EFFECTS OF SELENITE UPON THE CHEMICAL NATURE OF TISSUE ABNORMALITIES IN WHITE MUSCLE DISEASE OF LAMBS

by

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A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1960

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Date thesis is presented August 13, 1959

Typed by Verna Anglemier

ACKNOWLEDGMENT

Sincere gratitude is tendered the members of the faculty of Oregon State College, who inspired the author to overcome the many obstacles encountered during this period of graduate study.

Special appreciation is extended to Dr. J. E. Oldfield for his stimulating encouragement and advice, and for his assistance in the preparation of this manuscript.

The technical assistance in the laboratory by Mrs. Ngare Alexander is deeply appreciated; her pleasing personality turned the drudgery of laboratory work into a most pleasant interlude.

Gratitude is also expressed to Dr. O. H. Muth for his help in treating the animals and collecting tissues and for his histological diagnosis of white muscle disease, and to Mr. C. Dale Hays for preparing tissue sections for histological examination. Thanks are also due to Mr. Phil Williams, who fed and cared for the experimental animals. His interest in the project and his excellent management of the livestock were a large factor in the success of the experiments.

Finally, my humble thanks to the most deserving one of all, whose self-sacrifice, patience, understanding, and encouragement made this study possible: my wife, Gloria.

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INTRODUCTION

Muscular dystrophy has been reported in many species, including man, and although it has been studied for many years an absolute prevention or cure has not been established. One reason for the lack of resolution of this problem is probably the tendency to group as "muscular dystrophy" any abnormal condition of muscular tissue, including lesions, degeneration, necrosis, and calcification. It is quite likely that the dystrophies observed in different species under varied types of nutritional status are different specific entities, involving various metabolic abnormalities.

Dystrophy by definition implies defective or perverted nutrition. Thus, muscular dystrophy embodies many types of nutritional myopathies, or diseases of muscles caused by faulty nutrition.

White muscle disease (WMD) is a type of muscular dystrophy defined by Muth (46, p. 231-234) as "a specific myopathy of lambs and calves resulting when the dams have been fed during gestation on feeds, especially legumes, grown in certain areas". It may or may not be of the same etiology as related dystrophies occurring in a variety of

species in different parts of the world.

A method of prevention or cure of WMD would be of considerable economic importance to livestock producers in many parts of the world. In certain areas of Oregon there have been instances where 50 per cent or more of the lambs in a band have been affected, and losses of practically all of the calves in small herds kept on irrigated areas has been a common occurrence (44, p. 355).

The muscle degeneration observed in lambs affected with WMD is usually accompanied by the deposition of inorganic salts in the muscle fibers. The present study was designed in an attempt to elucidate the nature and cause of tissue changes, especially the mineral deposits in striated muscle tissue of lambs afflicted with experimentally produced white muscle disease.

Embodied in this study is a survey of the literature, sheep feeding trials, and chemical and histochemical determinations of skeletal and cardiac muscle tissue and blood plasma.

The review of literature includes a brief history of muscular dystrophy, clinical and histopathological characteristics, the use of vitamin E and selenium as protective agents, and a discussion of bone salt formation and pathogenic calcification of soft tissues.

The feeding trials include experimental production of WMD in lambs for two consecutive years (1957-58 and 1958-59). Ewes were fed dystrophogenic feeds during gestation and for a short time after parturition to produce the disease in their lambs, and the preventive effects of vitamin E and selenium through various routes of administration were studied.

Chemical analyses consisted of determinations of dry matter, ether extract, ash, cholesterol, calcium, phosphorus, magnesium, sodium, and potassium in skeletal and heart muscle, and glycoproteins and alkaline phosphatase activity in blood plasma.

Histochemical techniques were applied to muscle tissue sections in an effort to demonstrate enzymic control of calcification of soft tissues.

REVIEW OF LITERATURE

Clinical and Histopathological Characteristics of White Muscle Disease

According to Muth (44, p. 355-361), white muscle disease in lambs and calves may occur from before birth to several months of age, but is most frequently observed in lambs at 3 to 4 weeks and calves at 4 to 6 weeks of age. The disease is characterized by impaired voluntary movement and locomotion as a result of muscular degeneration. Other symptoms depend upon the location and extent of the myopathic lesions. Some individuals display a relaxation of the shoulder girdle and splayed toes. There may be a tendency to stand with feet placed closer together than normal, resulting in an arched back.

If the cardiac muscles are affected, dyspnea and general weakness occur, frequently followed by death. This often happens in conjunction with exercise when the young animals are turned out in the sunshine on warm spring days.

Upon necropsy, the heart, skeletal muscles or both display lesions which vary from small to large discolored areas having a light to a distinct bleached appearance. Hemorrhage and edema may be present. Skeletal lesions are usually bisymmetrical and may range in severity from

involvement of a few muscles to a degeneration of almost all of the striated musculature.

Histological studies of tissue in WMD cases show two distinct types of lesions. In one type the architecture of the muscle fibers may be retained but varying degrees of deposition of calcium salts occur. The calcification may be so extensive as to entirely fill the sarcolemma. In the other type of lesion there is usually little or no calcification. The muscle fibers lose their identity and decompose into an amorphous mass. This type of lesion is often termed a "hyaline degeneration" of muscle tissue (44, p. 355-361).

Historical Aspects of Muscular Dystrophy and White Muscle Disease

Muscular dystrophy of domestic animals was described in 1886 in Germany as a "wachsartige" degeneration in calves, sheep, and swine (28, p. 565-593). It was termed "Fischfleisch" or "Weissenfleisch" by the early workers and thought to be caused by a sarcosporidian which is a parasitic protozoan that invades the muscles and connective tissue of domestic animals.

The disease has an even longer history in the United States. It was reported in 1863 by Randall (58, p. 155-156), who described a condition which he called

"rheumatism". Randall's description seems to fit that of the "stiff-lamb disease" which was first studied at Cornell University in 1927 by Metzger and Hagan (40, p. 35-44). These investigators were unable to transmit the disease by inoculation, thus they concluded it was due to improper feeding and management.

On the basis of Metzger's conclusion, Willman et al. (84, p. 1-20) inaugurated a series of feeding trials in an attempt to determine the cause of stiff-lamb disease by producing it under experimentally controlled conditions. Farmers in upper New York state had reported that the disease seemed to be quite prevalent when the ewes were fed alfalfa hay and cull beans during gestation, so Willman based his experimental rations on these feeds and consistently produced the disease in lambs from ewes that were fed oats, barley, cull beans, and second cutting alfalfa hay. No stiff lambs resulted when the ewes were fed rations consisting of oats, wheat bran, corn silage, and mixed hay.

In Scotland, it has been a common practice to rear dairy calves on skim milk diets fortified with cod-liver oil. As a great deal of muscular dystrophy was observed in calves raised in this manner, Blaxter et al. (4, p. 34-50) initiated a program designed to determine the cause of the disease. The cod-liver oil was suspected and trials

were conducted using cod-liver oil at levels recommended for use in Great Britain. Groups of calves were fed 750 grams of dried skim milk and 0, 1, 2, or 4 ounces of cod-liver oil daily. A severe dystrophy was produced in all groups that were supplemented with cod-liver oil and the control calves developed normally. On the basis of the results of research carried out by the Cornell and Scotland groups, it was apparent that these myopathies had an origin from faulty nutrition.

Muscular dystrophy of domestic animals has been recognized in various parts of the world, including France, Germany, Scotland, New Zealand, Canada, and the United States (50, p. XXI-1 - XXI-4). In the United States it appears to be more prevalent in the northern areas where either rainfall or irrigation result in fairly high soil moisture during the forage growing season and where winter conditions make it necessary to feed cured roughages during a large part of the gestation period of most domestic animals.

Some type of muscular dystrophy has been reported to occur in at least nineteen different species of animals including all the common domestic farm animals and most commonly used experimental laboratory animals (20, p. 5).

White muscle disease was first observed in Oregon in 1928 (52, p. 1-15) and in 1953 the Oregon Agricultural

Experiment Station inaugurated an intensive research project to study the problem. The program developed cooperatively among the Departments of Veterinary Medicine, Dairy and Animal Husbandry, and Agricultural Chemistry. When the program was established it was designed around three main objectives: (1) To produce the disease experimentally under Oregon conditions, (2) to study in detail the nature of WMD and the chemical and histopathological effects on experimental animals, and (3) to effect a cure or prevention that would be of practical use to the livestock producers of Oregon.

Effects of Vitamin E on Muscular Dystrophy

It was learned early at Cornell that "stiff lamb disease" could be produced in lambs and calves from dams that had been fed, during pregnancy, rations composed largely of legume feeds such as alfalfa hay and cull beans (82, p.1-20). After experimenting with many different feeds Willman and coworkers (85, p. 128-130) learned that wheat germ meal and wheat bran seemed to help prevent the "stiff" lambs. Wheat germ meal and oats, creep fed to lambs, prevented stiff-lamb disease; or drenching lambs with wheat germ oil provided a high incidence of cure in stiff lambs. Wheat germ, reputed to be an excellent source of vitamin E, led Willman to try treatments with pure tocopherols and he met with considerable success.

Dystrophic lambs made rapid recovery when drenched with two milliliters of olive oil containing 140 milligrams D,L-alpha-tocopheryl acetate or injected subcutaneously with tocopherols. Thus, with the report of the protective action of tocopherols in 1945 from the Cornell Station many workers throughout the world began to study the therapeutic value of vitamin E in the treatment of muscular dystrophy.

At about this same time Schwarz (61, p. 109-116) discovered that vitamin E would cure nutritional liver necrosis in rats that had been produced by feeding European yeasts. This related the two diseases together and has had a great bearing on the recent work with nutritional myopathies. The importance of relating these two diseases together is discussed in detail in the next section of this dissertation.

Many other treatments have been proposed for the prevention of muscular dystrophy such as supplementation of the diet with sulfur containing amino acids, particularly cystine; potassium supplementation, and balancing the calcium-phosphorus ratio (20, p. 5-39). However, the great volume of experimental work indicated that avitaminosis E was one of the major factors involved (3, p. 1-21).

There is evidence, however, which suggests that WMD

is not an uncomplicated vitamin E deficiency, at least as it occurs in Oregon. WMD has been experimentally produced in calves (49, p. 211-214) and lambs (50, p. XXI-1 - XXI-4) by feeding alfalfa or Ladino clover hay from "white muscle areas" of central Oregon, whereas the incidence of WMD has been low when grass hays and grains have formed most of the ration. According to Morrison (43, p. 143) legumes contain more than adequate amounts of vitamin E and are better sources of the vitamin than grasses, but legumes are more prone to cause WMD than grass hays.

Recently the Cornell Station has expressed some doubt that their stiff lamb disease is the result of a simple vitamin E deficiency because blood tocopherol levels of all animals were quite similar regardless of the percentage of stiff lambs (30, p. 1051-1052). Further, in Oregon experiments, supplementation of the diets of ewes during pregnancy with vitamin E has not proved preventive against WMD in their lambs (45, p. 1090), however, the results of the present study indicate that massive doses of vitamin E given directly to the lambs provide protection from WMD.

Shortly following the revelation that WMD is more than a simple vitamin E deficiency other experiment stations began reporting similar results. Keith and Schneider (34, p. 519-522) in 1957 stated that afflicted calves and lambs were being produced from dams that were fed hay

mixtures that showed, upon analysis, total tocopherol levels of 80 to 154 micrograms per 100 grams hay. These levels are presumably sufficient to exclude a tocopherol deficiency as being responsible for the disease.

Hartley and Dodd (26, p. 61-66) have just published an account of the observations of WMD as it occurs in New Zealand. They are of the opinion that the availability of high-quality pasture during the late stages of pregnancy and throughout lactation suggests that, if vitamin E deficiency is associated with the New Zealand disease, complicating factors are present.

In the case of WMD in New Zealand, the presence of presumably adequate levels of tocopherols in the legume pastures on which ewes and lambs graze, and in the blood plasma of ewes with affected lambs, suggests the possibility of anti-vitamin E substances in the ewe's milk.

A new approach to the white muscle problem was generated by the work of Schwarz on liver necrosis in rats. The following is a discussion of the development of the discovery of the possible essentiality of an element hitherto unrecognized as having any beneficial function in the animal body.

Effects of Selenium on Muscular Dystrophy

Schwarz (61, p. 109-116) was studying dietary hepatic necrosis of rats in Germany and routinely produced the

disease experimentally by feeding rats European yeasts as the primary source of protein. In extending his liver degeneration studies in America, Schwarz discovered that brewers' yeasts would not produce the disease but that Torula yeast would (62, p. 818-823). Dam et al. (13, p. 493-494) suggested that Torula yeast may exhibit dystrophogenic properties because it contains from 4.5 to 5.3 per cent unsaturated fatty acids and brewers' yeast contains less than 0.01 per cent which would tend to produce a vitamin E deficiency. However, Gitler et al. (23, p. 399-408) demonstrated that the presence of a factor in yeast which prevents liver necrosis in rats was dependent on the growth media. Brewers' yeast lost the protective activity when grown on purified media and Torula acquired it when grown on beer wort.

These findings stimulated a series of experiments to determine the difference between the yeasts and Schwarz (63, p. 852-856) isolated a factor that contained the substance that protected rats from liver necrosis. This factor in brewers' yeast was not vitamin E or cystine, and was water soluble, stable against acid hydrolysis and was not identical with any one of the presently well-known vitamins. Schwarz termed this yeast fraction "Factor 3".

Stokstad et al. (75, p. 1160) working on exudative diathesis of chicks on the basis of Schwarz's results,

learned that Factor 3 would prevent this chick disorder. Exudative diathesis, an edematous condition of the breast, had a point in common with liver necrosis in rats in that vitamin E was effective in the cure and prevention of both maladies.

Scott et al. (68, p. 1155-1156) also working on exudative diathesis in chicks discovered that chicks fed Torula yeast required approximately 15 I.U. of vitamin E per pound of diet, but that many practical rations which contained less tocopherol have prevented all symptoms of vitamin E deficiency. These results further demonstrated not only that Factor 3 was something other than vitamin E but also that the etiology of exudative diathesis involved more than a simple vitamin E deficiency. Furthermore, Scott observed that the hexane-insoluble fractions of most feedstuffs had more Factor 3 activity than the hexane-soluble fractions, and being fat soluble, vitamin E would be concentrated in the hexane-soluble fractions.

The American Cyanamid Company became interested in Schwarz's Factor 3 and Stokstad (74, unpublished data) learned that the ash of kidney hydrolysates had Factor 3 activity when ashing was done in the presence of calcium oxide but was inactive when sulfuric acid was substituted. This indicated that the activity was due to an inorganic element which formed a volatile inorganic acid and a

non-volatile salt.

Schwarz and Foltz (65, p. 3292) in following up their investigation of Factor 3 found two chemically-related substances in different fractions, which they termed alpha- and beta-Factor 3. The alpha isomer was found to be water soluble, anionic, sensitive to reducing agents, and to lose its activity on dry ashing. The presence of a garlic-like odor upon the addition of alkali suggested the presence of selenium. Inorganic selenium salts were then tested and proved "remarkably effective" in protecting against necrotic liver degeneration. As little as 13.33 micrograms of sodium selenite per 100 grams of diet were completely protective. Potassium selenate was less effective, and potassium tellurite and sodium arsenate had no protective activity. Selenium, as sodium selenite, was 500-1000 times as active as vitamin E.

