MEMBRANE | SARCOPLASMIC RETICULUM | MYOFIBRILS | OBSERVED MANIFESTATIONS
---|---|---|---|---|---|---
Early Biochemical Lesion ?
Oxidative Phosphorylation

\[ \text{Na}^+ \text{and K}^+ \text{Pump} \]

K\(^+\) Leaks Out
Na\(^+\) and Ca\(^{2+}\) Enter Cell

Membrane Permeability
Dilatation
Loss of Ca\(^{2+}\)

Blood
Plasma CK elevated

Blood
Continued CK rise
Ultrastructure
Mitochondrial swelling, condensation
Sarcoplasmic reticulum swelling, vesicle formation, Triad
Cisternae swelling

Blood
Continued CK rise
Ultrastructure
Mitochondria Fragments
Sarcoplasmic Reticulum Fragments
Glycogen Bodies Accumulate
Histochemical
Changes in NADH-TR and ATPase Reactions

Blood
Peak in Plasma CK
Ultrastructure
Membrane Fragments Present
Myelin Figures Present
Sarcomere Dissolution
Z line Distortion
Histochemical
Changes in NADH-TR, ATPase and AcP
Histopathological
Myocyte Swelling
Myocyte Necrosis
Mononuclear Infiltrate
This study did not attempt to discover the underlying biochemical lesion caused by Se deficiency; however, the results suggest a biochemical lesion in the mitochondria. Such a lesion could result in diminished oxidative phosphorylation and would result in diminished ATP production and availability. Reduced ATP availability would lead to a reduced performance of the myocytic Na\(^+\) and K\(^+\) pump. Selective sarcoplasmic membrane permeability is regulated by the Na\(^+\) and K\(^+\) pump and the presence of elevated PCK as the earliest detectable change in development of NMD, even prior to any ultrastructural lesions, suggests increased permeability as a basic lesion in NMD. Increased sarcoplasmic membrane permeability would be a consequence of lowered Na\(^+\) and K\(^+\) pump effectiveness. A further consequence of the diminished capacity of the Na\(^+\) and K\(^+\) pump would be to allow rapid ionic fluxes both into and out of the cell (K\(^+\) leaks out, Na\(^+\) and Ca\(^+\) leak in). Such ionic changes within cells are known to result in mitochondrial condensation (Farber, 1981; Hill and LaVia, 1975). Mitochondrial condensation was observed as one of the early ultrastructural lesions in this study. Studies on hypoxic myocytes have shown that swelling of the sarcoplasmic reticulum is a result of the failure of the Na\(^+\) and K\(^+\) pump (Farber, 1981) and in this study, such swelling was an early ultrastructural change, particularly in the terminal sarcoplasmic reticular cisternae of the triads.

Another significant consequence of diminished ATP production is the inability of cells to regulate Ca\(^{+2}\), also the result of a diminished Na\(^+\) and K\(^+\) pump capacity. In the myocyte, this diminishment would allow Ca\(^{+2}\) to passively enter the sarcoplasm from both the extracellular interstitial fluids and from the intracellular sarco-
plasmic reticular cisternae. Calcium, a requirement for muscle contraction, is held at high concentrations within the sarcoplasmic reticular cisternae, being released only during muscle contraction (Ham and McCormack, 1979). The sequestration of Ca\(^{2+}\) within this compartment is dependent on the Na\(^+\) and K\(^+\) pump which in turn requires ATP. Calcium is known to activate sarcoplasmic lipases capable of acting on membrane lipids, particularly those of the lysosomes and mitochondria. The result of this activation would be an increased permeability of mitochondrial and lysosomal membranes. A consequence of this increased permeability is mitochondrial swelling (Farber, 1981; Hill and LaVia, 1975) which was also observed in this study as one of the early ultrastructural changes. Lysosomes, though present in myocytes, are infrequent and difficult to locate (Ham and McCormack, 1979), and lysosomal damage was not part of the early ultrastructural lesions observed in this study.

