ABSTRACT

THE ROLES OF VITAMIN E, SELENIUM, AND METHIONINE

IN DIETARY LIVER NECROSIS AND

NUTRITIONAL MUSCULAR DYSTROPHY IN THE PIG

by Robert L. Michel

Three experiments were conducted to study the pathology and pathogenesis of dietary liver necrosis (DLN) in the pig and to clarify the roles of vitamin E, selenium, the sulfur amino acids, and total dietary protein in the prevention of this disease. A total of 48 pigs was used in the 3 trials. A basal 6% protein diet contained Torula yeast as a source of protein and vitamin E-free lard as a source of fat. The various supplements employed included vitamin E at 2 levels, selenium, and methionine, each alone, and in several combinations. Other pigs were fed a diet containing the antioxidant ethoxyquin, and 6 pigs were fed rations containing sufficient Torula yeast to provide a 20% protein diet.

Dietary liver necrosis occurred consistently in pigs fed the basal 6% protein ration, or the basal 6% protein ration supplemented with low levels of vitamin E or with methionine. Supplementation with selenium, high levels of vitamin E, or additional protein completely prevented dietary liver necrosis during the periods of these trials. Lesions of DLN were observed in 1 of 4 pigs fed ethoxyquin.

Acute dietary liver necrosis appeared grossly as scattered red spots on the surfaces and in the parenchyma of affected livers. Chronic

changes were represented by irregularly shaped, roughened areas resulting from the contraction of scar tissue.

Microscopically the most striking features were necrosis of hepatocytes, pooling of blood in the necrotic lobules, and a characteristic limitation of the necrosis by the interlobular septa. Connective tissue and bile duct proliferation occurred as a sequel to necrosis.

Microscopic lesions of nutritional muscular dystrophy (NMD) were observed in some pigs of all groups except those fed the higher level of vitamin E or ethoxyquin. Muscle tissue of only 2 of 4 pigs fed ethoxyquin was examined for this lesion.

Ulcers or pre-ulcerous changes of the squamous epithelium of the stomach were observed in virtually all the pigs in these experiments. These lesions were somewhat less severe among pigs fed 20% protein rations.

Hydropic degeneration of hepatocytes and extreme atrophy of the thymus occurred consistently in pigs fed 6% protein diets.

Serum ornithine carbamyl transferase activity of pigs with dietary liver necrosis was generally higher than that of pigs free of DLN. However, none exceeded values reported as normal for pigs by one author.

Nuclear abnormalities in erythroid cells in bone marrow smears were relatively common among pigs maintained on vitamin E-deficient diets, whereas such lesions were extremely rare among the group fed a high level of vitamin E.

Growth rates were very poor in pigs fed 6% protein diets, but tocopherol deficiency appeared to have little effect on growth.

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By

Robert L. Louis Michel

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INTRODUCTION

Dietary liver necrosis (DLN) in the rat has been described by Gyorgy and Goldblatt (1939) and Schwarz (1951). Obel (1953) described a similar condition in the pig. The disease constitutes a significant clinical problem in commercial swine operations in some parts of the world, notably the Scandinavian countries, Germany, and New Zealand. A morphologically indistinguishable condition occurs in the Pacific Northwest of the United States and has been observed by veterinarians at Washington State University. There is a considerable volume of literature dealing with these field occurrences and with the experimental studies undertaken as a consequence of them. The research described herein was undertaken in order to study the pathogenesis and pathology of this disease, and to clarify the roles of vitamin E, selenium, methionine, and other dietary factors in its prevention.

LITERATURE REVIEW

Vitamin E Deficiency in Swine

The lesions of vitamin E deficiency in the pig, as in other species, may assume several forms and often overlap. So-called "yellow fat" may occur under conditions of high intake of unsaturated fats (Davis and Gorham, 1954). Other manifestations of vitamin E deficiency which have been reported include: anemia (Nafstad, 1965), muscular degeneration, female infertility (Adamstone et al., 1949), and necrosis of the liver (Obel, 1953). It is with necrosis of the liver that this treatise is mainly concerned.

History of Dietary Liver Necrosis in Swine

Field occurrences. Hutyra and Marek (1913), in Special Pathology and Therapeutics of the Disease of the Domestic Animals (3rd ed.) cite several early reports of necrotic liver conditions in swine. Among those mentioned were Semmer in Russia, who described the livers as enlarged, nodular, and marked with red blotches. Nonewitch isolated cocci from such livers and injected them into pigs. After 7 to 8 weeks the pigs died with liver lesions similar to those described by Semmer. Bradel made a histologic study of a porcine liver necrosis of unknown cause. He associated the condition with swine fever (hog cholera). Quin and Shoeman (1933) described field losses in Iowa from a condition which they termed "idiopathic hemorrhagic hepatitis of swine". Whether this was identical to dietary liver

necrosis is impossible to determine.

Obel (1953), in an extensive study of dietary liver necrosis ("hepatosis diaetetica") in swine, described the condition as encountered in commercial swine operations in Sweden. Up to 10% of the pigs presented for necropsy to the State Veterinary Medical Institute at Stockholm were affected. The clinical course was characteristically acute, with depression, vomiting, diarrhea, and bloody feces. Icterus was rare. Usually several in a litter were affected, most typically between 3 and 15 weeks of age.

Gross lesions included yellow fat, waxy degeneration of the skeletal muscles, ulceration of the esophageal area of the stomach, mucous colitis, and patchy, hemorrhage-like areas and fibrosis of the liver.

Microscopically the principal lesion was necrosis of the liver. The peculiar lobular distribution of the liver lesions was explained on the basis of the unique vasculature of that organ.