Until this time very little thought had been directed toward the idea that selenium might have a specific function in living organisms. In fact, most of the experimental work with selenium had been concerned with its toxic effects in excess, which caused "blind staggers" or "alkali disease" (78, p. 165-220) in domestic animals. However, in 1941 Poley and coworkers (56, p. 171) did report that subtoxic levels of selenium produced a positive growth response in chicks, but this statement went

unheeded in view of the overwhelming interest in the problems of selenium toxicity.

The first positive recognition that selenium may be an essential nutrient was made by Pinsent (55, p. 10-16) in 1954. She reported that selenite (equivalent to 3-8 millimicrograms of selenium per milliliter of media) and molybdate were essential for the formation of formic dehydrogenase by Escherichia coli. Selenite was not replaceable by selenate, tellurite, or tellurate; and molybdate was not replaceable by vanadate, chromate, tungstate, or uranyl ions in the media.

As the concept developed that selenium might possibly have an essential physiological role in animal life, many workers began investigating nutritional disorders from the selenium point of view. Levels of selenium from 0.08 to 5.0 parts per million and various forms such as selenite, selenate, selenocystine, selenocystathionine, and selenomethionine were tested and they all met with great success in the prevention of exudative diathesis in chicks (13, p. 493; 14, p. 494; 48, p. 601-618; 54, p. 617-620; 59, p. 590-593; 64, p. 621-625).

The success of these trials led Scott et al. (69, p. 387-402) to try Schwarz's Factor 3 on enlarged hock disorder in turkeys. From Dam's unsaturated fatty acid work (12, p. 193-211) and Schwarz's yeast work Scott set up

turkey rations consisting of Torula yeast and vitamin E-free lard which caused the enlarged hock disorder to occur. The addition of brewers' yeast or hexane-extracted brewers' yeast to the experimental rations prevented the disease from occurring. The hexane extracts of brewers' yeast offered no protection thus demonstrating that the protection was not provided by vitamin E, although tocopherol injections would cure the disorder in turkey poults. Factor 3 (presumed to be selenium) would now prevent or cure three seemingly unrelated diseases.

Eggert et al. (19, p. 1037) expanded the selenium work to nutritional studies with swine. Eighteen 2-3 week old pigs were divided into three lots which were fed a basal ration containing Torula yeast; basal plus 40 p.p.m. alpha-tocopherol acetate, and basal plus 1 p.p.m. selenium, as sodium selenite, respectively. Within 53 days 4 of the 6 controls died, and marked necrosis of the liver was observed. On the other hand, the pigs receiving vitamin E or selenium showed good growth and feed efficiency. When they were slaughtered after 57-66 days there was no evidence of liver necrosis.

The appearance in the literature of reports concerning the remarkable success with selenium in treatment of diseases which had been shown to respond to vitamin E prompted Muth, Oldfield, Schubert, and Remmert (45, p.

1090) to try both selenium and vitamin E as protective agents against white muscle disease in lambs in 1957-58 trials at the Oregon Station. A high incidence of white muscle disease occurred in the non-treated and tocopherol-treated lots but only three of sixteen lambs in the selenium-treated lot were diagnosed as positive for WMD. In these three, the WMD lesions were microscopic.

Concurrently Hogue (29, p. 1-8) at the Cornell Station carried out feeding trials designed to test the effectiveness of selenium in preventing muscular dystrophy in lambs. His reported results very closely paralleled the Oregon observations.

In the light of this recent experimental work with selenium it seems highly possible that selenium either has some direct physiological function, or that it may perhaps act indirectly in inhibiting some factor that precipitates tissue degeneration in many species of animals.

Conditions Under Which Selenium Has No Beneficial Effect

It seems that the response to treatment of muscular dystrophy is related to the cause. WMD, caused by the feeding of certain forages, responds to selenium and not always to vitamin E, but dystrophies produced by vitamin E-free diets or by addition of fish liver oils or purified unsaturated fatty acids to the rations show a response to

vitamin E supplementation.

Schwarz (66, p. 492) bioassayed various forms of selenium using a method of standardization of alpha-Factor 3 concentrate against liver necrosis in rats. A great difference in potency was observed with L-cystine, selenite, selenate, selenocyanate, selenocystine, selenocystathionine, and selenomethionine being quite active, whereas compounds such as phenyl selenide, 4-carboxybenzeneselenic acid, octafluoroselenophane, and 2-selenouracil were ineffective.

The protective effect of L-cystine is probably caused by selenium contamination since 2 micrograms of selenium were detected per gram of L-cystine. Torula yeast contains up to 1.6 micrograms of selenium per gram, so selenium in Torula yeast grown on purified substrate must be biologically unavailable.

Draper (17, p. 1419) produced characteristic symptoms of muscular dystrophy in six weeks in rabbits on a vitamin E-deficient diet. Twenty or 100 micrograms intramuscular injections of selenium, as sodium selenite, elicited no response in the symptoms of dystrophy, but oral administration of alpha-tocopherol induced a remission of symptoms. These results indicated that dystrophy of rabbits is of a different etiology than exudative diathesis of chicks and liver necrosis of rats. The disease was

produced by feeding a purified, tocopherol-free, diet consisting of glucose, corn starch, casein, vitamin E-free lard, cellulose, salts, and a complete vitamin mix minus vitamin E.

Hove et al. (31, p. 27-29) produced muscular dystrophy in rabbits on a vitamin E-deficient, soybean meal diet. Sodium selenite and selenocystine, when added to the basal diet, were both completely ineffective in prevention of the disease, whereas control rabbits fed the basal diet but supplemented with alpha-tocopherol grew at the normal rate of over 30 grams per day and remained in good health. Soybean meal itself is a relatively good source of Factor 3 as determined by rat assay against liver necrosis. It was concluded that, under the conditions imposed, rabbit muscular dystrophy is due to a lack of tocopherol alone.

Welch et al. (82, p. 1194) found selenium to be only partially effective in the prevention of muscular dystrophy in lambs. A dystrophic condition was produced in 88 per cent of the lambs from ewes fed vitamin E-deficient diets and fish liver oils; 60 per cent of the lambs from ewes supplemented with 0.5 parts per million selenium were afflicted, and no lambs were affected in the lot where the ewes were supplemented twice weekly with 0.5 grams of alpha-tocopherol.

Possible Physiological Functions of Selenium

Harris and coworkers (25, p. 686-688) tested the influence of various selenium compounds using the official bioassay procedure for determining vitamin E activity. Negative results were obtained with all the Factor 3-active preparations in preventing fetal resorption in rats. This finding clearly supported the concept that Factor 3 and vitamin E do not replace each other but are independent, essential dietary agents. A possible explanation of the phenomenon that simultaneous lack of both agents is the cause of certain fatal deficiency diseases has been proposed. It is based on the assumption that vitamin E and Factor 3 participate independently in alternate pathways of oxidation-reduction in the respiratory chains of intermediary metabolism.

Chernick et al. (11, p. 829-843) also suggest that selenium may take part in oxidation-reduction reactions, because in Factor 3-deficient diets normal respiration occurred in liver and muscle slices in vitro for about 30 minutes, then fell off rapidly, whereas control samples continued to use oxygen at a normal rate after 3 hours. A variety of substrates produced similar results, indicating involvement of more than one respiratory pathway.

Because of the synergistic effect of selenium and vitamin E there is a possibility that selenium is

associated in the respiratory chain as a metallic cofactor in an alternate pathway in the region of the cytochrome c reductase portion of the chain. Nason (47, p. 27-50) feels that tocopherol is strongly indicated as one of the active components of the terminal respiration chain in mammalian skeletal and heart muscle tissue. Moreover, tocopherol appears to function as a cofactor in the unidentified "Slater's Factor" of the DPN-oxidase and succinoxidase systems. Isooctane extraction of rat skeletal tissue decreased respiratory activity 75-95 per cent, and the activity was completely restored by addition of D-alpha-tocopherol. In case of vitamin E-deficiency a selenium-containing agent could allow electron transfer from cytochrome b or DPNH-dehydrogenase to cytochrome c without involving the Slater Factor.

However, Slater (72, p. 209-210) stated that although vitamin E may be involved in the respiration control mechanism, it does not fully satisfy the three criteria necessary for a substance to be considered as a component of the main respiratory chain of enzymes: (1) Alpha-tocopherol (in the form of alpha-tocopherylhydroquinone) is present in enzyme preparation in amounts commensurate with enzyme activity (1.7 micromoles per gram of protein), (2) removal of vitamin E by isooctane extraction results in enzyme inactivation and addition of alpha-tocopherol

restores activity, but (3) vitamin E has not been shown to undergo oxidation and reduction during the operation of the enzyme system at a comparable rate. Thus, evidence indicating alpha-tocopherol as a component of the respiratory chain is strongly suggestive but not yet conclusive.

The possibility that selenium may be an enzyme cofactor is substantiated by the demonstration of a strong affinity of selenium for protein by McConnell and Van Loon (37, p. 747, 750). Serum proteins from dogs were resolved by paper electrophoresis 24 hours after injection with $Se^{75}Cl_4$. There was an affinity which resulted in a fixation of selenium in the proteins.

Wilson and Bandurski (86, p. 975-981), working on the "activation" of sulfate in yeasts, postulated that ATP-sulfurylase and adenosine-5-phosphosulfokinase enzyme systems "activated" sulfate by forming sulfate esters with ATP. They proposed the following mechanisms:

$$ATP + SO_4^{=} \rightleftharpoons APS^{*} + PP$$

$$APS + ATP \rightleftharpoons PAPS^{**} + ADP$$

*APS = adenosine-5-phosphosulfate
**PAPS = 3-phospho-adenosine-5-phosphosulfate (activated sulfate)

Sulfite, selenate, chromate, molybdate, and tungstate also serve as substrates for sulfurylase, although stable adenylic acid-mixed anhydrides are formed only with sulfate and selenate. The reactivity of selenate in the sulfurylase

reaction suggests the possibility of a comparable selenylase system.

Possible Causes of Selenium Deficiency

Selenium deficiency may be produced by competitive inhibition besides being caused by simple low levels in the diet. The two main antagonists of selenium are arsenic and sulfur. Palmer et al. (53, p. 928-930) reported that arsenic compounds prevent selenite uptake by respiring yeast cells. Injected arsenite apparently blocks transport of selenite from the blood to the liver in the intact rat, but selenite uptake by kidney and spleen appears to be unaffected by arsenite.

There is quite a definite competition between selenium and sulfur. Shrift (70, p. 345-352) presented evidence that the toxicity of selenium is caused by competition with sulfur in formation of cystine and methionine. High intakes of glutathione, methionine, or feeding protein supplements reduce selenium toxicity. Fels and Cheldelin (22, p. 803-811) reported that DL-methionine was capable of partially reversing the toxic effect of selenate on yeast. Hurd-Karrer (33, p. 666-675) observed that the toxicity of plants from seleniferous soils could be overcome by the addition of sulfate to the soil.

In view of the above evidence it is not impossible

that a high concentration of sulfate in a low selenium diet could produce a selenium deficiency. This thought is being currently tested at the Oregon Station in conjunction with the white muscle disease program.

Sources of Selenium

In a series of experiments, Nesheim and Scott (48, p. 601-618) established that 0.17 parts per million selenium in the diet was the minimum level that would prevent exudative diathesis in chicks. The chicks were reared on Torula yeast which contained 0.09 parts per million selenium, and maximum growth was obtained with the additional supplementation of 0.1 parts per million selenium, as sodium selenite. When vitamin E was added to the diet only 0.04 parts per million selenium were required for maximum growth.

A tremendous growth response was observed in chicks when selenium was added to the ration. At 42 days of age, chicks on a Torula yeast diet weighed 426 grams when supplied with adequate vitamin E; chicks receiving selenium but no vitamin E weighed 699 grams, and chicks being fed both vitamin E and selenium weighed 684 grams. There were 40 chicks in each lot and at the 42 day period only 3 of the vitamin E lot were still living, whereas 36 of the selenium lot and 39 of the selenium plus vitamin E lot survived. This suggests that selenium and vitamin E do

not perform the same function, at least as far as growth is concerned, because the outstanding growth stimulus realized from selenium supplementation is not attained with vitamin E treatment.

On the basis of the dietary selenium levels reported above, certain feedstuffs should contain sufficient amounts of selenium to provide adequate protection from white muscle disease. Scott (67, p. 111) reported the selenium content of the following feeds based on a neutron activation analysis at Oak Ridge: Brewers' dried yeast, 0.60 parts per million; Torula yeast, 0.09; soybean meal, 0.60; linseed oil meal, 1.14; and oats, 0.03 parts per million. These reported levels would indicate that the oil meals, particularly linseed, should be excellent sources of selenium.

Proctor et al. (57, p. 1183) tested the effectiveness of linseed oil meal in prevention of white muscle disease in lambs. A known dystrophogenic ration of mixed trefoil-grass hay and raw cull kidney beans was fed to 64 pregnant ewes through the fall and winter of 1957-58. The addition of 0.25 pound per head daily of linseed oil meal to the basal ration was as effective as 1 part per million daily of selenium, as sodium selenite, in the prevention of WMD in the lambs.

The protective effects of soybean meal and linseed

oil meal when added to legume feeds from known white muscle areas of Oregon are currently being tested at the Oregon Station. The results of the 1958-59 trials indicate that the oil meals and inorganic selenite are equally effective in prevention of WMD in lambs.

Metabolic Alterations Associated With Muscular Dystrophy

Accompanying the onset of muscular dystrophy in an animal there is a generalized increase in metabolism. Hummel and Melville (32, p. 391-394) observed an increased oxygen consumption and an increase in glycolysis in muscle from rabbits afflicted with muscular dystrophy. Carpenter et al. (9, p. 162) reported an increased glycolysis in muscle from dystrophic rabbits but they noted that there was a decreased phosphate transfer from creatine phosphate to hexosemonophosphate and a decreased phosphoglucomutase activity. Also indicating increased glycolysis is the recent report by Blincoe (5, p. XLIII-2) that WMD causes an increase in lactic dehydrogenase activity in the blood sera of lambs from an average of 1152 units to 22,310 units. The units are defined as that activity of enzyme that will cause a decrease in optical density of 0.001 per minute.

An increase in fat metabolism is indicated by a marked increase in plasma cholesterol, phospholipid, and

neutral fat (41, p. 755-766; 42, p. 173-190). Deuel et al. (16, p. 14) noted that accompanying the increased plasma cholesterol was a simultaneous elevation in muscle cholesterol. In plasma, both free and esterified cholesterol increased markedly, but in muscle, free cholesterol showed the principal increase.

There is a great increase in the turnover rate of protein in dystrophic muscle. Young and Dinning (88, p. 743-747) reported an increase in skeletal muscle RNA and DNA which indicates an increase in the rate of protein synthesis. Muscle enzyme studies and analysis of dystrophic muscle indicate an accelerated protein degradation. There is an increased proteolytic activity (80, p. 257-260); dipeptidase activity is enhanced (81, p. 302-305), and free amino acid levels are greatly increased in dystrophic muscle (77, p. 553-555). Further evidence to demonstrate the increased turnover rate of proteins in skeletal muscle and bone marrow cells was provided by Sime et al. (71, p. 450) through the use of C^{14}-labeled glycine and formate. Dystrophic and control rabbits were injected with either glycine-1-C^{14} or sodium formate-C^{14} and killed 4 hours later. The dystrophic animals incorporated much more C^{14} into the protein of skeletal muscle than did the controls. Bone marrow cells from dystrophic and normal rabbits were incubated with the radioactive

glycine and formate and cells from dystrophic rabbits incorporated more C^{14} into protein than did the controls.