The final consequences of lipase mediated mitochondrial damage are denaturation of mitochondrial membranes and formation of myelin figures. If outer mitochondrial membranes are not affected by denaturation, then early stages of NADH breakdown to yield H\(^+\) ions can occur. However, the conformation of mitochondrial membranes would be altered which can affect cytochrome activity. Diplock (1974a,b; 1975) suggested that cytochrome P\(_{450}\) activity may require the stabilizing effect of Se as an antioxidant, and he demonstrated that without Se, the activity of P\(_{450}\) was reduced. This effect could also be operative with mitochondrial cytochromes. Loss of mitochondrial cytochrome activity would result in the buildup of H\(^+\) ions at the sites of initial NADH oxidation. Intense NADH-TR staining in atrophic myo-
fibers was seen in this study. Increased NADH-TR stainability depends on the presence of an excess $H^+$ ion concentration to reduce the tetrazolium dye to an insoluble precipitate. Conversely, if outer mitochondrial membranes are not preserved following denaturation, complete destruction of the mitochondria results with subsequent formation of myelin figures (Hill and LaVia, 1975). This stage of mitochondrial damage would lead to complete loss of any mitochondrial activity and result in focal loss of NADH-TR staining in myocytes (Dubowitz and Brooke, 1973). During this study, myelin figures were observed ultrastructurally and focal loss of NADH-TR stainability was demonstrated histochemically.

The action of lipases on lysosomes would result in the release of hydrolases and proteases within the myocyte. The release of intrafiber hydrolases would result in AcP positive foci within myofibers (Pearse, 1968, 1972). Such foci were seen in this experiment at acute stages of NMD and during recovery. The release of proteases would further promote breakdown of the sarcomere, leading to lysis and complete loss of the myofiber.

Lipases do not play a role in the damage to sarcoplasmic reticular membranes which are protected against calcium activated lipases (Farber, 1981). The loss of $Ca^{+2}$ however, would result in the collapse of the sarcoplasmic reticulum as discussed earlier, producing the observed vesicles. Additionally, the disruption of the sarcoplasmic reticulum would remove the membranous support around the myofibrils which would then allow the myofibrils to dissociate or spread apart. Dissociation of the myofibrils and subsequent sarcomere dissolution were observed ultrastructurally during this study at acute
stages of NMD. The dissociation of myofibrils would lead to reduced myofibril ATPase activity, most likely a result of altered protein conformation. Green (1975) demonstrated that alterations in sarcoplasmic reticular ATPase conformation will dramatically reduce its activity. Histochemical results from this study showed that in the atrophic fibers, there was a reduction in the ATPase activity. This reduction in ATPase stainability could be accounted for by conformational changes in the myofibrillar ATPase as a result of myofibrillar dissociation.

This study suggests that mitochondrial damage plays a key role in the pathogenesis of Se deficiency-induced NMD in lambs. Ladenstein et al. (1979) have shown that Se is an integral part of the GPx molecule, and Christophersen (1969) has demonstrated that GPx is present in mitochondria; however, the exact functional relationships between Se, GPx, and mitochondria have yet to be fully examined. Further research to elucidate these interrelationships may hold the key to fully understanding the pathogenesis of this disease.
LITERATURE CITED


APPENDIX

Routine Staining and Histochemical Techniques

1. Hematoxylin and Eosin (HE) for Paraffin Embedded and Frozen Specimens

The technique employed for routine Hematoxylin and Eosin staining of paraffin embedded and frozen biopsy specimens during this study is a modification to the technique employed by Armed Forces Institute of pathology (1968).

**Stock Solutions**

A. **Harris Hematoxylin Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin (light crystals)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Potassium alum</td>
<td>100.0 g</td>
</tr>
<tr>
<td>Mercuric oxide (red powder)</td>
<td>2.5 g</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

B. **Eosin Y Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin Y (yellowish)</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>80.0 ml</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>320.0 ml</td>
</tr>
</tbody>
</table>

**Procedures**

A. Section paraffin embedded or frozen biopsy at 6 μm.

B. Remove paraffin by immersion in xylene for 3 minutes; if frozen section proceed directly to step D.

C. Rinse sections in 100%, 95%, 50% ethanol solutions 2 minutes each.

D. Rinse in distilled water for 3 minutes.
E. Immerse sections in Hematoxylin solution for 2 minutes.
F. Rinse in distilled water until no additional stain appears to be removed.
G. Place in tap water for 1 minute.
H. Rinse in distilled water for 1 minute.
I. Place in Eosin Y solution for 2 minutes.
J. Rinse in 95% ethanol until no additional stain appears to be removed.
K. Rinse in two successive changes of 95% ethanol, three successive changes of 100% ethanol for 2 minutes each change.
L. Place in three successive changes of xylene for 2 minutes each change.
M. Mount in Permount (Fischer Products).

2. Hematoxylin and Eosin (HE) for Plastic Embedded Specimens
The technique employed, for routine Hematoxylin and Eosin staining of plastic embedded biopsy specimens, during this study is a modification of the technique for plastic sections utilized by the Armed Forces Institute of Pathology (1968).