Dodd and Newling (1960) described a field outbreak of dietary liver necrosis and nutritional muscular dystrophy in 40 of a herd of 200 pigs in New Zealand. The diet included fish liver oil supplements. There was necrosis and a notable lack of affinity for the ordinary tissue stains in the parenchymal cells of the liver. There were also degenerative changes in the intima of the hepatic arteries and in the biliary epithelium. Accompanying these changes were lesions of nutritional myopathy which were observed in both skeletal and heart muscle in many cases. The trouble ceased when the fish liver oil was deleted from the ration.

In the Annual Reports of the Ruakura and Wallaceville Animal Research Stations and the Whatawhata Hill Country Station (Anon.,

1963-1964) it was reported that sudden deaths among pigs occurred frequently as a result of diseases of the "mulberry heart disease - hepatosis diaetetica complex". Such outbreaks were commonly associated with barley feeding. On 2 occasions when barley meal was analyzed, it was found to be low in selenium (0.008 and 0.011 p.p.m.). Peroxide values were not excessive (less than 15 meq./100 Gm. of fat extractures). Oral administration of selenium appeared to be the best method of controlling the problem. Intestinal emphysema was reported as a common accompanying lesion. A histologically identical condition has been observed at the College of Veterinary Medicine, Washington State University, Pullman, Washington. These occurrences were also frequently associated with the feeding of barley (G. R. Spencer, unpublished data).

Experimental dietary liver necrosis in swine. Adamstone et al. (1949), in an investigation of the effects of vitamin E deficiency in swine, fed marine liver oils and rancid lard to 70-lb. gilts. They noted pool-like accumulations of blood and degeneration of the liver cords in one animal.

The Swedish worker Obel (1953) reported producing "hepatosis diaetetica" experimentally in pigs fed rations which included dried brewer's yeast (18%) as a source of protein and either cod liver oil (6%) or lard (6%) as a source of fat. The lesion was produced more consistently when the diet included cod liver oil. Alpha tocopheryl acetate administered at the rate of 150 mg. twice weekly prevented liver lesions in pigs on the lard diet but not in those fed cod liver oil. The liver lesions were also prevented by a 15% casein diet or

supplementation of the necrogenic diet with cystine (0.5%) or methionine (0.5%).

Hove and Siebold (1955) were among the early investigators of the effects of vitamin E-deficient diets in swine. They produced dietary liver necrosis in weanling pigs fed a 6% protein (soybean meal) ration with 2% cod liver oil. In addition to the typical changes of necrosis, they observed calcium deposits in the liver, ceroid pigment in the adipose tissue, and incipient cirrhosis in some of those which survived the necrotic process. There were no lesions suggestive of nutritional muscular dystrophy. They also described a failure of the cytoplasm of the hepatocytes to stain, and ascribed this to protein depletion. They considered cod liver oil essential to the pathogenesis of dietary liver necrosis.

The roles of vitamin E and selenium in swine nutrition were further investigated by Eggert and co-workers (1957). They fed a basal ration which included 40% Torula yeast, 5% vitamin E-free lard, 0.4% methionine, and 0.27% cystine. Four of 6 pigs on the basal ration died of dietary liver necrosis within 53 days. Two of the 4 pigs also had discolored fat. The addition of 40 p.p.m. of tocopheryl acetate or 1 p.p.m. of selenium as selenite completely controlled the disease.

Grant and Thafvelin (1958) fed pigs a hepatonecrogenic ration based on soya meal. The addition of 0.2 mg. sodium selenite per kg. to the deficient ration prevented liver necrosis. However, skeletal muscle degeneration and deposition of ceroid in adipose tissue were not prevented by the selenium.

Stowe (1962), in a study of the lesions of tocopherol deficiency in swine and mink, observed liver lesions which he described as

centrolobular hemorrhages. Alpha-tocopherol supplementation of the basal ration at the rate of 100 p.p.m. was adequate to prevent all lesions of the deficiency.

Stowe (1962) also cited Pellegrini (1958) as having produced fatal dietary liver necrosis and nutritional myopathy in swine by feeding a diet of 32 to 45% Torula yeast. Pellegrini found that cystine prevented the liver necrosis but not the muscle changes, whereas 50 mg. alpha-tocopheryl acetate and 0.45 p.p.m. sodium selenite prevented both conditions. It is interesting to note that the rates of gain were satisfactory in all groups.

Swahn and Thafvelin (1962) produced dietary liver necrosis in pigs experimentally by adding cod liver oil or heated maize or cottonseed oils to their diets. These rations also resulted in muscular lesions and microangiopathy. Yellow fat occurred in pigs fed cod liver oil. Pigs fed heated vegetable oils had a rise of serum glutamic-oxalacetic transaminase from a normal of approximately 22.5 $\pm$ 7.7 Kramer-Ordel units to values as high as 300 in 30 days. Supplementation of the vegetable oil diets with vitamin E or selenium prevented these changes except for a slight myopathy. Supplementation of the cod liver oil diet with selenium prevented all lesions of the deficiency except pigmentation of the fat. An interesting sidelight to this study was the fact that the livers of vitamin E-supplemented pigs had adequate levels of vitamin A despite the feeding of heated maize oil. Those on the basal ration or basal plus selenium had no detectable vitamin A in their livers.

Stokstad *et al.* (1958) fed pigs a vitamin E-deficient diet which included Torula yeast as a source of protein and vitamin E-free lard.

The pigs grew normally, but died suddenly with dietary liver necrosis. Some pigs also had edema, discolored fat, or hemorrhages of the gastrointestinal tract. They observed no changes in the hemoglobin values or red blood cell counts. Vitamin E or selenite supplementation prevented the liver necrosis.

Keahey (1963), in the course of a study on the relationships between protein malnutrition and infection, observed dietary liver necrosis in pigs fed a 6% crude casein diet which included commercial lard and 11.7 mg. dl-alpha-tocopherol per kg. of feed. The lesion did not appear in pigs fed higher levels of casein.