A rapid rate of protein catabolism could also be concluded from the high serum glutamic-oxalacetic transaminase (SGOT) activity reported by White and Hess (83, p. 541-544), Blincoe and Dye (6, p. 224-226), and Blincoe (5, p. XLIII-2). In studies of WMD in calves and lambs, Blincoe and Dye noted that SGOT activities underwent more than a ten-fold increase. Normal calves exhibited transaminase levels of about 57 units of activity and lambs from 57 to 128 units. Calves afflicted with WMD displayed an average activity of 1313 units and lambs an average of 1890 units. SGOT units are expressed as micrograms pyruvic acid liberated per milliliter of serum in 20 minutes at 25 degrees centigrade.

Bonetti et al. (8, p. 16) studied the morphology of dystrophic muscle with polarization and electron microscopy. They concluded that in the diseased muscle there is a decrease in myosin and actomyosin, and because of the loss of birefringence in the polarization microscope that the actin is permanently depolymerized.

In contrast to the theories that an increased metabolism is associated with muscular dystrophy, Cartan and Swingle (10, p. 235) feel that the disease may be promoted by an inhibition of glycolysis. They postulate that crops

grown on certain soils may be dystrophogenic because of the presence of toxic agents. An ethanol extract of hay consistently inhibited succinoxidase activity *in vitro* and extracts from dystrophogenic areas appeared to exert a greater inhibitory effect than those from hay obtained in areas where the disease was rare.

Calcium Deposition in Soft Tissues

The muscular lesions that result from white mucle disease are usually accompanied by a deposition of calcium salts in the muscle fibers. The salts are apparently precipitated within the sarcolemna of the fibers and not in the adjacent connective tissue (44, p. 355-361). Whether or not salt deposition can occur in muscle fibers by the same mechanism which operates in connective tissue is unknown, but the thought is worth consideration.

Salt deposition occurs in most types of connective tissue, and Strates and Neuman (76, p. 688-691) have established that collagens from skin, tendon, and bone of calves were equal in ability to induce crystal formation of hydroxy apatite. Apparently there is either some substance in bone cartilage that enhances the normal deposition of bone salt or there is an inhibitor in other types of connective tissue.

Sobel (73, p. 113-143) reported that the presence of cations inhibits calcification. Sodium is a good inhibitor

and potassium is a poor inhibitor of calcium deposition. A sodium concentration of 50 milliequivalents per liter against 150 milliequivalents per liter of calcium inhibited calcification in rachitic bone *in vitro*, but 400 milliequivalents per liter of potassium were required for the same degree of inhibition. An increase in calcium concentration overcomes the inhibition, probably by a common ion effect or by competition of cations for an enzyme system. Strontium, beryllium, and barium are also competitive inhibitors for some enzyme that is involved in the promotion of the formation of bone salt.

There is a close association between calcification and presence of acid-mucopolysaccharides, as indicated by a marked increase in intensity of toluidine blue or periodic acid-Schiff base (PAS) staining around cartilage cells right at the zone of transition from non-calcified to the calcified part of the matrix of cartilage, whereas the intercellular matrix remains relatively unstained. Rubin and Howard (60, p. 155-166) believe that mucopolysaccharides play a specific role in the calcifying mechanism, and that there is a chemical situation inthe matrix responsible for the conferring of the state of calcifiability on the tissue. It is postulated that this could be the liberation of substrate (chondroitin sulfate) from its linkages to matrix protein. Chondroitin sulfuric acid has

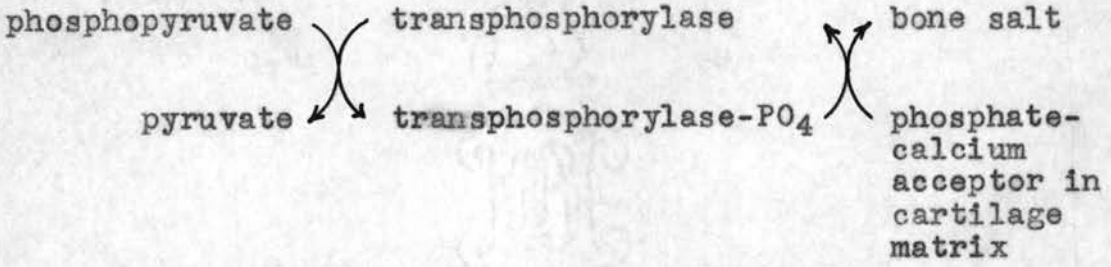
a binding power rather than catalytic action and in contrast to Sobel's theory of cation competition for enzyme, Rubin and Howard believe it is a substrate competition. That is, strontium and other cations compete to bind with chondroitin sulfuric acid. Gutman and Yu (24, p. 167-190) have suggested that chondroitin sulfuric acid is similar chemically to sulfonic acid cation exchange resins and could thus bind calcium.

Acid-mucopolysaccharides have been found in other tissues undergoing calcification, as in the periosteal insertion of tendons. There is also an increase in their concentration in the areas of pathogenic calcification such as are involved in calcified pericarditis, calcified bursitis, and renal stones of calcium phosphate; but not around calcium oxalate or ammonium urate stones, thus demonstrating a specificity for calcium and phosphate ions (24, p. 167-190).

The enzyme systems involved in the calcifying mechanism are rather obscure but it is commonly thought that phosphate is made available by hydrolysis of phosphate esters catalyzed by alkaline phosphatase, however Gutman and Yu have proposed a mechanism which does not utilize phosphatase systems. This alternate pathway was chosen because three glycolysis inhibitors, none of which will inhibit alkaline phosphatase, prevent calcification. The

inhibitors used were phlorizin which prevents phosphorylation of glucose from glycogen; iodoacetate which prevents formation of 1,3-diphosphoglyceraldehyde; and fluoride which inhibits enolase. It was shown that an active anaerobic glycolysis was necessary to obtain calcification of cartilage *in vitro*. Glycogen content in hypertrophied cartilage cells increased markedly and then disappeared with calcification. It was further postulated that the reaction ADP → ATP stores phosphate to prevent calcification until the matrix is ready for it.

The mechanism proposed for bone formation is presented below:



The above mechanism is substantiated by the work of Waldman (79, p. 203-220) on calcification of cartilage *in vitro*. It was suggested that alkaline phosphatase was necessary to prepare the calcifiable matrix. The tissue must then be made anoxic for calcification to take place, then enzyme systems such as phosphorylative glycolysis produce bone formation. Also, Blumgart *et al.* (7, p. 313) have reported that if cardiac muscle is made anoxic so

that its organic phosphates break down, the fibers become calcified.

Engfeldt *et al.* (21, p. 15-26) reported an increase in serum glycoproteins in cases of experimentally induced hyperparathyroidism, and Oppenheimer *et al.* (51, p. 882-886) have shown an increase in serum glycoproteins in rabbits afflicted with muscular dystrophy. The reason for this increase is not well understood. It could be the result of an accelerated protein degradation, or it might well indicate an increased synthesis or transportation of mucoproteins to the site of damaged tissue to act as a matrix for the deposition of calcium salts.

In the present study on white muscle disease of lambs there will be an attempt to demonstrate that the pathogenic calcifying mechanism in muscle tissue is either similar to mechanisms involved in normal bone salt deposition or is an entirely different metabolic process. The problem was approached in a variety of ways. Calcium, magnesium, phosphorus, sodium, potassium, and cholesterol levels in muscle tissues were determined and ratios of abnormal amounts of calcium and phosphorus were calculated and compared to the calcium-phosphorus ratio of bone to establish the nature of pathogenic deposits. Plasma protein-bound hexoses were determined to ascertain if there is an active transport of glycoproteins through the

blood stream. Kidney tissue was analyzed for calcification as an indication of the possible involvement of a secondary hyperparathyroidism. Also, histochemical techniques were applied to sections of muscle tissues to discover whether or not there is an increase in the concentration of chondroitin sulfate in the regions of calcification in muscle fibers.

METHODS AND MATERIALS

White muscle disease in lambs is a serious problem and is of great economic importance to the livestock producers of Oregon. For these reasons a program was established to study in detail the nature and effects of WMD and to effect a cure or prevention of the disease. To do this, it was necessary to produce WMD experimentally, using natural feedstuffs, under controlled conditions. The major objectives of the present study were to determine the protective effects of selenium and vitamin E by various routes of administration and to attempt to establish the cause and nature of salt deposits in the pathogenic soft tissue calcification that usually accompanies WMD. These objectives were accomplished by (1) feeding pregnant ewes legume roughages that were known to produce WMD in the lambs, specifically Ladino clover from Jefferson County, Oregon, and to treat certain lots with selenium or vitamin E; and (2) to analyze plasma and muscle tissue from afflicted and treated lambs to determine resulting abnormalities.

Encompassed in this study are the results of feeding trials for two successive years designed to produce WMD in lambs under experimentally controlled conditions by feeding legume hays from "white muscle areas" of Oregon.

The two trials are designated as the 1957-58 experiment and the 1958-59 experiment.

Mature ewes of mixed breeds were fed the dystrophogenic feeds the latter three months of gestation and for a six week post-partum period. Facilities, consisting of a shed divided into a series of pens, were provided by the Oregon State College Department of Veterinary Medicine. The shed was open to the weather on one side and provided with outside runs. Inside and outside areas were littered with wood shavings that were renewed frequently. Fresh water and iodized block salt were provided ad libitum.

In the 1957-58 experiment, as reported herein, 60 ewes were randomly assigned to five lots of 12 ewes each and in the 1958-59 trial similarly discussed, there were four treatments of 12 ewes each.

1957-58 Experiment

The feeds and treatments for the experimental groups in the 1957-58 trial are listed in Table I.

The alfalfa and Ladino clover hays fed the control lot were grown in areas where WMD is not a serious problem and the oats had proved to have no protective effect in previous trials. The hay fed the basal experimental lots originated from an irrigated area in Jefferson County, Oregon, where the clover is grown for seed and was the

Table I

1957-58 WMD Experiment

Feeds and Treatments

Daily feed per head except as indicated	Lot I	II	III	IV	V
Alfalfa hay (Union Station) (lbs.)	2				
Ladino clover (Western Oregon) (lbs.)	2				
Ladino clover (Jefferson County, Oregon) (lbs.)		4	4	4	4
Oats (lbs.)	0.25	0.25	0.25	0.25	0.25
Parenteral vitamin E (I.U./wk)			770		
Oral vitamin E (I.U.)				100	
Selenium (p.p.m.)					0.1

first cutting removed to stimulate flower production of the plant, and was graded "U.S. No. 1 green leafy". Hay originating on the same ranch proved capable of eliciting the disease in a high percentage of lambs when experimentally fed to their dams the previous year.

In addition to the basal experimental ration, each ewe in lot III was injected intramuscularly each week with 770 I.U. of vitamin E and D-alpha-tocopheryl polyethylene glycol 1,000 succinate[1]. Ewes in lot IV were each fed 100

[1] Supplied by Distillation Products Industries, Rochester, N.Y.

I.U. of vitamin E per day in the oats as D-alpha-tocopherol acetate (Myvamix)[2], and those in Lot V were fed 0.1 parts per million of selenium (calculated for the entire ration) in the oats as sodium selenite. A preliminary trial indicated that the stated dosage of D-alpha-tocopheryl polyethylene glycol 1,000 succinate was sufficient to maintain satisfactory blood levels in ewes on clover hay.

One of each pair of twins born in lots I and II was killed within the first three days after birth. Surviving lambs were killed at approximately 6 weeks of age. All lambs were necropsied and tissues were selected for histopathological and chemical study.

The semitendinosus muscle and heart were taken for chemical analysis with the tissues being placed in plastic bags and frozen until analyzed. In addition, small blocks of each tissue were fixed in neutral buffered formalin for microscopic diagnostic studies by Dr. O. H. Muth of the Veterinary Medicine Department.

1958-59 Experiment

This trial involved four lots of ewes all of which were fed a basal ration of Ladino clover chaff from Jefferson County and oats. Feeds and treatments in the

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[2] Supplied by Distillation Products Industries, Rochester, N.Y.

1958-59 experimental year are shown in Table II.

Table II

1958-59 WMD Experiment

Feeds and Treatments

Daily feed per head except as indicated	Lot I	II	III	IV
Ladino clover chaff (lbs.)	4	4	4	4
Oats (lbs.)	0.25	0.25	0.25	0.25
Parenteral selenium (mg., total)		1.4		
Oral vitamin E (I.U., total)			2000	
Oral selenium to ewes (p.p.m.)				0.1

Lot I was used as a control and received only the basal ration. The ewes in lot II received no treatment but the lambs were given 1.40 milligrams of selenium, as aqueous sodium selenite, by subcutaneous injection in two doses, one at birth and one at two weeks of age. The initial injection contained 0.28 milligrams of selenium in 1.0 milliliter of normal saline and the final dose contained 1.12 milligrams of selenium in 4.0 milliliters of normal saline.

As in lot II, the ewes in lot III received only the basal ration but the lambs were drenched at birth and again at 4 days of age with a vitamin E-water emulsion. Each dose contained 1000 I.U. of vitamin E.

The ewes in lot IV received 0.1 parts per million of the total ration daily of selenium, as sodium selenite, for the latter two thirds of gestation and for a six week post-partum period. The selenite was carefully premixed and dispersed with the oats.

All the lambs were weighed at birth, two weeks, and six weeks of age and the weights were recorded. All surviving lambs were sacrificed at six weeks of age and tissues were collected for analysis. Two lambs in lot I were killed at five weeks because they were so severely affected that survival to six weeks seemed improbable.

The semitendinosus muscle, heart, and kidneys were retained and frozen pending chemical analysis. Approximately 200 milliliters of blood was collected by venipuncture in citrated bottles. Of this, 50 milliliters was centrifuged and the plasma was pipetted into plastic vials and frozen.

Portions of the semitendinosus and heart were fixed in neutral buffered formalin solution, processed, cut at 6 micra, stained with von Kossa's silver nitrate technique to demonstrate calcium (39, p. 404), and counterstained with alcian blue 8GS. The sections were exposed to sunlight for one hour in 5 per cent silver nitrate, rinsed in distilled water, immersed in sodium thiosulfate for 3 minutes and washed thoroughly. The sections were then

placed in a 1 per cent aqueous solution of alcian blue 8GS for 40 seconds, rinsed in distilled water and mounted in permount. Alcian blue was used as a counterstain because it is specific for mucopolysaccharides, according to Lillie (36, p. 787).