Stock Solutions

A. Mayer's Hematoxylin Solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin (dark crystals)</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium iodate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Ammonium alum</td>
<td>50.0 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Chlora hydrate</td>
<td>50.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>
3. Gomori's Trichrome (Trich)

The technique employed, for demonstration of nuclear and connective tissue alterations in frozen biopsy specimens, during this study is a modification of that utilized by the Armed Forces Institute of Pathology (1968) as described by Dubowitz and Brooke (1973).

Stock Solutions

A. Gomori's Trichrome Solution

Fast Green (FCF)  
Chromotrope 2R  

0.3 g  
0.6 g
Phosphotungstic acid 0.6 g
Glacial acetic acid 1.0 ml
Distilled water 100.0 ml

(Adjust pH to 3.4 utilizing 1 N Sodium hydroxide)

B. Harris' Hematoxylin Solution
See section 1.A Stock solutions

Procedures
A. Section frozen biopsy at 6 μm.
B. Immerse in Hematoxylin solution for 5 minutes.
C. Rinse in distilled water until no additional stain appears to be removed.
D. Immerse in Gomori's trichrome solution for 10 minutes.
E. Rinse in distilled water until no additional stain appears to be removed.
F. Rinse in two successive changes of 95% ethanol, three successive changes of 100% ethanol for 2 minutes each change.
G. Place in three successive changes of xylene for 2 minutes each change.
H. Mount in Permount.

4. Periodic Acid Schiff's Reaction (PAS)
The technique employed, for the demonstration of glycogen in frozen biopsy specimens in muscle as described by Dubowitz and Brooke (1973), is a modification of that which is utilized by the Armed Forces Institute of Pathology (1968).

Stock Solutions
A. Calcium Formal Solution
Calcium chloride 1.0 g
Formaldehyde (10% aqueous) super saturated with calcium carbonate 100.0 ml

B. Schiff's Reagent

Basic fuchsin 1.0 g
Potassium metabisulfate 1.0 g
Hydrochloric acid (1 N) 20.0 ml
Distilled water 100.0 ml

(Place in the dark for 48 hours, and then mix with 20.0 g activated charcoal powder and filter with Whatman #1 filter paper)

C. Harris' Hematoxylin Solution

See section 1.A. Stock solutions

Procedures

A. Section frozen biopsy at 6 μm.

B. Fix sections in calcium formol solution which has been pre-chilled to 4°C and adjusted to a pH of 7.0 with 1 N NaOH or 1 N HCl, for 5 minutes.

C. Rinse in three changes of distilled water, 1 minute total time.

D. Immerse in 1.0% periodic acid for 10 minutes.

E. Immerse in Schiff's reagent for 10 minutes.

F. Wash in running tap water for 10 minutes.

G. Immerse in Harris' Hematoxylin solution for 30 seconds.

H. Rinse in tap water for 1 minute.

I. Rinse in distilled water for 1 minute.
J. Rinse in two successive changes of 95% ethanol, three successive changes of 100% ethanol for 2 minutes each change.

K. Place in three successive changes of xylene for 2 minutes each change.

L. Mount in Permount.

5. Oil Red O Method for Fats and Oils (ORO)

The technique employed, for the routine demonstration of fats and oils in frozen biopsy specimens, during this study was a modified technique from that employed by Dubowitz and Brooke as described by Cardinet and Holliday (1979).

Stock Solutions

A. Oil Red O solution

   Absolute isopropyl alcohol saturated with
   Oil Red O Powder 60.0 ml
   Distilled water 40.0 ml

B. Harris' Hematoxylin solution

   See section 1.A Stock Solutions.

Procedures

A. Section frozen biopsy specimen at 6 μm.

B. Fix sections in a 1.0% aqueous osmium tetroxide solution for 5 minutes.

C. Rinse in three successive changes of distilled water, 2 minutes each change.

D. Immerse in Oil Red O solution for 30 minutes.

E. Rinse in distilled water by agitating for 1 minute.

F. Mount in a 1 part gelatin 1 part glycerin solution.
6. Adenosine Triphosphatase Reaction (ATPase)

The technique employed for the demonstration of adenosine triphosphatase present as calcium dependant ATPase in myosin myofilaments, during this study was the same as that described by Dubowitz and Brooke (1973) and Pearse (1972), as modified by Cardinet (1979).