Experimental Dietary Liver Necrosis in the Rat

In 1935 Weichselbaum published the first report on dietary liver necrosis in the rat. He fed diets deficient in cystine and methionine and added cod liver oil as a source of vitamins A and D. Supplementation with cystine or methionine prevented the lesion. Cystine, and to a lesser extent methionine administration would also cause regression of the early signs of the disease. Subsequently, Gyorgy and Goldblatt (1939) observed the condition, again in the course of an experiment in which cod liver oil was a part of the ration. They published the first clear description of the liver necrosis lesions.

Glynn *et al*. (1945) employed a diet in which pure amino acid mixtures replaced other protein sources. They also concluded that dietary liver necrosis is a manifestation of cystine deficiency.

Himsworth and Linden (1949) were among the first to demonstrate the protective effect of alpha-tocopherol against dietary liver necrosis. They saw no necrosis in 8 rats fed 15 to 20 mg. alpha-tocopherol twice

weekly. Necrosis occurred in 8 of 11 controls on the deficient ration.

During the early period of investigation into the nature of this liver necrosis, there was confusion between liver necrosis as the result of nutritional deficiencies and an entirely distinct nutritonal problem characterized by fatty changes and fibrosis (Gyorgy and Goldblatt, 1942). In 1942 Daft *et al*. reported proof that these were separate entities and that the latter condition was primarily the result of choline deficiency. Methionine, a source of methyl groups in the biosynthesis of choline, could also prevent fatty degeneration and fibrosis. By contrast, dietary liver necrosis was preventable with tocopherol or the sulfur amino acids, methionine or cystine.

Gyorgy and Goldblatt (1949) were among the first to call attention to the fact that tocopherol deficiency was enhanced by the inclusion of highly unsaturated fats (e.g., cod liver oil) in the diet. Abell and Beveridge (1951) noted that all the reliably necrogenic experimental rations had contained cod liver oil as a source of vitamins A and D.

Fite (1954) published a pathologic study of dietary liver necrosis. He observed that the structural changes appeared suddenly and that there was little or no gross or microscopic evidence of disease until the acute necrosis occurred.

Gyorgy *et al*. (1951) demonstrated that some antimicrobial agents, such as chlortetracycline, when administered *per os* to rats on necrogenic diets, significantly delayed, but did not prevent, the onset of dietary liver necrosis. They interpreted this as evidence that the necrosis occurred as the result of the absorption and transport to the liver of toxic substances elaborated by intestinal microorganisms. The deficient liver was less able to carry out its normal function of

detoxification, and necrosis was the result. They theorized that the antibiotic temporarily suppressed the intestinal microflora but resistant strains eventually developed.

Schwarz (1958b) demonstrated that such antioxidants as ethoxyquin and diphenyl-p-phenylenediamine (DPPD), when included in the diet at adequate levels, would completely protect rats from dietary liver necrosis.

Schwarz and Foltz (1957), aware that in addition to cystine and tocopherol there was a third factor which would prevent necrosis of the liver in rats, reported that the essential component of this poorly understood "factor 3" was selenium. Two years later Schwarz et al. (1959) reported that cystine, available from commercial sources, was contaminated with traces of selenium. It was their contention that the protective effect of cystine against dietary liver necrosis was due solely to its selenium content. Chemically pure cystine would delay but would not prevent the occurrence of dietary liver necrosis in the rat.

Schwarz (1965) found that liver slices from vitamin E-deficient rats decline in oxygen consumption after 30 to 60 minutes of incubation. This was characteristic of the latent (prenecrotic) stage of dietary liver necrosis. This occurred in whole homogenates as well as in liver slices, but not in isolated mitochondria. Also at this stage, degenerative changes of the mitochondria could be demonstrated by electron microscopy. Simon's metabolites (p.15) but not alpha-tocopherol, when added to such preparations in vitro, prevented respiratory decline. This phenomenon bore no relationship to the rate of peroxide formation in the preparation. Schwarz concluded that trace element atoms, present in whole homogenates or liver slices, blocked active sulfhydryl groups

of essential enzymes. In support of this he pointed to the protective effect of such chelating agents as ethylenediaminetetraacetate (EDTA). Schwarz speculated that the tocopherols functioned in the body as quinones, identical to, or closely resembling Simon's metabolites. In this form they might oxidize the vulnerable sulfhydryl groups to less labile disulfides, and thus prevent the respiratory decline of dietary liver necrosis.

Olson and Dinning (1954) also found abnormalities of some of the enzymes of the citric acid cycle in rats with dietary liver necrosis. McLean (1963) noted that liver slices from vitamin E-deficient rats were unable to reaccumulate potassium removed by leaching in cold saline. Such slices also had a reduced rate of oxygen uptake. He speculated that the deficient tissues were unable to stand the stress of cooling, and the result was a disturbance of certain mechanisms involved in ion movement. McLean suggested that in the bodies of deficient animals other stresses may produce effects on these mechanisms similar to the effects of cooling *in vitro*, thus precipitating deficiency signs. He pointed out that there may be a high intracellular sodium and low potassium in dystrophic muscles of vitamin E-deficient animals.

Serum Enzyme Activity in Dietary Liver Necrosis

Musser *et al*. (1966), studied the changes in serum enzyme activity in human beings with various liver diseases. They recommended a combination of glutamic-oxalacetic transaminase (GOT), alkaline phosphatase (AP) and ornithine carbamyl transferase (OCT) determinations as diagnostic aids in hepatic disease.

Orstadius et al. (1959) noted that serum glutamic-oxalacetic transaminase, serum glutamic-pyruvic transaminase (GPT), and serum ornithine carbamyl transferase were all elevated in dietary liver necrosis of swine. By contrast, only GOT and GPT activities increased in nutritional muscular dystrophy. They pointed out that muscle tissue has little OCT, and therefore recommended this determination as highly specific for liver disease. The bromsulfalein excretion test and icteric index determinations were unreliable indicators of dietary liver necrosis.