Chemical Analyses

Complete proximate analyses were carried out on the feedstuffs used in the experiments. Per cent dry matter was determined by weighing a sample of finely ground feed, drying at 105 degrees centigrade for 24 hours, cooling to room temperature in a dessicator and reweighing. Crude protein, crude fat, crude fiber, and total ash were determined by the official methods of the Association of Official Agricultural Chemists (1, p. 368, 371, 372, and 368, respectively). Approximately one gram of feed was ashed in a platinum crucible and the ash dissolved in 0.1 N HCl and transferred quantitatively to a volumetric flask and diluted to 25 milliliters. The mineral analyses were subsequently carried out on the ash solutions. Total phosphorus was obtained by the method of Fiske and Subbarow (27, p. 579-581) and calcium, magnesium, sodium, and potassium were determined by flame photometric analysis as outlined by Denson (15, p. 1-10). A hydrogen fuel, Beckman model DU flame photometer with photomultiplier

attachment was used for these analyses.

In addition, samples of legume roughages fed in these trials and of other similar roughages known to be innocuous from a WMD standpoint were submitted to Dr. P. R. Stout, University of California, Berkeley, for selenium and molybdenum analyses by X-ray fluorescence.

The heart, skeletal muscle, and kidney tissues were all analyzed with the same techniques. Dry matter was determined by trimming away all external fat and connective tissue with a scalpel and then dicing the sample into one quarter inch cubes. After mixing the diced pieces, 10 grams of the sample were weighed into a previously weighed and dessicated evaporating dish and dried at 75 degrees centigrade in a vacuum oven for 24 hours, cooled in a dessicator and reweighed.

The percentage of crude fat was determined by extracting the dried sample with 40 milliliters of diethyl ether on a Goldfisch extraction apparatus for a minimum of 4 hours. After extraction, the ether extract was transferred with ether rinses into a 100 milliliter beaker and evaporated to dryness. The beaker was covered with Parafilm and stored in the freezer. The tissue sample was redried in the vacuum oven for one hour and cooled in a dessicator before reweighing.

Total ash was obtained by ashing the dry, fat free sample in a platinum crucible at 600 degrees centigrade for 5 hours. After being weighed the ash was dissolved in 0.1 N HCl, transferred quantitatively to a volumetric flask and diluted to 25 milliliters. The solution of soluble chlorides was stored in a 15 dram plastic vial.

The lipid material obtained by ether extraction was dissolved in reagent grade chloroform and transferred with chloroform rinses into a volumetric flask and diluted to 25 milliliters. Total muscle cholesterol was estimated by analysis of the chloroform solution with the Reinhold and Shiels modification of the Myers-Wardell cholesterol method (27, p. 536-538).

Mineral analyses were carried out as previously described for the forage samples. For quantitative interpretation of the flame photometric ignition luminosities of the various metals, standard curves were established for each metal. Minerals causing background interference or suppression of luminosity were added to the standard solutions in approximately the same concentrations as they occur in mammalian tissues to balance out the interference (20, p. 45). In the determination of calcium and magnesium it is of the utmost importance to add sufficient phosphorus to obtain maximum suppression of luminosity. The

compositions of the standard solutions are reported in Table III.

Table III

Composition of Flame Photometric Standard Solutions

Constituents in p.p.m.	Standard Solutions			
	Calcium	Magnesium	Sodium	Potassium
Calcium	0-80	30	1.4	.56
Magnesium	80	0-160	4	1.6
Sodium	320	320	0-80	8
Potassium	1280	1280	640	0-800
Phosphorus	200	200	200	200

All solutions in 0.1 N HCl

Calcium and magnesium were determined directly from the stock ash solutions. For the sodium and potassium analysis the stock solutions were diluted 1:5 before aspiration through the burner. The photometer adjustments are presented in Table IV, however, they may be considered as only approximate, for reference purposes, because of the variation between machines, burners, flame heights, etc.

Blood plasma analyses included determination of alkaline phosphatase activity in ewe's blood just prior to lambing, and of plasma protein-bound hexoses in lamb's blood at time of slaughter. The ewe's blood was drawn

Table IV

Adjustments for Beckman Model DU Flame Photometer

	Calcium	Magnesium	Sodium	Potassium
Wavelength (mμ)	556	285.4	592	774
Slit Width (mm)	0.03	0.05	0.02	0.0175
Sensitivity (turns from clockwise limit)	5	4.5	7	6
Tube	Photomultiplier	Photomultiplier	Photomultiplier	Red sensitive
Power Unit Sensitivity	5	5	1	1
Zero Suppression	2	2	2	2

Oxygen pressure set at 12 p.s.i.
Hydrogen pressure set at 4 p.s.i.

from the jugular vein; citrated as it was collected, then centrifuged and the plasma was frozen overnight. The phosphatase activity was determined the following day. The Bodansky method (27, p. 584) modified to permit the use of the Fiske-Subbarow procedure for the determination of phosphate liberated was utilized for the determination of alkaline phosphatase activity.

The lamb's blood was collected and stored as previously described and the Weimer and Moshin modification of the Lustig and Langer method for serum glycoproteins (87, p. 290) was used on the plasma. The analysis of

protein-bound hexoses is assumed to be an estimation of blood serum glycoproteins.

Statistical analyses of the data were made using the Student t-test as described by Li (35, p. 127-130).

RESULTS AND DISCUSSION

1957-58 Experiment

Diagnosis of WMD was made on recognition of characteristic gross or microscopic lesions, or both. The results of the diagnoses are reported in Table V.

Table V

1957-58 WMD Diagnosis of Lambs[3]

Lot	Ration	Affected	Non-affected
I	Control	1	17
II	Basal Experimental	11	4
III	Basal plus parenteral vitamin E	11	4
IV	Basal plus oral vitamin E	16	4
V	Basal plus oral sodium selenite	3	13

From the low incidence of WMD in lot V, it appears that selenium has a strong protective effect while vitamin E given pre-natally offers very little, if any, protection against WMD, under the conditions of the experiment. None of the lambs in lots I and V diagnosed as affected displayed any gross characteristics of the disease. When killed, these lambs were fat and clinically healthy, but

[3] Diagnosis by Dr. O. H. Muth, Department of Veterinary Medicine, Oregon State College.

tissue sections from them showed the development of microscopic lesions. The three affected lambs in lot V were the last lambs born in the lot and one of these, No. 69, was sacrificed 22 days after the termination of selenium feeding. The other two lambs, No's. 71 and 72, were killed 34 days after selenium feeding had ceased and were members of a triplet group. The ewes were fed the dystrophogenic feeds during this period. It is possible that there is a continuous daily requirement for selenium and that the amounts the ewes were receiving were inadequate for triplet lambs.

Table VI reports the average values by lot of the results of chemical analysis of muscle tissues (analysis of tissues on an individual lamb basis are tabulated in Appendix No's. 1-10).

The average dry matter, ether extract, and ash contents of the tissues all correlate closely and do not show any significant difference among treatments. The cholesterol contents and levels of inorganic constituents of the skeletal muscle of lot V compare favorably with those of lot I. Lots II, III, and IV show great increases over normal in cholesterol, calcium, phosphorus, and sodium values. Further, there is only a slight increase in magnesium levels and quite a large decrease in potassium content in the same groups.

Table VI

1957-58 Muscle Tissue Analysis

Lot	Dry Matter %	Ether Extract %	Ash %*	Cholesterol mg%**	Ca	P	Mg	Na	K
					mg/100 gm dry, fat-free tissue				
				Skeletal Tissue					
I	21.51	8.80	5.34	312	29.5	673	120	371	1800
II	19.56	9.02	5.98	472	474.4	827	140	709	1251
III	21.73	12.26	5.96	407	388.8	862	138	491	1559
IV	20.59	14.37	5.55	460	290.0	702	118	590	1318
V	22.26	11.45	5.20	335	26.6	676	118	413	1638
				Cardiac Tissue					
I	20.96	6.30	5.43	447	32.4	691	128	485	1584
II	19.92	8.52	6.26	461	284.6	830	131	599	1412
III	22.48	8.30	5.53	475	129.2	747	123	516	1475
IV	20.73	8.26	5.59	414	202.0	731	125	509	1462
V	20.74	6.17	5.34	406	24.8	760	116	428	1506

* Per cent of dry, fat free tissue.

** mg% on dry matter basis.

It appears that calcium deposition in the muscles of the lambs on vitamin E therapy is not quite as severe as in the basal diet group, lot II. Lots III and IV had an average muscle calcium of 388.8 and 290.0 as compared with 474.4 milligrams per 100 grams in lot II. A similar trend toward the normal occurs with the other constituents reported, except for the high value of 862 milligrams of phosphorus per 100 grams dry, fat-free tissue in lot III. This would indicate that although pre-natal vitamin E does not prevent the affliction it does seem to reduce the severity or alter the nature of the lesions. The differences are not as great in the cardiac tissue as they are in skeletal muscle except for the increases in calcium and phosphorus. Heart cholesterol is only slightly increased in affected animals. Also, the ionic balance is only slightly upset with a small increase in sodium and a slight decrease in potassium.

To obtain a truer picture of the mineral imbalances caused by WMD, each lot was divided into two groups: affected and non-affected lambs as diagnosed by histological examination of muscle and cardiac sections. The lot means and standard deviations for affected and non-affected lambs are presented in Table VII.

The cholesterol and mineral levels in the muscles of the lambs diagnosed as affected in lots I and V appear

quite normal. In lots II and III there is a 20 fold increase in average calcium content in the skeletal muscle. Lot IV shows a 10 fold increase. There is approximately a 10 fold increase in calcium levels in lots II, III, and IV in the cardiac tissue.

The extent of calcium deposition in the muscles of affected lambs varies greatly as may be seen in Table VII by the standard deviations of the calcium levels. The calcium values in the skeletal muscles of affected lambs in lots II, III, and IV have standard deviations ranging from 814 to 1383 milligrams per 100 grams dry, fat-free tissue. The standard deviations of calcium in cardiac tissue vary from 210 to 632. Apparently, the amount of calcium deposition varies with the nature or severity of the lesions.

As striking as these differences appear they are not statistically significant because of the variability. The calcium levels of skeletal muscle of affected lambs in lot II range from 24.8 to 4771.6 milligrams per 100 grams; phosphorus, 558 to 2391; magnesium, 93 to 332; sodium, 560 to 1237; and potassium, 464 to 1773 milligrams per 100 grams tissue. The calcium, phosphorus, magnesium, sodium, and potassium values for cardiac tissue in lot II range from 17.8-1652.0, 248-1148, 105-161, 376-914, and 796-1690 milligrams per 100 grams, respectively. These analyses

Table VII
1957-58 WMD Experiment
Mineral Levels in Muscle Tissue of Affected and Non-Affected Lambs as Diagnosed by Histological Examination

Lot No.	Ca	P	Mg	Na	K	Cholesterol mg/100 gms dry tissue
			mg/100 gms of dry, fat free tissue			
			Affected			
			Skeletal Tissue			
I	27.5	558	94	279	1924	224
II	637 ± 1383	953 ± 500	147 ± 65	818 ± 252	1126 ± 386	524 ± 199
III	488 ± 1341	902 ± 654	146 ± 68	494 ± 283	1529 ± 314	424 ± 236
IV	296 ± 814	715 ± 393	122 ± 37	620 ± 289	1286 ± 642	465 ± 143
V	22.8 ± 5.0	759 ± 46	116 ± 23	504 ± 28	1541 ± 78	372 ± 81
			Cardiac Tissue			
I	30.3	361	133	457	1651	497
II	374 ± 521	793 ± 253	134 ± 16	627 ± 198	1343 ± 297	462 ± 113
III	158 ± 210	786 ± 246	132 ± 15	518 ± 131	1473 ± 189	474 ± 111
IV	299 ± 632	710 ± 317	126 ± 41	501 ± 148	1446 ± 225	418 ± 91
V	19.9 ± 5.1	719 ± 74	103 ± 4	403 ± 48	1410 ± 144	340 ± 82
			Non-Affected			
			Skeletal Tissue			
I	29.6 ± 13.5	680 ± 193	122 ± 30	376 ± 79	1793 ± 203	317 ± 137
II	28.1 ± 6.6	480 ± 301	120 ± 13	409 ± 34	1594 ± 131	329 ± 73
III	25.1 ± 5.7	684 ± 137	109 ± 14	482 ± 153	1669 ± 229	345 ± 97
IV	21.4 ± 12.6	658 ± 178	102 ± 13	484 ± 199	1430 ± 153	444 ± 293
V	27.6 ± 10.7	656 ± 226	119 ± 11	392 ± 107	1659 ± 191	326 ± 152
			Cardiac Tissue			
I	32.5 ± 13.2	710 ± 215	127 ± 17	486 ± 58	1580 ± 178	444 ± 139
II	29.7 ± 10.8	934 ± 80	123 ± 14	521 ± 23	1604 ± 88	459 ± 109
III	22.2 ± 11.7	602 ± 243	89 ± 54	508 ± 74	1480 ± 150	479 ± 85
IV	36.2 ± 37.0	806 ± 148	122 ± 19	536 ± 91	1519 ± 271	402 ± 48
V	25.9 ± 8.0	774 ± 257	119 ± 15	433 ± 60	1528 ± 510	421 ± 109

substantiate the observations by Muth (44, p. 355-361) that there are two distinct types of lesions produced as a result of WMD. These are histologically distinguishable and calcium deposition occurs in one type and not in the other. In fact, of the tissues analyzed, only about one third of the animals diagnosed as affected showed extensive calcification.

In order to observe the differences between the two types of muscle lesions, the data from the tissue analyses from all the lambs diagnosed as positive for WMD were pooled regardless of lot numbers. These pooled data were divided into two categories; one group included the tissues showing extensive calcification and the other group included those which had apparently normal calcium levels. This subdivision was made on the basis of calcium analysis of the tissues. The average calcium levels and standard deviations of lot I were taken as normal values. The mean ($\bar{y}$) of the control lot plus 1.96 times the standard deviation (s) would include 95 per cent of all observations having normal calcium levels. For skeletal muscle, $\bar{y} + 1.96s = 56.0$ milligrams per cent; and for cardiac tissue, $\bar{y} + 1.96s = 58.4$ milligrams per cent. It was assumed that all tissues having calcium levels above these values had some degree of pathological calcification and the affected tissues were divided accordingly into one of

two groups: those showing abnormal calcium deposition and those showing normal calcium content.

These two groups were analyzed statistically by the Student t-test with the hypothesis that the population means of each group are equal to the population means of lot I. The average values of each group and the results of the statistical analysis are reported in Table VIII.

Table VIII

1957-58 WMD Experiment

Averages and t-values of the Pooled Data of Affected Animals

	Tissues Showing Abnormal Calcium Deposition		Tissues Showing No Abnormal Calcium Deposition	
	mg%	t	mg%	t
	Skeletal Tissue			
Calcium	997.1	2.596*	28.3	-.339
Phosphorus	1056	2.282*	665	-.230
Magnesium	156	1.801	118	-.764
Sodium	840	6.812**	462	1.804
Potassium	1042	-7.010**	1567	-3.145**
Cholesterol	538	3.323**	397	2.048*
	Cardiac Tissue			
Calcium	513.6	3.108**	30.5	-.474
Phosphorus	864	2.163*	658	-.709
Magnesium	140	1.471	120	-.351
Sodium	646	3.502**	448	-1.707
Potassium	1356	-2.856**	1480	-1.500
Cholesterol	420	-.569	457	.345

* Significant difference from lot I ($P < .05$)
** Significant difference from lot I ($P < .01$)

According to Table VIII there is no significant difference between the magnesium levels of the affected lambs and control lambs. Therefore, the main cation in the salts that are deposited in the muscle tissue is probably calcium.