Stock Solutions

A. Incubation Medium (Substrate)

Adenosine triphosphate 15.0 mg
Sodium barbitol 412.4 mg
Calcium chloride 199.8 mg
Distilled water 100.0 ml

B. Preincubation Medium (pH 4.2, 4.5)

Sodium acetate 1.94 g
Sodium barbiturate 2.94 g
Distilled water 100.00 ml

To 50 mls of this solution add:

0.1 M Hydrochloric acid 100.00 ml
Distilled water 80.00 ml

adjust to a pH of 4.5 or 4.2 with 0.1 M Hydrochloric acid.

C. Calcium Formol solution

See section 4.A Stock Solutions

D. Eosin Y Solution

See section 1.B Stock Solutions

Procedures

A. Section frozen biopsy at 6 μ, cut 3 sections to be run at pH 9.8, 4.5, 4.2 respectively; and an additional 9 discards
for test sections, 3 for each pH.

B. Fix sections in calcium formol solution which has been prechilled to 4°C and adjusted to a pH of 7.0 with 1 N NaOH or 1 N HCl, for 5 minutes.

C. Rinse in three successive changes of distilled water, 1 minute total. Sections to proceed at routine pH 9.8, proceed to step E.

D. Immerse sections for preincubation procedures in preincubation media of pH 4.2 and 4.5 respectively for 15 minutes.

E. Immerse all sections in the incubation media, removing test sections at 15 minute internals and proceed with step F through J examining the sections for correct color intensity. Then proceed with F through 0.

F. Rinse in 1.0% calcium chloride for 1 minute.

G. Immerse in 2.0% cobaltous chloride for 2 minutes.

H. Rinse in three successive changes of distilled water, 1 minute total.

I. Immerse in 2.0% ammonium sulfide for 3 minutes.

J. Rinse in three successive changes of distilled water, 1 minute total.

K. Immerse in Eosin Y solution for 3 minutes.

L. Rinse in three successive changes of 95% ethanol, 1 minute total.

M. Place in three successive changes of 100% ethanol for 2 minutes each change.
N. Place in three successive changes of xylene for 2 minutes each change.

O. Mount in Permount.

7. Nicotinamide adenine Dinucleotide - Tetrazolium Reductase (NADH-TR)
   The technique employed for the demonstration of diaphorases and tetrazolium reductases present at the mitochondria in frozen biopsies, during this study is a modification of that described by Barka and Anderson (1963).

Stock Solutions

A. Incubation Medium (Substrate)
   Para-nitro blue tetrazolium chloride 100.0 mg
   0.1 M Tris-HCL Buffer pH 7.4 100.0 ml

   Adjust solution to a pH of 7.4 and add 10.0 mg/10.0 ml of DPNH-β-Nicotinamide adenine dinucleotide just prior to use.

B. Calcium Formol solution
   See 4.A Stock Solutions

Procedures

A. Section frozen biopsies at 6 μm.

B. Fix sections in calcium formol solution which has been prechilled to 4°C and adjusted to a pH of 7.0 with IN NaOH or IN HCL, for 3 minutes.

C. Immerse in incubation medium for 1 hour.

D. Rinse in three successive changes of distilled water, 1 minute total.
E. Rinse in two successive changes of 95% ethanol, three successive changes of 100% ethanol for two minutes each change.

F. Place in three successive changes of Xylene for 2 minutes each change.

G. Mount in Permount.

8. Esterase

The technique employed for the demonstration of nonspecific esterases in frozen biopsies during this study is the same as that reported by Pearse (1968).

Stock Solutions

A. Incubating medium (Substrate)

- 4.0% Sodium nitrite 0.8 ml
- Pararosaniline hydrochloride 2.0 g
- Distilled water 40.0 ml
- Concentrated hydrochloric acid 10.0 ml

Filter with Whatman #1 filter paper and add to a solution of:

- 0.1 M Tris HCL Buffer pH 7.4 20.0 ml
- 1.0% alpha-Napththol acetate 0.5 ml

Bring to a pH of 7.2 with 0.1 N NaOH and filter with Whatman #1 filter paper.

Procedures

A. Section frozen biopsy at 6 μm.

B. Immerse sections incubation medium for 1 hour.

C. Wash in running tap water for 10 minutes.
D. Rinse in 95% ethanol for 1 minute.
E. Rinse in distilled water for 1 minute.
F. Immerse in Harris' Hematoxylin solution for 30 seconds.
G. Rinse in tap water for 1 minute.
H. Rinse in distilled water for 1 minute.
I. Mount in a 1 part gelatin 1 part glycerin solution.