Wretlind et al. (1959), employing Reichard's technic (Reichard, 1957), determined the plasma ornithine carbamyl transferase activity in 19 normal pigs. They reported a mean normal value of 5.93 units with a standard deviation of $\pm$ 2.42.

Vitamin E

History. The essential nature of vitamin E in nutrition was reported by Evans and Bishop (1922) of the University of California. They noted reproductive failure in female rats as a result of fetal resorption. The condition could be prevented by the feeding of fresh lettuce, dried alfalfa, or additional butterfat. Neither orange juice (vitamin C) nor cod liver oil (vitamins A and D) were effective in the prevention of this disorder.

The name "tocopherol" was suggested to Evans by George M. Calhoun, a professor of Greek at Berkeley. The term is derived from the Greek words tocos, child birth, and phero, to bear or carry. The suffix "-ol" indicates the alcoholic nature of the compound.

To date, numerous tocopherols have been isolated, differing in the number and placement of methyl groups on the basic chromane ring (Emerson et al., 1937). Of these, the one designated as alpha-tocopherol is the most abundant in nature and the most active biologically (Dam, 1957).

Since the original description of infertility in the rat by Evans and Bishop, a great variety of vitamin E deficiency manifestations have been noted in many different species. Among the better known of these are myopathies in the pig (Lannek et al., 1960), in the lamb (Metzger and Hagan, 1927), in the calf (Schofield, 1953), and in the guinea pig and the rabbit (Goettsch, 1931). Other well known diseases associated with vitamin E deficiency include: exudative diathesis in the chick (Dam and Glavind, 1939), fat pigmentation in the mink (Hartsough and Gorham, 1949) and the rat (Mason et al., 1946), degeneration of the testes in the rat (Mason, 1940), and necrosis of the liver in the rat (Gyorgy and Goldblatt, 1949) and in the pig (Obel, 1953). This is by no means a complete list. Vitamin E deficiency has never been characterized as a clear-cut clinical or experimental entity in man. Whether human beings can indeed suffer such a deficiency is questionable.

Metabolic role of vitamin E. The precise metabolic role or roles of vitamin E are not known. The diverse manifestations of vitamin E deficiency render improbable the finding of a simple, single biochemical explanation of its function. However, it has been possible to demonstrate, by in vitro studies of the chemical behavior of the tocopherols, and by in vivo trials using experimental rations, some of the more likely mechanisms of action of this unique nutrient.

Much of the research to elucidate the function of vitamin E has related to the well known ability of the tocopherols to undergo oxidation-reduction reactions *in vitro* to form the corresponding quinones. This fact suggested that vitamin E might serve as a biochemical antioxidant to protect essential labile compounds in the body, such as fatty acids, vitamins, and enzymes, from oxidation by hydrogen peroxide or molecular oxygen.

Among the leading proponents of this theory are the Danish worker Dam and Tappel of the University of California. Dam (1957), in a comprehensive review of the subject, concluded that the principal effect of an antioxidant in protecting fats is the neutralization of free radicals which represent the initial stage in an autoxidative chain reaction. The succeeding stages are the formation of peroxidic free radicals, hydroperoxides, keto- and hydroxy- compounds, and polymerization products. In the reaction with the free radical, the antioxidant itself is converted to a free radical form. In this form it is unstable and is thus irreversibly destroyed. Although alpha-tocopherol is actually the least active *in vitro* antioxidant of the known tocopherols, its greater activity *in vivo* may be due to its greater tendency to accumulate in the tissues. Although commercially the tocopherols are often converted to the acetate ester to increase their stability in storage, only the free phenolic forms are active as antioxidants. Therefore, the esters must first be hydrolyzed in the body. The antioxidant effect of the tocopherols *in vitro* is enhanced by the presence of ascorbic and phosphoric acids (Dam, 1957). The latter observation is interesting in light of apparent successes in the treatment of nutritional myopathies of lambs and calves with phosphoric acid (Schofield, 1953).

Olcott and Emerson (1937) did not agree that the free hydroxyl group is necessary for antioxidant activity. They found that the allophonates of the tocopherols also act as antioxidants.

Tappel (1962) stated that vitamin E is the principal lipid antioxidant in the animal body. He espoused the theory that damage to the lipid components of the mitochondrial, microsomal, and lysosomal membranes was the fundamental disorder in vitamin E deficiency. This occurred as the result of lipid peroxidation which was in turn initiated by free radical chain reactions. Tappel and Zalkin (1959a) found that tocopherol added to in vitro preparations of isolated mitochondria markedly reduced peroxidation and at the same time increased the stability of DPNH-cytochrome C reductase. In a companion paper (Tappel and Zalkin, 1959b) they reported that mitochondria contained about 25% lipids, mainly unsaturated, and that they were subject to hematin-catalyzed oxidative deterioration in the presence of molecular oxygen. They suggested that vitamin E, normally present in mitochondria, stabilized the unsaturated lipids.

Pollard and Bieri (1959), of the National Institutes of Health, found no difference in DPHN-cytochrome C reductase activity in heart muscle preparations from normal and vitamin E-deficient chicks. Alpha-tocopherol could not be demonstrated in the preparations from the deficient birds in contrast to those from the controls which did contain alpha-tocopherol.