The calcium, phosphorus, sodium, and cholesterol levels of the skeletal tissue showing salt deposition are significantly greater than the controls. The potassium content is significantly less.

In the cardiac tissue showing calcification there is a significant increase in calcium, phosphorus, and sodium, and a significant decrease in potassium. However, as may be noted in Appendix Tables 6-10, the incidence of affected heart muscle was lower than of affected skeletal, and the extent of the heart lesions was less. In conjunction with these observations, it has been the experience of Muth (44, p. 356) that although both calves and lambs may be affected in a similar manner, cardiac injury occurs more frequently in calves and there is a higher incidence of skeletal lesions in lambs with the hearts remaining unaffected.

The increases in calcium and phosphorus are probably caused by the deposition of calcium phosphate salts in the muscle fibers. The increased sodium and decreased potassium levels indicate an increased membrane permeability

or a loss of the control of the cellular potassium retention mechanism, or both.

There is no significant difference in cholesterol levels in heart muscle. The increased cholesterol in the skeletal muscle is probably indicative of an increased fat metabolism of some sort in the voluntary musculature.

As a matter of conjecture, the calcium phosphate deposition could occur by either of two different mechanisms. There could be an increase in available inorganic phosphate because of a pathogenic increase in phosphatase activity. Through the common ion effect and the low solubility product of calcium phosphate there could be a passive crystallization of salts in the muscle fibers. Phosphate pressure could pull calcium ions out of the lipoprotein complex in the cell membranes and precipitate them in the sarcoplasm of the fibers. The loss of calcium ions would result in an increased permeability of the cell membranes which would cause an increase in intracellular sodium ions and also result in a leaking out of potassium ions into the extracellular fluids.

On the other hand, the salt deposition could be an active process caused by a pathogenic alteration or induction of certain enzyme systems, resulting in a salt deposition mechanism similar to that involved with bone salt formation. This thought is taken into consideration

in the 1958-59 trials.

There is an ionic imbalance, as indicated by the alterations in the sodium and potassium levels. This imbalance would result in a tremendous upset in the osmotic pressures in the muscles. A deranged osmotic balance could cause many different alterations in muscle metabolism. As a matter of fact, a wide variety of metabolic upsets have been reported in conjunction with muscular dystrophy.

It is interesting to note in Table VIII that in skeletal muscle there is a significant decrease in potassium and increase in cholesterol even when there was no apparent salt deposition in the fibers. The lowered potassium indicates that calcium deposition is not responsible *per se* for the ionic imbalance although the many manifestations of WMD may all be interrelated.

In an effort to define further the nature of the salt deposits in pathogenic muscle fibers in WMD, the calcium-phosphorus ratio was determined for the excess calcium and phosphorus in the affected animals showing calcium deposition in the heart and skeletal muscles. The average calcium and phosphorus levels of the control lot were subtracted from the average values for the affected tissues showing calcification. These figures were

then divided by the atomic weights of calcium and phosphorus to obtain atomic ratios. The calcium-phosphorus ratio for the mineral content above the normal amounts of skeletal muscle was 2.00:1 and for heart muscle the ratio was 2.045:1. The calcium-phosphorus ratio calculated from the average values for the calcium and phosphorus content of bone as reported by Morrison (43, p. 1045) is 2.074:1. These values all correspond very closely so it appears that the soft tissue salt deposits are very similar in nature to bone salt. It would appear that the salts could not be entirely tricalcium phosphate but are probably a combination of tricalcium phosphate, calcium carbonate, and perhaps a small amount of calcium citrate. This assumption is based on the molecular composition of bone salt. If this is true then pathogenic salt deposition must be enzymatically controlled and not just a passive precipitation. Of course, further research would be necessary before such an assumption could be considered valid.

1958-59 Experiment

The proximate analyses of the experimental feedstuffs (see Appendix, Table 11) compare very favorably with the average values reported by Morrison (43, p. 1004, 1058). Except for phosphorus the mineral levels in the

experimental Ladino clover and oats compare closely to normal average values (43, p. 1096, 1101). Phosphorus levels are low in the experimental feeds, the Ladino clover chaff contained 0.15 per cent phosphorus as compared to an average of 0.29 per cent phosphorus in Ladino clover hay as reported by Morrison. This results in the relatively wide calcium-phosphorus ratio of 8.4:1 as compared to an average ratio of 5.3:1 in Ladino clover hay. The low phosphorus is probably not significant because the chaff was allowed to mature more than hay in order to harvest seed, hence the roughage would be lower in phosphorus than most hays.

The selenium and molybdenum analyses[4] of pertinent legumes are very interesting (see Table IX).

Table IX

Selenium and Molybdenum Content of Dystrophogenic and Non-dystrophogenic Legumes

	Selenium p.p.m.	Molybdenum p.p.m.
Non-dystrophogenic alfalfa hay from Union, Oregon	0.48	3.68
Dystrophogenic legumes from Jefferson County, Oregon		
Ladino clover chaff	0.05	0.22
Ladino clover clippings	0.05	0.28

[4] Analyses by Dr. Perry R. Stout, Kearney Foundation of Soil Science, University of California, Berkeley.

The selenium content of the Ladino clovers was below the limits of detectability, whereas the 0.48 parts per million selenium in the Union alfalfa would appear more than adequate to prevent a selenium deficiency. The results of this selenium assay substantiate the hypothesis that a selenium deficiency is a causative agent in the development of white muscle disease.

Even more striking is the difference in molybdenum levels between the Union alfalfa hay and the Ladino clovers. The 3.68 parts per million of molybdenum in the alfalfa is more than 10-fold greater than the levels found in the clovers. What possible connection molybdenum might have with WMD is not known, but this possibility will be investigated further.

The preliminary diagnosis of the 1958-59 lambs by physical appearance and the presence of macroscopic lesions at time of slaughter is presented in Table X.

Table X

1958-59 Preliminary* WMD Diagnosis of Lambs

Lot	Treatment	Affected	Non-Affected	Questionable
I	Control	16	2	2
II	Parenteral selenite (lambs)	0	17	
III	Vitamin E drench (lambs)	0	18	
IV	Oral selenite (ewes)	0	18	

* Confirmation will be made on the basis of histological examination of tissues.

Complete protection was afforded by either feeding selenium to the ewes or by injection of it in the lambs. Complete protection was also provided by oral vitamin E when given to the lambs in massive doses, however in the 1957-58 trials injection or feeding of vitamin E to the ewes offered no protection against WMD.

Although drenching the lambs with vitamin E prevents the onset of WMD it does not result in appreciable growth stimulation. On the other hand, supplementation with selenium proved to be a tremendous growth stimulus. As presented in Table XI, the total average gain for the first six weeks of life in the lambs receiving no treatment was 15.2 pounds. The vitamin E-supplemented lambs gained an average of only 17.3 pounds, but the selenium treated lambs made more dramatic gains. The injected lambs gained an average of 20.8 pounds or 37 per cent greater than the control lambs and lambs from ewes fed selenium gained an average of 27.2 pounds, which is an increase of 79 per cent over the controls.

The lambs in lot IV made outstandingly greater gains than all the other lots including lot II. Although birth weights are similar for all lots, one may speculate that the increased gains in lot IV over lot II are probably due to some delayed effect as a result of the advantage of receiving adequate amounts of selenium during embryonic

Table XI

1958-59 Lamb Growth Data

Lot	Average total 6-week gain (lbs.)	t
I	15.2	
II	20.8	2.958*
III	17.3	.868
IV	27.2	4.420*

* Significantly different from Lot I ($P < .01$).

development. The increased gains over controls in both lots II and IV are highly significant. The growth of lambs in lot III where the lambs received vitamin E supplementation was not significantly different from that of the control lambs.

Others have recently suggested that, in certain specific circumstances, selenium may act as an effective growth stimulant to domestic animals. McLean et al. (38, p. 29-30) have reported growth responses to selenium treatment in New Zealand similar to those observed in this study. They obtained highly significant increase in growth in lambs from both WMD-affected and non-affected areas with either injected or oral supplementation of selenium (as selenite). Growth response ranged from 12 to 40 per cent. In all, some 800 lambs have been treated

and weighed under trial conditions over a five month period, including all types of lambs ranging from those suffering from "ill-thrift" to fat, thrifty lambs. There was growth stimulus in varying degrees in all groups studied. Accumulation of such data as these and the local findings strongly suggests the addition of selenium to the list of elements considered as essential nutrients for livestock.

Table XII contains the average values and standard deviations obtained by chemical analyses of skeletal muscle, heart muscle, and kidney tissue. As in the 1957-58 trials, the percentages of dry matter, crude fat, and ash are all well within a normal range.

There is an outstanding difference in cholesterol levels in the skeletal muscle. The control lot shows approximately a 65 per cent increase over the selenium injected lot and well over a 100 per cent increase in comparison to the groups administered vitamin E or oral selenium. In the heart muscle there is a real difference between the cholesterol content of lot I and the treated groups but the difference is not as great as in skeletal muscle. Very little difference was expected in heart cholesterol in view of the 1957-58 experiment in which no significant difference was found in this item between affected and non-affected animals, although there were

considerable differences in skeletal muscle cholesterol. Of course, greater increases in cholesterol induced by an accelerated fat metabolism might be expected in skeletal muscle than in heart muscle since the voluntary muscles are a more active site of lipid deposition than cardiac tissue (18, p. 472).

In general appearance, the muscular lesions in the lambs afflicted with WMD in the 1958-59 experiment were not quite as severe as they were in the 1957-58 experiment. This observation was borne out in the mineral analysis of muscle tissues of affected lambs. The soft tissue calcification was not quite as extensive and the ionic imbalance was somewhat less severe. This was especially true in heart muscle. The calcium levels were several times greater in the control lot than in the treated groups but the phosphorus content of the affected and non-affected lambs were almost equal in skeletal muscle and in heart muscle the phosphorus was actually lower in the affected lambs.

There was apparently little calcification in the kidney tubules as evidenced by similar calcium levels in kidney tissue of all four lots, although the kidney phosphorus of the affected lambs is much greater than the levels in the treated groups.

As in the 1957-58 trials, there was very little

Table XII
1958-59 WMD Experiment
Average Values of Tissue Analysis

Lot No.	DM %	Crude Fat %	Ash %*	Cholesterol mg/%**	Ca	P	Mg	Na	K
					mg/100 gm dry, fat free tissue				
					Skeletal Tissue				
I	18.72	11.28	6.41	855±337	423±1187	787±557	153±51	647±283	1276±266
II	20.60	9.01	5.35	517±159	57.6±10.6	750±58	165±20	443±150	1540±235
III	20.99	6.88	5.50	388±135	47.0±10.0	783±56	135±23	331±148	1663±186
IV	21.42	9.88	5.31	414±67	69.4±11.8	743±46	121±14	307±151	1511±219
					Cardiac Tissue				
I	19.25	4.07	5.67	682±95	160.9±359.3	630±162	137±12	558±194	1290±156
II	19.92	3.38	5.38	545±168	60.4±12.9	796±74	155±15	494±73	1376±65
III	20.30	4.30	5.31	573±158	42.4±10.4	856±90	138±28	477±96	1285±109
IV	19.76	4.51	5.42	560±65	64.4±8.6	861±39	123±7	430±56	1305±70
					Kidney Tissue				
I	20.30		6.11		119±10	839±157	139±16		
II	19.86		6.42		103±15	604±186	152±12		
III	19.72		6.32		97±25	498±149	107±19		
IV	19.99		6.29		119±16	337±43	94±10		

* per cent of dry, fat free tissue
** mg% of dry tissue

difference in the soft tissue magnesium levels.

There was a substantial increase in sodium levels and a decrease in potassium values in the skeletal muscle of the control lambs over that in the treated groups, but in the cardiac tissue the differences are only slight.

The statistical significance of the differences in the chemical composition of muscle and kidney tissues listed in Table XII is presented in Table XIII.

The statistical analysis of data presented in Table XIII was made by the Student t-test in testing the hypothesis that the population mean of each treatment group is equal to the mean of the control population.

Except for the cardiac tissue of lots II and III the differences in per cent ash are non-significant. This indicates generally that as calcium salts are deposited in muscle tissue there is a corresponding loss of other minerals. The decreased potassium probably accounts for the major percentage of these losses.

The cholesterol content of both skeletal and cardiac muscle of all treatment animals is significantly less than the control. Although the differences in heart muscle appeared unimpressive quantitatively in Table XII, they proved to be statistically significant.

The phosphorus levels in skeletal muscle of the treated lambs are not significantly different from those

Table XIII
1958-59 WMD Experiment
t-values for Tissue Analyses

Lot	Ash	Cholesterol	P	Ca	Mg	Na	K
			Skeletal Tissue				
II	1.097	3.471**	.244	1.147	-.799	2.458*	-2.952**
III	1.008	5.193**	.026	1.264	1.313	4.027**	-4.900**
IV	.959	4.066**	.247	.934	1.966	3.534**	-2.395*
			Cardiac Tissue				
II	2.100*	2.957**	-3.533**	1.043	-3.745**	1.165	-1.869
III	2.655*	2.517*	-4.970**	1.316	-.128	1.516	.112
IV	1.542	3.632**	-4.821**	.931	3.513**	2.222*	-.312
			Kidney Tissue				
II	-1.786		3.884**	3.619**	-2.629*		
III	-1.305		6.469**	3.433**	5.158**		
IV	.862		9.806**	.064	7.952**		

* Significantly different from basal group levels ($P < .05$)
** Significantly different from basal group levels ($P < .01$)

of the affected animals and in heart muscle phosphorus levels are actually significantly higher in the non-affected animals. The average phosphorus contents in kidney tissue of all the treated lots show a highly significant increase over the untreated lambs.

The differences between affected and non-affected lambs in calcium and magnesium values in skeletal and heart muscle are non-significant except for the magnesium content of cardiac tissue in lots II and IV. Although in these two instances there is a significant difference in magnesium values, they should probably be disregarded because of the lack of consistancy, that is, the magnesium content of heart muscle of lambs that were injected with selenium were significantly higher than untreated animals while the magnesium levels of lambs from ewes that were fed selenite were significantly lower than the control lambs.

The non-significant differences in the calcium levels are due to the great variation; calcium content ranged from normal values of about 50 to 5180 milligrams per 100 grams of tissue. The differences are real because in skeletal muscle there was about a 10 fold increase in the white muscle animals and in cardiac tissue the calcium content of the control lot is about 200 per cent greater than the treated lots. The variability of calcium levels

further substantiate the hypothesis by Muth (44, p. 357-358) that two distinct types of muscular lesions result from the onset of WMD and calcification occurs in only one type.

In kidney tissue, except for one case, both calcium and magnesium levels show a highly significant increase in the affected lambs. The elevated magnesium levels in kidney and normal magnesium content of muscle tissue in lambs affected with WMD indicate that salt deposits in the renal tubules may differ from muscle calcification in that they contain considerable magnesium phosphate as well as calcium phosphate. A slightly more alkaline condition in the kidneys than in muscle would tend to precipitate out magnesium phosphate.

In skeletal muscle the affected lambs showed a highly significant increase in sodium and decrease in potassium, whereas, in general the differences were non-significant in heart muscle. This would seem to indicate that the skeletal muscles suffered a great osmotic imbalance while the heart remained fairly normal in ionic balance.