10. Acid Phosphatase

The technique employed for the demonstration of nonspecific acid phosphatases in frozen biopsies during this study is as described by Barka and Anderson (1963).

Stock Solutions

A. Incubating Medium (Substrate)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthol AS-BI phosphoric acid</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>N, N-dimethyl foramide</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>0.2 M Sodium acetate</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>15.0 ml</td>
</tr>
</tbody>
</table>

Add to the above:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0% Para-rosanaline hydrochloride</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>4.0% Sodium nitrite</td>
<td>0.8 ml</td>
</tr>
</tbody>
</table>

Bring to a pH of 5.0 with 1 N Sodium hydroxide, and filter with Whatman #1 filter paper.

B. Harris' Hematoxylin Solution

See section 1.A Stock Solutions

Procedures

A. Section frozen biopsy at 6 μm.
B. Immerse sections in incubating medium for two hours at 37°C.
C. Rinse in tap water for 10 minutes.
D. Rinse in distilled water for 1 minute.
E. Immerse in Harris' Hematoxylin solution for 15 seconds.
F. Rinse in distilled water for 1 minute.
G. Rinse in tap water for 1 minute.
H. Rinse in distilled water for 1 minute.
I. Mount in a 1 part gelatin 1 part glycerin solution.
### Histologic Features

**Histologic Features**

**SIX WEEKS AFTER PEAK PCK**
- Few multinucleated cells present
- Mitochondrial swelling observed
- Mitochondria
- Myofibrils
- Sarcoplasm
- Sarcoplasmic Reticulum

**FOUR WEEKS AFTER PEAK PCK**
- Few multinucleated cells present
- Myofibrils
- Sarcoplasm
- Sarcoplasmic Reticulum

**TWO WEEKS AFTER PEAK PCK**
- Few multinucleated cells present
- Myofibrils
- Sarcoplasm
- Sarcoplasmic Reticulum

**ONE WEEK PRIOR TO PEAK PCK**
- Few multinucleated cells present
- Myofibrils
- Sarcoplasm
- Sarcoplasmic Reticulum

**SIX WEEKS PRIOR TO PEAK PCK**
- Few multinucleated cells present
- Myofibrils
- Sarcoplasm
- Sarcoplasmic Reticulum

**TWO WEEKS PRIOR TO PEAK PCK**
- Few multinucleated cells present
- Myofibrils
- Sarcoplasm
- Sarcoplasmic Reticulum

**Table 1. Summary of Histologic, Histochemical and Immunohistochemical Changes in Selenium-Deprived Lambs During Developmental, Peak and Recovery Stages of Nutritional Muscular Dystrophy**

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>Histologic Features</th>
<th>Histochemical Features</th>
<th>Immunohistochemical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two weeks prior to peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Recovery stage</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
</tbody>
</table>

**Table 2. Summary of Histologic, Histochemical and Immunohistochemical Changes in Selenium-Deprived Lambs During Developmental, Peak and Recovery Stages of Nutritional Muscular Dystrophy**

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>Histologic Features</th>
<th>Histochemical Features</th>
<th>Immunohistochemical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two weeks prior to peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Recovery stage</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
</tbody>
</table>

**Table 3. Summary of Histologic, Histochemical and Immunohistochemical Changes in Selenium-Deprived Lambs During Developmental, Peak and Recovery Stages of Nutritional Muscular Dystrophy**

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>Histologic Features</th>
<th>Histochemical Features</th>
<th>Immunohistochemical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two weeks prior to peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Recovery stage</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
</tbody>
</table>

**Table 4. Summary of Histologic, Histochemical and Immunohistochemical Changes in Selenium-Deprived Lambs During Developmental, Peak and Recovery Stages of Nutritional Muscular Dystrophy**

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>Histologic Features</th>
<th>Histochemical Features</th>
<th>Immunohistochemical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two weeks prior to peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Recovery stage</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
</tbody>
</table>

**Table 5. Summary of Histologic, Histochemical and Immunohistochemical Changes in Selenium-Deprived Lambs During Developmental, Peak and Recovery Stages of Nutritional Muscular Dystrophy**

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>Histologic Features</th>
<th>Histochemical Features</th>
<th>Immunohistochemical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two weeks prior to peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Peak PCK</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
<tr>
<td>Recovery stage</td>
<td>Few multinucleated cells present</td>
<td>Few interstitial foci</td>
<td>Few interstitial foci present</td>
</tr>
</tbody>
</table>