Tappel's views are supported by the fact that many of the conditions associated with vitamin E deficiency occur only if the diet includes significant amounts of easily oxidizable, i.e., unsaturated fats. Indeed, most of the manifestations of vitamin E deficiency in all

species are more readily produced experimentally if such fats are included in the diet. Deficiency states may occur under such conditions even when the diet contains relatively high levels of vitamin E (Evans and Burr, 1927). Examples of diseases which require the presence of greater amounts of dietary polyunsaturated fats for their occurrence are encephalomalacia of chicks (Century and Horwitt, 1958; Kummerow, 1964), and "yellow fat" disease of mink (Mason and Hartsough, 1951). An example of a deficiency state which is produced more readily when relatively high levels of polyunsaturated fats are fed is nutritional muscular dystrophy (NMD) of pigs (Lannek et al., 1961). Cod liver oil is often employed in experimental rations because of its high content of polyunsaturated fats. McCoy et al. (1938) demonstrated that if cod liver oil were first hydrogenated, it did not enhance vitamin E deficiency in the experimental production of nutritional muscular dystrophy of rabbits.

Csallany and Draper (1962) lent further support to the antioxidant theory of vitamin E function. They demonstrated the conversion of labeled alpha-tocopherol to a quinone in the body. Similarly, Simon et al. (1956) reported the isolation of a quinone from urine of subjects following oral administration of large doses of dl-alpha-tocopherol. The quinone in the urine was conjugated as a glucuronide. The isoprenoid side chain of alpha-tocopherol had been shortened by 13 carbons and the terminal methyl group had been oxidized to a carboxyl group. A gamma lactone of this compound was also isolated. These two molecules have come to be known as "Simon's metabolites".

The antioxidant concept of vitamin E function is supported by numerous reports wherein structurally unrelated compounds with antioxidant activity have prevented deficiency signs in animals fed vitamin E-deficient diets. Crider et al. (1961), using diphenyl-p-phenylenediamine (DPPD) or ethoxyquin (EQN) maintained rats on vitamin E-deficient diets through normal reproduction. These compounds did not prevent the disappearance of tocopherol from the tissues. They concluded, therefore, that the action of DPPD or ethoxyquin was not due simply to a sparing or protective effect on tocopherol, but that they actually replaced the vitamin biologically. Singsen et al. (1955) prevented encephalomalacia in chicks fed 2% cod liver oil by supplementing the ration with DPPD (0.025%).

Schwarz (1958b)maintained rats on a hepatonecrogenic diet by supplementing with ethoxyquin (0.25%) or DPPD (0.02%). He did not believe the antioxidants prevent liver necrosis simply by stabilizing dietary fats because liver necrosis was readily produced with fat-free rations and his basal ration was stable against rancidity. This stability was due to antioxidants in Torula yeast which he employed as a protein source.

Bieri and Anderson (1960) found that peroxide formation in incubated tissue homogenates was prevented when adequate supplements of tocopherol were fed to chicks and rats on vitamin E-deficient diets. There was considerable peroxide formation in the tissues of unsupplemented animals.

Bunyan et al. (1963) used the thiobarbituric acid test (Wilbur et al., 1949) as an index of lipid peroxidation in tissue homogenates. This is a colorimetric test which depends for color development on the

breakdown of peroxides to malonaldehyde. They found little correlation between the inhibition of lipid peroxidation and protection from dietary liver necrosis. They concluded that either lipid peroxides were not increased in necrotic livers or that the peroxides were not broken down to malonaldehyde.

Green (1962) likewise concluded that the antioxidant activity of the tocopherols in the reduction of lipid peroxidation could not alone explain their functions in prevention of the many manifestations of vitamin E deficiency.

Bouman and Slater (1957), using horse heart muscle preparations, presented evidence that alpha-tocopherol was intimately involved with the respiratory chain. They speculated that the molecule might act catalytically to transfer phosphate groups from phosphoric acid to adenosine diphosphate. Thus, the essential effect of vitamin E deficiency was an uncoupling of oxidative phosphorylation. It is known that this effect increases respiratory chain activity by reducing the inhibition ordinarily exerted by the accumulation of adenosine triphosphate. They supported this theory by pointing out that oxygen consumption is increased in dystrophic muscle.

An increasing number of scientists believe that, although vitamin E is unquestionably active as a biological antioxidant, it has other, specific functions which characterize it as a true vitamin. Scott (1962), at Cornell University, in a review of vitamin E in poultry nutrition, pointed out that while this vitamin is the most effective, but not the only, antioxidant for the prevention of encephalomalacia, it serves also in a specific role, interrelated with selenium, to prevent exudative diathesis as reported by Patterson et al. (1957).

Analytical Methods for Vitamin E Determination

Emmerie and Engel (1938) reported a colorimetric method for the quantitative determination of alpha-tocopherol in certain foodstuffs, such as wheat germ oil and cottonseed oil. The technic was based on the ability of tocopherol to reduce ferric to ferrous iron. In the presence of alpha, alpha' dipyridyl, the ferrous ion formed a colored salt which could be measured colorimetrically. In a subsequent paper (Emmerie and Engel, 1939) they reported wider adaptation of the method for the analysis of biologic materials which contained significant levels of interfering substances, such as vitamin A and carotenoids. They separated these from tocopherol by adsorption on Floridin XS earth, eluting the tocopherol with benzene. The basic principle of the Emmerie-Engel colorimetric determination using ferric chloride and alpha, alpha' dipyridyl is still widely employed for vitamin E determination in feeds and tissues.

A micro-technic for the determination of total tocopherols in blood serum or plasma was described by Quaife et al. (1949). The procedure was based on the Emmerie-Engel reaction. Light absorption due to carotenoids was measured at 460 mμ, and a correlation was made for the contribution of the carotene to the Emmerie-Engel reaction color at 520 mμ.

Bro-Rasmussen and Hjarde (1957) reported a method for separating alpha-tocopherol from other tocopherols and vitamin A. They eluted alpha-tocopherol from a column of secondary magnesium phosphate using petroleum ether containing 2% ethyl ether. The other compounds could then be eluted by increasing the percentage of ethyl ether in the eluant.