A preliminary plasma alkaline phosphatase analysis was determined on blood taken from ewes just prior to lambing. No significant difference was found between the control and selenium supplemented groups so the analysis was pursued no further. The results of the plasma

inorganic phosphate and alkaline phosphatase activity analyses for the two groups of ewes are presented in Table XIV.

Table XIV

1958-59 WMD Experiment

Plasma Inorganic Phosphate and Alkaline Phosphatase Activity in Ewes Prior to Birth of Lambs

Control			Selenium Supplemented		
Ewe No.	Inorg. Phosphate mg %	Phosphatase Activity*	Ewe No.	Inorg. Phosphate mg %	Phosphatase Activity*
26	2.82	2.46	37	5.79	1.24
27	4.12	1.59	38	4.06	1.78
30	4.27	3.98	39	4.87	1.42
33	3.45	7.29	42	4.73	1.64
35	4.10	2.21	46	4.44	5.95
36	2.94	1.65	48	4.00	1.26
Ave.	3.62 ± .64	3.20 ± 2.18		4.65 ± .66	2.22 ± 1.84

* Unit phosphatase activity = mg P liberated from β-glycerophosphate by 100 ml plasma in 1 hr. @ 37°C.

In an effort to determine the cause of calcification of muscle fibers in lambs afflicted with WMD, blood plasma protein-bound hexoses were determined. It was assumed that an increase in plasma protein-bound hexoses in affected animals would be indicative of an increase in plasma glycoproteins, more specifically chondroitin sulfuric acid. Also, histochemical techniques were applied to tissue sections in an attempt to demonstrate an

increase in concentration of chondroitin sulfate within the muscle fibers.

Although Winzler (87, p. 286) hypothesized that an increase in serum glycoprotein is a reflection of accelerated tissue destruction and that the glycoproteins arise from depolymerization of the ground substance of connective tissue, the possibility must not be overlooked that in types of myopathy in which muscle calcification occurs there may be an active transport of mucoproteins to the site of calcification, and that mucopolysaccharides act as a matrix for the deposition of calcium salts. Rubin and Howard (60, p. 155-166) have proposed that acid-mucopolysaccharides, specifically chondroitin sulfuric acid, are actively involved in the calcifying mechanism in bone. A similar system may become activated in pathogenic calcification of soft tissues.

The average plasma glycoprotein values and standard deviations and the results of statistical analyses of the data are presented in Table XV.

Table XV

1958-59 WMD Experiment

Plasma Protein-Bound Hexoses

Lot	mg hexose per 100 ml	t
I	112.2 ± 12.3	
II	104.6 ± 14.4	1.615
III	106.5 ± 13.8	1.260
IV	96.8 ± 7.3	3.688*

* Significantly different from Lot I ($P < .01$)

The plasma analyses show a trend towards an increase in protein-bound hexoses in animals affected with WMD, however the glycoprotein levels in lot I are only significantly higher than the values reported for lot IV. The average values for all four lots are somewhat lower than the values reported by Blincoe (5, p. XLIII-4). The lower values may be due to differences in ages of the lambs and to differences in stages of progression of the disease. The lambs studied by Blincoe were obtained through the cooperation of ranchers in western Nevada and displayed extreme clinical symptoms of stiffness, whereas in the present study only 5 out of 20 lambs in the control lot showed any outward signs of the disease. Blincoe reported highly significant increases in serum glycoprotein in lambs affected with WMD. The affected lambs had an

average of 222 ± 23 milligrams of hexose per 100 milliliters serum and non-affected lambs showed an average of 139 ± 19 milligrams per 100 milliliters. On the basis of these results it would seem desirable to pursue this possibility further.

In an effort to determine whether pathogenic calcification of soft tissues is caused by passive precipitation or is under enzymic control, sections of muscle tissue were stained with the von Kossa silver nitrate technique and then counterstained with the phthalocyanin dye, alcian blue 8GS. Silver nitrate is specific for calcium salts (39, p. 404), staining them dark brown or black. Alcian blue is specific for mucopolysaccharides (36, p. 787) and even thought by Attwood (2, p. 214) to stain only chondroitin and mucoitin sulfuric acid.

Chondroitin sulfate is intimately involved in bone salt formation probably in a calcium binding capacity (60, p. 155-166). It was felt that the demonstration of an increase in concentration of chondroitin sulfate in the regions of calcification inmuscle tissue would indicate enzymic control of calcium deposition. The muscle fibers in the sections stained a pale blue probably due to a small amount of mucopolysaccharides in the muscle tissue. Therefore, the areas immediately surrounding the calcium deposits were scrutinized closely to determine if there

was an increase in intensity of the blue coloration adjacent to the salt crystals. The slides were scanned under low power (10X objective) and then the calcified areas were studied in detail under high, dry power (45X objective).

The results of the study were encouraging but inconclusive. In many areas of calcification there appeared to be a more intense blue coloration and in some instances the differences in color intensity were vividly distinct. However, in many regions of salt deposits no increase of color intensity could be discerned. It may be that the chondroitin sulfate acts as a base for the crystal lattice of the salts to build upon. If so, then the polysaccharides would be localized on one side of the salt crystals and demonstration of increased concentrations would depend upon the tissue sections being cut through the proper portion of the muscle fibers and not all sections would show the increased color intensity.

Of the 20 animals in the basal experimental lot, 13 showed calcification in varying degrees and in 7 of the lambs no calcium deposits could be observed. Four of the 13 exhibiting calcification had only isolated specks of calcium salts scattered throughout the muscle fibers and 9 sets of tissue sections were extensively calcified. In many cases the sarcoplasm of entire muscle fibers was

displaced by calcium salts.

The results of this initial histochemical study suggest that the hypothesis concerning enzymic control of pathogenic calcification is worthy of further investigation.

SUMMARY

The annual losses of lambs from white muscle disease in Oregon were of sufficient magnitude to instigate the launching of a scientific investigation of the problem several years ago. The program was designed to study in detail the nature and effects of WMD and to effect a cure or prevention of the disease. The present study was carried out in conjunction with this project and the major objectives were to determine the protective effects of selenium and vitamin E by various routes of administration and to attempt to establish the cause and nature of salt deposits in the pathogenic soft tissue calcification that usually accompanies WMD. These objectives were accomplished by (1) feeding pregnant ewes legume roughages that were known to produce WMD in the lambs and administering vitamin E or selenium to specified groups, and (2) to analyze blood plasma and muscle tissue from affected and non-affected lambs to determine resulting abnormalities.

Embodied in this study are the results of feeding trials for two consecutive years termed the 1957-58 and 1958-59 experiments. In the 1957-58 trial 60 ewes were randomly assigned to five lots of 12 ewes each and given the following treatments: non-dystrophogenic alfalfa and Ladino clover hay and oats; basal diet of dystrophogenic

Ladino clover hay and oats; basal plus parenteral vitamin E administered to the ewes; basal plus oral vitamin E; and basal plus oral sodium selenite (mixed with the oats).

The 1958-59 experiment included four lots of 12 ewes each which received the following treatments, respectively: basal ration of dystrophogenic Ladino clover chaff and oats; basal plus parenteral selenium (as sodium selenite) administered to the lambs; basal plus vitamin E drench (given to the lambs at birth); basal plus 0.1 p.p.m. selenium (as sodium selenite) mixed in the oats.

The lambs were weighed at birth, two weeks, and six weeks of age. The two selenium treated lots showed increases in rate of gain over the control lot that were highly significant, whereas in the vitamin E supplemented group the growth rate was not significantly different from the control lambs.

The feedstuffs used in the experiments were subjected to a proximate and mineral analysis. There were no noticeable deviations in proximate constituents from normal values in the dystrophogenic legumes. In the mineral analysis, however, there was a wide calcium-phosphorus ratio and the selenium and molybdenum levels were very low in the WMD producing feeds.

The lambs were slaughtered at six weeks of age, necropsied and tissues were selected for histopathological

and chemical study. Chemical analyses consisted of determinations of dry matter, ether extract, ash, cholesterol, calcium, phosphorus, magnesium, sodium, and potassium in skeletal and heart muscle, and plasma glycoproteins and alkaline phosphatase activity in blood plasma.

Histochemical techniques were applied to muscle tissue sections in an effort to demonstrate enzymic control of calcification of soft tissues.

Neither injection of vitamin E into pregnant ewes or supplementing it in the diet provided any degree of protection from WMD in the lambs, whereas selenium supplementation caused almost complete prevention of the disease. However, protection from WMD was provided by drenching the lambs with massive doses of vitamin E at birth.

Administration of vitamin E to the ewes did alter the nature of the muscle lesions because the content of muscle inorganic constituents did not deviate as far from normal as the values observed in lambs from untreated ewes.

When the results of muscle tissue analysis from lambs in the 1957-58 experiment were subjected to statistical analysis no significant differences were observed between the treated and untreated lots because of great variability. This was to be expected in view of the reports that two different types of muscle lesions occur in lambs affected with WMD. Calcium salts are deposited in the

muscle fibers in one type and no muscle calcification occurs in the other type. However, when affected animals were divided into two groups, one including tissues showing calcification and the other including those which had apparently normal calcium levels, highly significant differences were obtained. There were significant increases in calcium, phosphorus, sodium, and cholesterol levels in muscle tissue, except for normal cholesterol values in heart muscle. Potassium levels were significantly decreased and magnesium contents remained normal. Thus, the deposition of calcium phosphate and other calcium salts, but not magnesium salts, are indicated. The calcium-phosphorus ratios of abnormal salt deposits are very similar to the calcium-phosphorus ratio of bone salt, which indicated that normal bone and pathogenic calcium deposits are similar in nature.

The increased sodium and decreased potassium levels suggest an upset of the ionic balance of muscle cells, and the elevated cholesterol content of skeletal muscle is indicative of an increased fat metabolism.

The results of the 1958-59 experiment were similar to those of the 1957-58 trials. Selenite, either injected in the lambs or fed to the ewes, offered complete protection against WMD, and drenching the lambs with massive doses of vitamin E also gave complete protection. Selenium

treatment produced a dramatic growth response in the lambs but vitamin E did not.

Similar elevated levels of calcium, phosphorus, sodium, and cholesterol, and decreased potassium values were observed.

Kidney tissue was analyzed for calcium, phosphorus, and magnesium in the 1958-59 trials. The values were all significantly increased in the kidneys from affected lambs which suggests that magnesium as well as calcium salts are deposited in the renal tubules.

Alkaline phosphatase determinations on blood plasma from ewes showed no significant difference between treated and untreated groups.

Determinations of plasma glycoproteins on lambs plasma indicated a trend towards an increase in glycoproteins with the affliction of WMD. The mucoprotein levels in affected lambs were significantly higher than those in the lambs from ewes fed selenite. The increases are probably caused by an increased protein catabolism and mobilization of mucopolysaccharides from connective tissue or there may be an active transport of mucoprotein to the sites of calcification in muscle tissue.

Muscle tissue sections were stained specifically to demonstrate an increase in concentration of mucopolysaccharides, presumably chondroitin sulfuric acid, in the

regions of calcification in the muscle tissues. A positive demonstration would suggest that pathogenic salt deposition is under enzymic control in a mechanism similar to that involved in bone salt formation. The results of the study were encouraging but inconclusive. Several instances were observed in which it appeared there were definite increases in chondroitin sulfate concentration, but in many calcified regions no differences could be discerned.

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A P P E N D I X

Appendix Table 1
Lot I--1957-58 Muscle Tissue Analysis

Lamb No.	Diagnosis	Skeletal Tissue				Cardiac Tissue			
		% DM	% EE*	% Ash*	mg% Cholesterol*	% DM	% EE	% Ash	mg% Cholesterol
14	-	21.61	8.90	5.18	304	20.50	5.00	5.56	373
15	-	20.84	5.85	5.54	292	18.86	3.08	6.01	153
30	-	alive							
94	-	23.45	14.75	5.16	138	22.12	7.12	4.96	534
43	-	20.77	3.22	5.42	313	20.39	7.44	5.66	549
48	-	22.24	11.82	5.16	211	22.16	14.38	5.11	448
49	-	20.30	2.68	5.83	292	20.58	3.62	5.56	280
54	-	22.66	15.49	4.19	262	24.19	5.42	5.76	470
55	-	21.80	8.79	5.38	196	20.32	3.97	5.13	502
74	-	22.01	13.01	5.37	248	21.60	5.33	5.32	336
75	-	22.96	8.79	5.29	234	20.77	5.10	5.48	333
76	-	21.08	6.62	5.67	420	20.68	4.37	5.66	638
79	-	alive							
80	-	23.27	17.84	5.40	296	21.09	3.41	5.18	349
84	-	23.26	13.11	4.74	240	20.30	8.32	4.72	636
85	-	20.88	5.50	5.53	430	21.56	10.36	5.53	543
86	-	alive							
87	†	21.15	6.10	5.28	224	20.36	2.87	5.42	497
88	-	19.13	5.08	5.68	536	19.71	8.71	5.76	651
90	-	19.83	2.69	5.75	701	21.67	8.70	5.28	385
91	-	19.98	8.14	5.57	270	20.42	6.14	5.64	374
Mean		21.51	8.80	5.34	312	20.96	6.30	5.43	447

* Ether Extract is reported as percentage of dry tissue. Ash is reported on dry, fat free basis. Cholesterol is reported as mg/100 gms dry tissue.

Appendix Table 2
Lot II--1957-58 Muscle Tissue Analysis

Lamb No.	Diagnosis	Skeletal Tissue				Cardiac Tissue			
		% DM	% EE*	% Ash*	mg% Cholesterol*	% DM	% EE	% Ash	mg% Cholesterol
1	+	20.97	6.71	5.28	306	20.65	10.00	5.94	411
2	-	20.04	7.94	5.96	307	18.91	9.78	5.88	554
5	+	18.79	12.33	12.73	707	19.41	2.75	5.54	368
20	-	21.49	2.93	5.46	268	19.52	3.23	5.66	406
21	+	17.74	13.38	5.84	946	19.13	2.52	10.18	452
31	+	23.55	21.79	4.80	408	25.33	26.12	5.28	534
32	-	20.84	1.59	5.00	306	21.48	7.84	5.48	331
33	+	20.92	12.68	4.90	400	21.31	12.01	5.15	583
38	+	20.94	15.19	6.75	753	16.35	4.44	5.41	497
39	-	19.72	5.59	5.92	436	20.47	12.79	5.62	544
57	+	18.59	8.49	4.37	308	20.96	17.66	6.16	245
63	+	18.11	10.99	5.60	481	18.48	1.88	5.29	329
70	+	18.14	7.64	5.47	438	21.76	4.93	6.95	547
77	+	18.02	5.61	5.58	499	18.14	7.65	7.00	507
78	+	15.56	2.42	6.10	523	16.90	4.18	8.31	608
Mean		19.56	9.02	5.98	472	19.92	8.52	6.26	461

* Ether Extract is reported as percentage of dry tissue. Ash is reported on dry, fat free basis. Cholesterol is reported as mg/100 gms dry tissue.