This purification procedure was suitable when quantitative colorimetric determinations were to be done using the Emmerie-Engel technic.

Dicks and Matterson (1961) reported a procedure for the determination of alpha-tocopherol in tissues. They employed the Bro-Rasmussen-Hjarde technic of secondary magnesium phosphate column chromatography. However, they modified the method with respect to the saponification step and the colorimetric determination with ferric-chloride-dipyridyl.

Duggan (1959) described a technic for the determination of plasma tocopherols based on the fact that tocopherols exhibit ultraviolet fluorescence. He reported good correlation with chemical methods.

Edwin and co-workers (1960) described a procedure for the determination of tocopherols in tissues. After extraction and separation from the saponifiable lipids, tocopherols were further purified by Floridin earth column chromatography and finally isolated by 2-dimensional paper chromatography. It was their contention that this technic avoided the problem of false high values to the Emmerie-Engel colorimetric determination which may occur due to other reducing substances in the tissues. They reported a 90% recovery of added tocopherols.

Mason (1942) studied the distribution of vitamin E in the tissues of the rat. He concluded that the amount of tocopherol in the liver was the best index of the dietary level and body stores of the vitamin. The total amount of stored vitamin E in all the tissues of the rat, however, represented only a small fraction of that ingested. From this Mason inferred that the tocopherol was either poorly absorbed or rapidly degraded. Quaife and Harris (1944), using a method for tocopherol determination based on the Emmerie-Engel reaction, reported the normal plasma range for man as 0.90 to 1.59 mg.% with 1.20 mg.% as the average.

Selenium

Prior to the 1950s the element selenium was thought to have biological significance only as a poison. Forage crops with high selenium content were responsible for numerous losses among livestock in the central plains and Rocky Mountain states (Draize and Beath, 1935; Moxon and Rhian, 1943). In 1954, however, Pinsent (1954) reported that certain bacteria of the coli-aerogenes group required selenite for the biosynthesis of formic dehydrogenase. The organisms did not have an absolute requirement for selenite, however, in that they were capable of carrying out all essential oxidations without this enzyme.

Numerous subsequent reports were concerned with the use of selenium compounds in treatment of diseases associated with vitamin E deficiency. Among the conditions which were reported to be preventable or treatable with selenium were dietary liver necrosis in swine (Eggert et al., 1957) and rats (Schwarz and Foltz, 1957), exudative diathesis in chicks (Patterson et al., 1957), nutritional muscular dystrophy (white muscle disease) of lambs and calves (Muth et al., 1959), and the corresponding condition in swine (Lannek et al., 1961).

Despite these examples of the interrrelationship of selenium and vitamin E, no one has been able to explain in biochemical terms the exact mechanism of this relationship. Bieri (1961) suggested that selenium in some form serves as an antioxidant and can substitute for vitamin E in this capacity. In a later paper, however (Bieri, 1959), he pointed out that although lipid oxidation is reduced in homogenates of liver and muscle from selenium supplemented chicks, there was no reduction when selenium was added directly to the preparation in vitro.

This was interpreted as evidence that selenium compounds do not act directly as lipid antioxidants.

Welch et al. (1960) produced nutritional myopathy in lambs by feeding fish liver oils to the dams. The plasma tocopherol levels of the dams were reduced. The condition could be prevented if the dams were fed supplementary vitamin E. The addition of sodium selenite to the rations of the dams was much less effective in controlling NMD in the young.

Oldfield et al. (1960), at Oregon State University, noted that white muscle disease in the lamb could be prevented by giving selenium or large doses of vitamin E at birth, but the growth rate was better in the selenium-supplemented group. The growth-stimulating effect of selenium was even more pronounced if it were given prenatally to the dam. They concluded that selenium is essential for normal growth in lambs.

In discussing the role of selenium in nutrition, Schwarz (1960) stated that although it frequently serves to prevent the occurrence of the signs of vitamin E deficiency, it is also an essential micronutrient in its own right. As evidence for this view he mentioned poor growth in rats and chicks on diets adequate with respect to vitamin E but deficient in selenium. Schwarz (1958a) propounded the theory that vitamin E and selenium were each essential to the proper function of two different but equivalent metabolic pathways. Either one of these pathways was sufficient to maintain the animal's health. Thus, such conditions as dietary liver necrosis represented a simultaneous deficiency of vitamin E and selenium.

Green et al. (1961) reported that both selenium and vitamin E supplementation increased tissue ubiquinone. In tocopherol-deficient rats ubiquinone levels in the uterus were reduced. Administration of vitamin E resulted in increased uterine ubiquinone and correction of the accompanying resorption sterility. Selenium supplementation did not affect ubiquinone levels in uterine tissue and did not prevent resorption sterility.

Tappel (1962), in discussing the relationship of selenium to vitamin E, speculated that dietary inorganic selenium compounds are incorporated by the body into unknown organic antioxidants. Such selenium antioxidants are about 500 to 2000 times as effective as alpha-tocopherol on a molar basis for the prevention of lipid peroxidation and these compounds react with free radical intermediates to break chain reactions.

Olcott et al. (1961) reported that selenomethionine had strong antioxidant properties in vitro as indicated by its ability to control lipid oxidation and rancidification. They suggested that the failure of selenium supplements to prevent some of the diseases due to vitamin E deficiency could be explained on the basis of different degrees of availability in different tissues.

Bunyan et al. (1963) found that reduction in lipid peroxidation is not the essential mechanism by which selenium protects rats from necrosis of the liver. The lesion could be prevented with dietary levels of selenium lower than that required to control peroxidation in tissues.

Sondergaard et al. (1958) studied the role of selenium in the diet of the tocopherol-deficient rat. They concluded that selenium would not prevent the following manifestations of vitamin E deficiency in the rat:

1. Decreased liver storage of vitamin A.
2. Depigmentation of the incisors.
3. Peroxidation and discoloration of body fat.
4. Increased erythrocyte fragility.

Nutritional Muscular Dystrophy (NMD) in Pigs

Adamstone et al. (1949), at Illinois, were among the early investigators of the importance of vitamin E in swine rations. Feeding natural diets which contained rancid lard and marine liver oils, they observed degenerative lesions of the muscles in addition to liver necrosis and reproductive failures. Forbes and Draper (1957), at the same institution, also observed muscle degeneration in pigs fed low vitamin E rations with 5 to 10% cod liver oil. Dodd and Newling (1960), in New Zealand, reported nutritional muscular dystrophy in pigs affected with dietary liver necrosis. The ration contained fish liver oil. Losses ceased when the addition of oil to the feed was discontinued. Thafvelin (1960) demonstrated that nutritional muscular dystrophy could occur in pigs on rations containing no fats other than vegetable fats.

Lannek et al. (1961) fed a vitamin E-deficient diet containing stripped lard, casein, and brewer's yeast to pigs. No NMD was observed. If, however, the lard were replaced with cod liver oil, muscle degeneration did occur. Sodium selenite was said to have a curative effect. However, Swahn and Thafvelin (1962) reported that selenium provided pigs only partial protection from NMD when diets contained fish liver oils or oxidized vegetable oils.

Grant and Thafvelin (1958) fed a known hepatonecrogenic diet to newly weaned pigs to determine the effect of selenium supplementation under such conditions. Although liver necrosis was completely prevented, ceroid deposition in adipose tissue and degeneration of skeletal muscle did occur. Thus, selenium apparently exerted a preventive action on some but not all of the manifestations of vitamin E deficiency in the pig. Selenium has also been found ineffective or only partially effective in preventing NMD in rabbits (Hove et al., 1958).

Hematologic Changes in Vitamin E-Deficient Pigs

Nafstad (1965) studied the blood and bone marrow of vitamin E-deprived pigs. She fed a ration which included 10% casein and either 4 or 5% cod liver oil. After 4 weeks, deficient pigs had reduced hemoglobin values and packed cell volumes. There was also an increased total leukocyte count due principally to a neutrophilia. There was hypercellularity of the bone marrow involving mainly the erythroid elements. Multinucleated cells representing all stages of the erythropoietic series were observed. Vitamin E supplementation effectively prevented these changes. Supplementation with 0.25 p.p.m. sodium selenite or with a combination of methionine, lysine, and threonine did not influence the occurrence of these changes in the blood and blood-forming tissues.

Gastric Ulcers

The problem of gastric ulcers in swine has received a great deal of attention in recent years. Although the so-called "peptic ulcer" of the glandular epithelium occurs in swine, lesions of the esophageal portion of the stomach are far more common (Muggenberg et al., 1964).

Reese and co-workers (1966a) reviewed several studies on the incidence of this condition. They cited reports indicating that the incidence of ulcers and preulcerous lesions may exceed 50%. The changes apparently begin as a hypertrophic parakeratosis of the squamous epithelium which progresses to erosion, ulceration, and finally to chronic inflammatory changes. Severe, even fatal, hemorrhages may occur (Muggenberg et al., 1964).

Many attempts have been made to relate the incidence of gastric ulcers in pigs to various dietary or environmental influences. Nafstad (1967a), in Norway, studied the effects of various dietary factors on ulcer development. She concluded that the incidence of lesions was not influenced by the level of protein or fat, thiamine supplementation of the ration, or addition of tryptophan, lysine, and methionine. Vitamin E appeared to have a mildly mitigating effect. Nafstad et al. (1967b) reported that rations containing high levels of resh or oxidized unsaturated fats were consistently ulcerogenic. Vitamin E had a slight protective effect. Increasing the level of protein, amino-acids, or selenium did not alter the incidence of lesions. In a study of the effects of different proteins and fats on the occurrence of ulcers, Nafstad et al. (1967c) found that a diet containing saturated fat (hydrogenated coconut oil) was far less ulcerogenic than one containing unsaturated fat (cod liver oil). Similarly, the incidence of ulcers was markedly reduced when soybean oil meal was substituted for part of the casein used as a source of protein. If the casein were eliminated and replaced with soybean meal (36%), ulcers were completely prevented. The authors speculated that soybean oil meal may possess buffering properties which casein does not.

Jensen (1946) studied the effects of tocopherols on the incidence of gastric ulcers in rats maintained on a diet deficient in vitamin A. Ulcers developed in a large percentage of vitamin A-deprived rats. Vitamin E supplementation substantially reduced ulcer formation. However, if large doses of alcohol were included in the rations, the apparent protective effect of vitamin E was overcome.

Reese et al. (1966a) studied the effects of various nutritional and environmental factors on the occurrence of gastric ulcers in swine. They fed antibiotics, arsenilic acid, dry skim milk, fluid milk, previously heated ground soybeans, 15% soybean oil, the water soluble B group of vitamins, and the fat soluble vitamins A, D, E, and K. They observed no change in the over-all incidence of ulcers as a result of adding any of these to the ration. In a companion study (Reese et al., 1966b) they found that oats had a marked protective effect when added to corn or wheat rations. If the diet consisted of 85% oats, no lesions were observed. By contrast, all pigs fed a 76% corn ration had lesions of the esophageal portions of their stomachs, and, in 53% of these animals, the changes had progressed to the erosion or ulcer stage.

Nuwer et al. (1965) fed pigs a highly ulcerogenic diet containing gelatinized corn. They found no effect on the incidence of ulcers from feeding a number of different additives, including oxytetracycline, oxytetracycline plus copper sulfate, vitamin A, tocopherol, tocopheryl acetate, menadione, methionine, and an antihistamine.