Appendix Table 3
Lot III--1957-58 Muscle Tissue Analysis

Lamb No.	Diagnosis	Skeletal Tissue				Cardiac Tissue			
		% DM	% EE*	% Ash*	mg% Cholesterol*	% DM	% EE	% Ash	mg% Cholesterol
6	+	22.89	19.94	4.68	336	19.53	6.87	5.34	291
89	-	23.80	22.10	5.35	309	21.96	9.09	5.09	576
93	+	21.78	17.20	5.18	334	21.24	10.73	5.70	393
23	-	20.19	3.47	5.97	455	20.71	3.96	5.71	418
24	+	20.81	9.96	5.31	433	21.02	4.98	5.25	588
25	+	23.30	11.50	4.96	354	21.05	15.32	5.64	500
29	+	21.65	6.62	5.22	282	22.53	8.14	5.13	201
28	+	17.90	12.50	5.54	391	20.99	5.73	6.61	510
40	+	22.50	10.19	4.84	327	20.11	5.19	5.34	472
46	+	22.76	9.12	4.99	387	21.88	16.64	6.36	637
47	+	22.02	16.28	16.30	1124	21.40	4.45	5.25	436
52	+	21.90	15.06	4.73	393	19.88	6.04	5.26	622
60	-	18.64	4.48	5.29	272	17.04	3.28	5.30	442
95	+	24.13	13.18	5.11	305	22.71	15.76	5.44	362
96	+	spoiled--discarded							
Mean		21.73	12.26	5.96	407	22.48	8.30	5.53	475

* Ether Extract is reported as percentage of dry tissue. Ash is reported on dry, fat free basis. Cholesterol is reported as mg/100 gms dry tissue.

Appendix Table 4
Lot IV--1957-58 Muscle Tissue Analysis

Lamb No.	Diagnosis	Skeletal Tissue				Cardiac Tissue			
		% DM	% EE*	% Ash*	mg% Cholesterol*	% DM	% EE	% Ash	mg% Cholesterol
3	+	19.91	13.73	9.91	683	20.56	7.61	5.64	467
4	+	22.81	7.88	5.00	418	22.36	12.62	4.99	484
45	+	21.00	20.06	5.60	476	20.64	3.84	5.15	604
8	+	22.57	5.07	6.61	456	20.47	6.83	6.12	425
16	+	20.20	24.24	5.23	640	20.26	7.99	5.35	562
26	+	15.30	5.12	5.87	399	21.30	9.41	10.12	388
27	+	19.64	8.34	6.08	624	21.16	3.67	6.00	345
34	+	21.94	33.03	5.25	411	21.94	9.24	5.60	265
35	-	17.55	2.88	5.99	857	17.85	2.78	6.32	393
36	-	18.50	3.63	4.63	352	20.02	11.38	4.55	352
37	+	21.10	29.81	5.39	685	21.30	8.95	5.44	370
41	+	23.11	16.42	5.15	251	20.24	8.39	5.38	372
50	+	25.74	38.46	5.13	305	20.56	6.73	5.19	448
51	+	19.19	16.80	5.10	302	19.62	15.45	5.27	385
67	-	20.63	10.10	4.41	169	20.50	7.95	5.36	396
68	-	21.38	5.49	5.19	396	20.40	4.96	5.36	468
82	+	lost							
81	+	spoiled--discarded							
83	+	19.98	13.06	3.84	463	21.94	15.12	3.62	408
92	+	20.20	4.55	5.56	392	21.95	5.70	5.12	324
Mean		20.59	14.37	5.55	460	20.73	8.26	5.59	414

* Ether Extract is reported as percentage of dry tissue. Ash is reported on dry, fat free basis. Cholesterol is reported as mg/100 gms dry tissue.

Appendix Table 5
Lot V--1957-58 Muscle Tissue Analysis

Lamb No.	Diagnosis	Skeletal Tissue				Cardiac Tissue			
		% DM	% EE*	% Ash*	mg% Cholesterol*	% DM	% EE	% Ash	mg% Cholesterol
44	-	22.60	8.34	5.02	329	21.08	6.93	5.38	465
66	-	23.45	14.54	5.18	301	21.04	2.05	5.31	369
12	-	23.30	14.37	5.25	195	22.89	8.19	5.36	424
13		Born dead--no tissue							
61	-	22.19	6.31	5.19	283	20.68	4.67	5.63	536
18	-	19.98	1.41	5.86	292	20.32	7.66	5.73	473
19	-	21.63	1.90	5.23	316	20.02	13.63	5.29	370
53	-	21.28	6.67	5.04	411	20.48	7.04	5.56	595
56	-	24.98	18.15	5.04	262	21.19	4.62	5.31	324
97	-	25.25	24.58	4.98	323	21.48	10.98	5.17	445
59	-	19.34	4.23	5.71	797	20.35	4.12	5.53	424
62	-	22.68	9.46	4.88	253	21.20	5.16	5.30	333
64	-	23.90	12.02	4.87	209	21.62	6.36	5.16	537
65	-	20.92	6.81	5.61	266	19.36	1.99	5.37	178
69	+	21.78	18.80	5.26	385	20.36	5.12	4.63	354
71	+	22.42	26.00	5.06	285	19.39	7.61	5.26	413
72	+	20.40	9.78	5.01	446	20.40	2.64	5.44	252
73	-	alive							
Mean		22.26	11.45	4.62	335	20.74	6.17	5.34	406

* Ether Extract is reported as percentage of dry tissue. Ash is reported on dry, fat free basis. Cholesterol is reported as mg/100 gms dry tissue.

Appendix Table 6
Lot I--1957-58 Mineral Analysis of Muscle Tissue

Lamb No.	Skeletal Tissue					Cardiac Tissue				
	Ca	P	Mg	Na	K	Ca	P	Mg	Na	K
	mg/100 gms of dry, fat free tissue					mg/100 gms of dry, fat free tissue				
14	18.6	280	117	460	1886	28.3	345	130	460	1279
15	18.9	590	121	342	1803	38.5	690	149	532	1831
30	alive									
94	17.4	573	131	306	1618	40.9	307	122	568	1358
43	43.2	882	117	362	1787	35.5	957	142	436	1765
48	55.5	694	123	312	1761	54.8	747	123	469	1470
49	49.6	650	124	371	1990	56.4	738	123	566	1709
54	14.1	606	120	296	1221	10.4	587	101	453	1529
55	19.9	556	117	317	1911	20.1	726	119	523	1369
74	21.6	845	147	385	2091	41.0	840	136	505	1556
75	24.6	529	99	242	1744	15.5	928	99	500	1574
76	24.6	1052	151	408	2065	12.6	549	141	376	1822
79	alive									
80	15.9	549	119	341	1720	38.0	620	138	478	1373
84	19.7	567	132	356	1821	21.6	470	94	578	1451
85	30.3	779	107	505	1768	37.7	1059	154	391	1658
86	alive									
87	27.5	558	94	279	1924	30.3	361	133	457	1651
88	42.3	880	138	513	1760	32.2	958	143	449	1730
90	42.3	955	93	504	1589	44.4	874	131	513	1789
91	44.4	576	119	376	1945	25.1	677	119	469	1605
Mean	29.5	673	120	371	1800	32.4	691	128	485	1584

Appendix Table 7

Lot II--Mineral Analysis of Muscle Tissue

Lamb No.	Skeletal Tissue					Cardiac Tissue				
	Ca	P	Mg	Na	K	Ca	P	Mg	Na	K
	mg/100 gms of dry, fat free tissue					mg/100 gms of dry, fat free tissue				
1	236.7	812	123	573	1198	297.0	945	143	442	1603
2	33.3	870	133	429	1773	44.5	1047	113	554	1497
5	4771.6	2391	332	1033	464	66.8	864	133	441	1675
20	22.4	563	104	360	1551	26.8	878	121	503	1659
21	167.5	730	132	903	1209	17.8	520	136	409	1513
31	72.8	780	139	580	1425	26.2	248	139	376	1513
32	22.4	241	116	416	1461	51.8	876	116	516	1569
33	24.8	841	115	560	1730	31.4	899	133	543	1606
38	480.5	558	158	605	1454	28.7	957	105	571	1543
39	34.2	244	127	433	1593	35.6	933	143	510	1690
57	134.8	564	93	805	809	387.9	671	140	812	1032
63	214.5	894	171	933	1037	106.8	778	124	786	1014
70	270.1	950	109	1164	1006	1016.2	1008	152	807	796
77	30.2	941	140	606	1438	479.0	680	110	799	1312
78	599.9	1030	110	1237	615	1652.0	1148	161	914	1162
Mean	474.4	827	140	709	1251	284.6	830	131	599	1412

Appendix Table 8

Lot III--Mineral Analysis of Muscle Tissue

Lamb No.	Skeletal Tissue					Cardiac Tissue				
	Ca	P	Mg	Na	K	Ca	P	Mg	Na	K
	mg/100 gms of dry, fat free tissue					mg/100 gms of dry, fat free tissue				
6	35.8	815	123	500	1230	50.9	848	122	613	1449
89	21.2	839	121	312	1897	14.5	323	118	407	1511
93	138.8	688	116	346	1752	398.5	953	123	378	1579
23	31.6	635	112	524	1671	16.4	718	124	475	1612
24	26.3	433	119	318	1810	42.0	414	146	438	1522
25	28.6	828	132	350	1661	52.5	932	136	563	1831
29	50.8	827	136	338	1808	25.1	856	116	426	1299
28	253.5	895	99	1168	878	603.8	798	150	838	1089
40	14.7	804	115	317	1734	14.6	286	117	429	1352
46	24.2	274	132	255	1769	420.1	1071	140	579	1451
47	4520.9	2790	342	886	1625	26.9	951	124	441	1489
52	19.1	786	111	421	1356	25.7	924	159	450	1611
60	22.4	578	93	609	1439	35.6	766	27	642	1316
95	254.7	781	176	532	1194	81.6	615	124	545	1534
96	spoiled--discarded									
Mean	388.8	862	138	491	1559	129.2	747	123	516	1475

Appendix Table 9

Lot IV--Mineral Analysis of Muscle Tissue

Lamb No.	Skeletal Tissue					Cardiac Tissue				
	Ca	P	Mg	Na	K	Ca	P	Mg	Na	K
	mg/100 gms of dry, fat free tissue					mg/100 gms of dry, fat free tissue				
3	2977.5	1831	210	1227	576	141.3	996	134	499	1524
4	44.3	281	124	294	1673	52.9	395	130	385	1567
45	28.0	320	114	601	1765	25.8	540	119	410	1551
8	32.8	426	137	388	1875	17.9	704	78	506	1496
16	46.6	930	131	882	1065	41.2	356	104	450	1592
26	848.3	466	50	615	428	2423.6	1490	242	833	1253
27	952.1	946	159	878	886	340.5	919	132	428	1387
34	56.9	858	141	792	1153	78.4	654	132	612	1625
35	22.5	651	101	723	1409	16.6	750	137	562	1839
36	36.8	433	111	570	1229	91.7	625	94	623	1182
37	26.2	507	116	802	1319	124.6	284	102	512	1755
41	11.5	696	119	316	1599	32.9	731	121	542	1458
50	13.9	870	112	488	1633	35.6	970	160	436	1435
51	50.3	811	117	802	1281	112.4	815	133	750	1255
67	6.1	678	85	302	1491	19.7	894	124	551	1486
68	20.2	868	113	340	1589	17.0	955	132	407	1570
82	lost									
81	spoiled--discarded									
83	30.2	618	73	384	1154	10.6	533	66	297	822
92	15.6	455	112	216	1599	53.4	554	107	351	1525
Mean	290.0	702	118	590	1318	202.0	731	125	509	1462

Appendix Table 10

Lot V--Mineral Analysis of Muscle Tissue

Lamb No.	Skeletal Tissue					Cardiac Tissue				
	Ca	P	Mg	Na	K	Ca	P	Mg	Na	K
	mg/100 gms of dry, fat free tissue					mg/100 gms of dry, fat free tissue				
44	13.7	814	117	359	1460	19.3	968	114	537	205
66	28.4	876	142	345	1665	18.9	392	110	402	1624
12	35.3	343	104	343	1842	22.9	470	119	441	1421
13	Born dead--no tissue									
61	28.2	352	113	479	1822	25.1	965	131	425	2639
18	28.7	845	112	415	1561	21.8	574	111	288	1746
19	15.4	776	115	436	1815	12.8	879	135	424	1561
53	43.9	304	119	412	1334	32.0	255	112	464	1477
56	14.1	806	123	294	1484	28.4	978	85	432	1463
97	27.9	842	102	277	1830	34.9	915	127	517	1595
59	35.8	362	117	693	1637	41.4	966	120	467	1564
62	12.2	785	135	311	1566	27.5	938	148	405	1620
64	41.1	786	124	365	1549	18.4	948	122	416	1320
65	33.0	642	124	370	2000	33.3	820	117	412	1626
69	18.6	810	131	472	1476	14.0	783	100	364	1280
71	28.4	748	127	525	1628	22.0	736	107	457	1564
72	21.5	719	90	515	1519	23.6	638	101	389	1385
73	alive									
Mean	26.6	676	118	413	1638	24.8	760	116	428	1506

Appendix Table 11

1958-59 Experimental Feeds

% on Dry Matter Basis	Madras Ladino Clover Chaff	Oats
Dry Matter	87.09	87.46
Crude Fat	2.42	4.74
Crude Protein	12.86	12.02
Crude Fiber	25.55	13.78
Ash	9.29	3.16
Calcium	1.26	.06
Magnesium	.58	.02
Phosphorus	.15	.15
Sodium	.12	.004
Potassium	3.09	.44

Appendix Table 12

1958-59 WMD Experiment--Lamb Growth Data

Lot I				Lot II			
Lamb No.	Birth Weight	6 Wk. Weight	Total Gain	Lamb No.	Birth Weight	6 Wk. Weight	Total Gain
101	10.0	24.0	14.0	126	10.0	34.0	24.0
102	9.25	-		127	8.0	32.0	24.0
103	8.0	23.0	15.0	128	7.5	28.5	21.0
104	8.5	26.5	18.0	129	11.5	41.0	29.5
105	12.0	24.0	12.0	130	7.5	38.0	30.5
106	8.5	19.5	11.0	131	11.5	39.0	27.5
107	8.5	18.5	10.0	132	9.5	25.0	15.5
108	10.0	21.5*		133	9.0	20.0	11.0
109	8.25	14.0	5.75	134	7.5	28.0	20.5
110	10.5	28.0	17.5	135	9.5	29.0	19.5
111	12.0	25.0	13.0	136	12.0	-	
112	8.0	22.0	14.0	137	11.5	33.5	22.0
113	10.0	27.5	17.5	138	11.0	26.0	15.0
114	12.0	34.5	22.0	139	7.75	22.0	14.25
115	11.0	32.5	21.5	140	8.5	26.0	17.5
116	12.0	32.0*		141	7.0	-	
117	9.5	27.5	18.0	142	7.0	-	
118	10.5	31.5	21.0				
119	10.5	24.0	13.5				
120	9.75	-					

Appendix Table 12, continued

Lot III				Lot IV			
Lamb No.	Birth Weight	6 Wk. Weight	Total Gain	Lamb No.	Birth Weight	6 Wk. Weight	Total Gain
151	9.0	28.5	19.5	176	-	-	
152	5.0	17.0	12.0	177	12.0	46.0	34.0
153	8.0	-		178	8.0	-	
154	11.0	40.0	29.0	179	11.5	57.0	45.5
155	8.0	26.0	18.0	180	7.5	27.0	19.5
156	12.0	39.0	27.0	181	8.75	30.0	21.25
157	7.25	22.0	14.75	182	13.5	47.0	33.5
158	6.75	-		183	7.75	-	
159	10.5	25.0	14.5	184	8.25	-	
160	9.5	28.0	18.5	185	7.0	-	
161	10.5	21.0	10.5	186	7.5	24	16.5
162	9.0	20.0	11.0	187	10.5	30	19.5
163	7.0	23.5	16.5	188	7.25	-	
164	8.5	31.0	22.5	189	8.0	-	
165	10.25	10.0	0	190	5.0	-	
166	8.5	21.0	12.5	191	12.5	45.5	33.0
167	10.0	44.0	34.0	192	9.75	30.0	20.25
168	11.5	-		193	10.0	38.5	28.5

* Lambs 108 and 116 were weighed and slaughtered at 5 weeks of age because they were so severely affected survival to 6 weeks was improbable. The weights are not included in the analysis of data.

Appendix Table 13
1958-59 Skeletal Tissue Analysis

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot I - Control					
101	16.47	14.93	5.98	1130	738	113.6	152	1015	1236
102	No tissue								
103	17.08	10.54	5.70	1110	728	107.9	124	800	1187
104	17.33	4.94	4.98	633	706	132.2	146	969	883
105	20.32	9.79	4.66	562	480	69.6	144	253	1199
106	20.20	7.29	5.45	436	588	94.4	144	310	1689
107	19.86	7.02	5.78	470	599	103.5	146	275	1750
108	17.11	15.58	5.50	1293	838	73.3	127	1062	1047
109	15.86	6.81	6.00	1219	756	98.3	127	820	1378
110	20.58	9.56	9.55	993	1139	1337.9	227	493	1381
111	18.43	14.97	4.88	988	526	34.0	166	512	1158
112	20.44	19.46	5.45	918	650	90.4	147	723	1173
113	19.04	16.20	5.53	882	612	80.4	118	618	1265
114	20.55	18.36	4.90	861	534	93.4	108	388	1281
115	19.86	11.87	5.59	609	598	101.2	145	367	1704
116	19.96	15.07	20.68	909	3008	5179.9	337	1122	795
117	18.79	10.88	5.34	840	582	81.0	140	542	1362
118	18.79	10.67	5.59	741	663	94.0	136	462	1554
119	18.43	10.12	5.21	791	566	88.9	131	602	1257
120	16.49	0.34	5.06	865	644	65.5	148	961	946

Appendix Table 13, continued

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot II - Injected Na_2SeO_3					
126	21.82	5.57	5.30	306	809	63.7	201	270	1642
127	23.14	11.99	5.48	320	725	61.2	143	293	1670
128	19.25	6.86	5.34	394	826	56.2	175	384	1719
129	24.18	17.98	5.05	317	821	49.9	182	303	1681
130	20.18	10.59	5.32	627	728	73.4	146	543	1524
131	25.97	12.13	4.94	500	604	60.4	155	494	1382
132	17.51	6.20	5.21	640	763	63.6	145	574	1145
133	19.07	6.61	5.26	695	752	48.2	167	695	1365
134	19.88	11.48	5.47	687	796	52.7	167	598	1341
135	20.59	7.10	5.61	410	662	66.9	149	324	1868
136	No tissue								
137	21.45	9.10	5.35	407	738	73.5	135	251	1775
138	19.00	5.62	5.80	499	786	52.2	179	335	1838
139	18.81	8.12	5.36	730	762	35.0	195	517	1411
140	17.62	6.81	5.41	703	714	48.9	168	616	1193
141	No tissue								
142	No tissue								

Appendix Table 13, continued

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot III - Oral Vitamin E					
151	23.54	13.40	5.06	301	772	32.7	119	227	1575
152	22.23	7.14	5.38	401	854	39.8	127	229	1687
153	No tissue								
154	20.67	7.03	5.29	141	753	46.2	155	255	1754
155	21.39	7.90	5.34	325	760	38.7	125	244	1706
156	21.75	4.41	5.26	351	788	29.2	123	232	1744
157	21.68	6.41	5.32	350	842	54.7	133	269	1738
158	No tissue								
159	19.51	4.45	5.98	540	881	52.7	136	330	1800
160	20.88	7.91	5.60	408	690	48.3	120	307	1698
161	20.86	8.04	5.52	367	795	49.4	123	371	1581
162	20.43	5.51	5.70	447	776	52.9	111	427	1739
163	21.30	5.66	5.62	391	756	62.5	179	323	1859
164	21.57	7.58	5.80	372	726	50.4	135	262	1618
165	19.68	1.53	5.69	270	876	45.4	107	588	1279
166	20.71	4.50	5.68	409	708	59.8	188	257	1790
167	21.09	4.76	5.29	354	777	32.5	133	220	1848
168	18.61	13.87	5.47	782	780	56.3	151	755	1197

Appendix Table 13, continued

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot IV - Oral Na_2SeO_3					
176	No tissue								
177	22.54	11.78	5.20	349	733	61.9	108	236	1597
178	No tissue								
179	22.86	25.85	5.17	457	791	61.9	118	218	1522
180	21.54	6.59	5.37	338	831	71.8	102	227	1678
181	21.12	5.58	5.51	376	737	64.2	148	237	1642
182	20.84	7.18	5.15	349	698	64.0	130	290	1471
183	No tissue								
184	No tissue								
185	No tissue								
186	20.73	7.30	5.45	475	712	64.6	116	324	1568
187	20.58	7.42	5.37	531	708	68.0	109	355	1430
188	No tissue								
189	No tissue								
190	No tissue								
191	18.50	7.84	5.27	474	787	101.8	134	714	932
192	22.38	7.79	5.31	369	695	65.9	129	212	1660
193	23.08	11.45	5.30	418	740	69.6	115	253	1611

Appendix Table 14
1958-59 Cardiac Tissue Analysis

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot I - Control					
101	19.56	2.96	5.52	569	689	96.2	145	488	1414
102	No tissue								
103	18.92	3.41	5.54	800	629	94.6	163	520	1475
104	19.46	4.31	5.40	735	610	94.9	144	544	1287
105	19.40	2.41	5.91	569	634	342.9	134	354	1072
106	20.35	7.90	5.42	728	602	90.0	116	496	1407
107	18.48	3.37	5.70	601	689	88.8	134	474	1318
108	19.33	5.35	7.02	554	942	487.5	142	876	1139
109	17.27	3.78	6.08	845	670	174.3	150	724	1295
110	18.65	4.05	5.77	600	648	107.3	150	522	1446
111	18.64	3.46	5.22	697	613	90.9	127	452	1138
112	19.08	8.67	5.62	837	604	108.0	135	520	1374
113	18.77	2.92	5.77	666	676	104.9	136	477	1534
114	19.68	3.11	4.96	623	652	67.3	115	321	1227
115	19.70	2.98	5.54	691	656	105.7	144	397	1334
116	20.00	3.55	6.32	664	744	370.6	137	805	1160
117	19.56	3.64	5.51	677	575	112.1	115	469	1309
118	19.17	5.34	5.47	599	655	78.1	136	528	1328
119	18.57	4.05	5.51	655	643	112.7	137	515	1366
120	21.12	2.05	5.53	840	744	131.2	137	1122	893

Appendix Table 14, continued

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot II - Injected Na_2SeO_3					
126	19.80	5.39	5.12	558	853	58.4	154	395	1423
127	19.68	3.07	5.31	557	906	48.5	172	486	1263
128	20.36	4.22	5.08	403	774	51.0	175	528	1257
129	21.15	2.34	5.07	432	916	69.6	137	428	1330
130	19.86	3.31	5.36	667	809	43.0	161	566	1398
131	20.42	5.07	5.46	644	684	40.7	175	583	1391
132	21.29	3.00	5.42	520	754	75.0	144	407	1472
133	19.58	2.71	5.42	553	791	71.8	137	576	1339
134	19.29	3.39	5.53	627	780	72.9	146	469	1340
135	19.67	3.19	5.54	596	733	53.4	160	542	1421
136	No tissue								
137	20.12	2.78	5.32	687	865	69.9	145	381	1436
138	19.14	4.34	5.49	739	849	72.3	146	591	1410
139	19.06	2.41	5.65	597	736	72.8	135	511	1435
140	19.46	2.07	5.53	655	692	45.8	178	457	1348
141	No tissue								
142	No tissue								

Appendix Table 14, continued

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot III - Oral Vitamin E					
151	19.77	7.32	4.85	638	834	24.6	120	392	1167
152	20.60	1.79	4.84	370	916	24.9	122	383	1109
153	No tissue								
154	19.69	4.37	5.38	459	858	35.8	102	464	1361
155	26.60	9.66	5.28	986	1090	49.2	91	283	1092
156	20.50	2.14	4.96	387	950	36.8	128	539	1279
157	21.03	5.06	5.08	499	874	53.8	125	451	1303
158	No tissue								
159	19.74	2.97	5.30	633	823	43.7	179	509	1278
160	19.38	4.31	5.41	574	780	36.3	128	496	1301
161	20.53	4.56	5.45	661	891	44.1	153	419	1306
162	21.02	3.59	5.29	608	859	48.5	142	636	1305
163	19.46	4.35	5.49	675	816	51.9	163	471	1416
164	19.41	3.79	5.74	462	804	50.7	161	495	1356
165	17.64	3.16	5.63	330	833	49.0	100	693	1499
166	19.54	3.64	5.33	657	810	48.8	175	487	1307
167	19.37	4.18	5.39	596	902	25.8	142	414	1330
168	20.51	3.92	5.48	629	664	54.3	171	501	1153

Appendix Table 14, continued

Lamb No.	D.M.	E.E.	Ash	Cholesterol	P	Ca	Mg	Na	K
				Lot IV - Oral Na_2SeO_3					
176	No tissue								
177	19.82	3.48	5.34	589	883	70.9	116	380	1393
178	No tissue								
179	20.55	4.18	5.11	442	884	60.3	128	370	1241
180	19.48	13.57	5.09	489	931	42.9	116	401	1164
181	19.81	2.27	5.39	582	846	61.0	119	414	1272
182	20.68	3.00	5.50	567	820	66.3	120	417	1329
183	No tissue								
184	No tissue								
185	No tissue								
186	18.76	3.87	5.65	563	894	66.5	138	511	1377
187	19.00	3.26	5.70	633	872	73.8	119	518	1329
188	No tissue								
189	No tissue								
190	No tissue								
191	19.96	5.48	5.53	624	798	69.4	128	457	1277
192	20.42	2.44	5.27	490	837	65.8	117	365	1301
193	19.08	3.55	5.59	617	846	67.4	130	472	1367

Appendix Table 15
1958-59 Kidney Analysis

Lamb No.	D.M.	Ash	P	Ca	Mg	Lamb No.	D.M.	Ash	P	Ca	Mg
	Lot I - Control						Lot II - Injected Na_2SeO_3				
101	20.04	6.38	925	117.0	151	126	19.92	6.21	507	111.9	171
102	No tissue					127	19.50	6.89	964	139.8	145
103	20.96	6.22	538	140.8	144	128	21.08	6.63	588	101.9	124
104	20.08	6.24	626	117.6	142	129	22.65	5.96	604	92.2	155
105	27.17	3.85	580	76.1	113	130	18.65	6.45	546	86.5	163
106	19.88	6.48	912	138.6	126	131	18.54	6.32	546	86.1	134
107	19.74	6.26	826	119.4	132	132	20.31	6.39	510	117.6	157
108	20.54	6.24	854	121.0	108	133	18.99	6.53	573	88.0	162
109	20.44	6.23	850	124.5	146	134	20.04	6.39	468	92.6	160
110	20.42	6.36	924	118.3	140	135	19.80	6.53	498	108.0	143
111	21.58	5.48	836	99.9	134	136	No tissue				
112	18.97	6.30	856	135.6	152	137	20.37	6.28	530	96.9	151
113	17.86	6.53	1008	130.2	146	138	20.37	6.29	475	117.8	154
114	19.75	5.97	996	104.5	165	139	19.25	6.46	1092	98.8	154
115	20.14	6.11	620	119.7	117	140	18.54	6.56	549	97.9	160
116	21.17	6.14	1143	111.0	130	141	No tissue				
117	19.84	6.28	850	125.2	142	142	No tissue				
118	19.70	6.38	862	119.6	140						
119	17.18	6.58	903	120.0	168						
120	No tissue										

Appendix Table 15, continued

Lamb No.	D.M.	Ash	P	Ca	Mg	Lamb No.	D.M.	Ash	P	Ca	Mg
	Lot III - Oral Vitamin E						Lot IV - Oral Na_2SeO_3				
151	20.06	6.13	585	76.0	93	176	No tissue				
152	20.30	6.36	356	122.8	88	177	20.93	6.00	274	107.0	90
153	No tissue					178	No tissue				
154	20.35	6.21	485	75.1	106	179	20.04	6.01	277	107.0	98
155	20.61	6.12	585	69.4	127	180	20.73	6.27	318	114.3	77
156	20.77	6.22	510	83.9	122	181	20.45	6.15	344	110.4	94
157	21.49	6.24	566	83.2	117	182	21.70	6.15	414	114.1	79
158	No tissue					183	No tissue				
159	19.35	6.55	444	80.4	118	184	No tissue				
160	19.59	6.39	330	119.7	81	185	No tissue				
161	19.79	6.51	508	92.2	114	186	19.82	6.55	362	130.7	97
162	18.81	6.48	425	122.7	87	187	19.01	6.50	352	125.8	109
163	19.26	6.48	526	136.2	96	188	No tissue				
164	19.80	6.43	312	124.3	82	189	No tissue				
165	17.93	5.98	928	71.0	107	190	No tissue				
166	19.20	6.23	580	65.7	147	191	19.07	6.59	326	128.1	100
167	18.02	6.46	499	93.2	132	192	18.43	6.60	328	152.4	103
168	20.14	6.33	326	132.7	102	193	19.72	6.07	376	95.8	91

Appendix Table 16
1958-59 WMD Experiment - Plasma Protein-Bound Hexoses

Lot I		Lot II		Lot III		Lot IV	
Lamb No.	mg/100 ml	Lamb No.	mg/100 ml	Lamb No.	mg/100 ml	Lamb No.	mg/100 ml
101	108.5	126	98.5	151	115.0	176	no plasma
102	no plasma	127	91.0	152	107.5	177	94.5
103	106.0	128	93.0	153	no plasma	178	no plasma
104	115.5	129	99.5	154	94.0	179	105.0
105	113.0	130	119.5	155	94.0	180	102.5
106	98.5	131	95.5	156	104.5	181	97.0
107	97.5	132	117.0	157	128.0	182	100.5
108	103.5	133	145.5	158	no plasma	183	no plasma
109	106.5	134	100.0	159	88.5	184	no plasma
110	112.5	135	97.5	160	92.5	185	no plasma
111	95.0	136	no plasma	161	105.0	186	87.5
112	125.0	137	95.0	162	102.5	187	90.0
113	115.0	138	105.0	163	134.5	188	no plasma
114	117.0	139	106.5	164	126.0	189	no plasma
115	110.0	140	100.5	165	no plasma	190	no plasma
116	143.5	141	no plasma	166	104.5	191	84.5
117	104.0	142	no plasma	167	104.0	192	103.0
118	115.5			168	96.5	193	103.0
119	133.0						
120	no plasma						