Rothenbacher et al. (1963) surveyed the problem of gastric ulcers among swine in Michigan. They reported that both bacteria and fungi could commonly be demonstrated in the "fibrino-necrotic membranes" often associated with gastric ulcers. These organisms were regarded as

secondary invaders, since they were not observed in the deeper structures of the affected areas.

* * *

Thus, it is clear that although there has been a considerable amount of investigation of the problem of dietary liver disease, there is as yet an incomplete understanding of the roles of various dietary factors in combatting these diseases, and of the interrelationships among these factors.

MATERIALS AND METHODS

This dissertation describes 3 experiments which were done at Michigan State University during the period from February 1966 through the spring of 1967. Although the 3 trials were similar in many respects, there were some important differences which are explained in the following general description of materials and methods.

Experimental Animals

Young Yorkshire-cross pigs were used as the experimental animals in all experiments. Except for pigs numbered C-1 through C-18, which were bought from a local farmer, all pigs were farrowed at the Veterinary Research Farm at Michigan State University. They were identified by ear notching and assigned particular experimental rations by random selection. Supplemental iron was administered before beginning each experiment. Pigs were observed at least twice daily for obvious clinical signs. They were weighed at the beginning and end of each experiment and at intervals during the course of each experiment. Final weight was determined at the time of euthanasia or, in the cases of pigs which died for other reasons, as soon after death as practicable.

A single litter consisting of 7 males and 5 females was used for Experiment I. They were started on the experimental regimen when 16 days of age.

A single litter of 6 male and 6 female pigs was used in Experiment II. They were 29 days of age when confined in individual cages and started on the experimental rations.

Pigs from 3 different litters were used in Experiment III. One litter of 7 males and 3 females (pigs C-1, C-3, C-5, C-7, C-10, C-12, C-14, C-16, C-17, C-18) were 20 days of age at the start of the experiment. Another litter of 4 males and 4 females (pigs C-2, C-4, C-6, C-8, C-9, C-11, C-13, C-15) were 18 days of age at the start of the experiment. A 3rd litter of 2 males and 4 females (pigs C-19 through C-24) were 25 days of age at the start of the experiment. These last 6 were all fed a basal ration containing 20% protein.

Housing

Excepting C-19 through C-24, all pigs were maintained in individual metal metabolism cages in a heated, isolated room. Throughout Experiment III pigs C-19 through C-24 were maintained in ordinary pens with a concrete floor. At first pigs C-19 and C-20 were kept in one pen while C-21 through C-24 were kept in an adjoining pen. In hosing down the floors some of the excreta were washed into the adjoining pens and the pigs thus had access to the wastes from animals on other experimental regimens. It was thought that the feces and urine of C-19 and C-20 might contain enough selenium to jeopardize the experiment. Therefore, on the 12th day of the trial, C-19 and C-20 were moved to a different room, and pigs C-21 and C-22 were separated from C-23 and C-24 and placed in the pen vacated by C-19 and C-20.

Rations and Feeding Practices

All pigs were fed twice daily. Those in individual cages were fed and watered in separate bowls. An effort was made to keep water available at all times,but some of the caged pigs consistently emptied their bowls between waterings, particularly during the night. The pigs

which were penned on the floor were fed using one pan for each 2 pigs. Note was made of pigs which failed to eat all of the feed provided, and the amount was adjusted accordingly.

During Experiments I and II, feed was prepared in quantities sufficient to last 1 to 2 weeks. Mixing was accomplished with a Hobart mixer in 7 kg. batches. The mixed preparation during these 2 trials was stored in galvanized containers at room temperature. Unused stripped* lard in opened cans was refrigerated until used the next time feed was mixed.

During Experiment III feed was mixed at intervals of one week or less except at the beginning of the trial, when sufficient feed was prepared for 17 days. Feed was kept at refrigerator temperatures in polyethylene bags. The standard Michigan State University pig grower which was fed to pigs C-17 and C-18 was kept at room temperature.

The basal ration is given in TABLE 1.

The 20% protein ration is given in TABLE 2.

The standard Michigan State University pig grower ration is given in TABLE 3.

The diets fed in Experiment III were analyzed for alpha-tocopherol content and the results are given in TABLE 4.

Supplements

The experimental supplements employed in these trials included vitamin E, selenium, methionine and ethoxyquin. Vitamin E was supplied as dl-alpha-tocopheryl acetate.* It was administered at the rate of

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*Vitamin E free.
**Rovimix E-100W, Hoffman La Roche.

TABLE 1. Basal ration, Experiments I, II, and III.

Torula yeast	12%
Cerelose	72%
Stripped lard	5%
Mineral mix*	6%
Cellulose	5%
Vitamin mix**	25 ml./kg.

* Mineral mix:

$CoCO_3$	1.00
KI	0.02
$CuSO_4$	1.00
$MnSO_4 \cdot H_2O$	1.00
$FeSO_4 \cdot 2H_2O$	7.00
$ZnSO_4 \cdot H_2O$	4.00
$MgCO_3$	20.00
KCl	100.00
$CaCO_3$	125.00
Cerelose	131.00
$NaHCO_3$	250.00
$CaHPO_4 \cdot 2H_2O$	360.00
	1000.00 Gm.

** Vitamin mix:

Thiamine mononitrate	0.300 Gm.
Riboflavin	0.600 Gm.
Pyridoxine	0.200 Gm.
Calcium pantothenate	3.000 Gm.
Niacin	4.000 Gm.
Para-amino benzoic acid	1.300 Gm.
Biotin	0.005 Gm.
Folic acid	0.026 Gm.
Inositol	13.000 Gm.
Ascorbic acid	8.000 Gm.
Choline chloride	130.000 Gm.
Vitamin B_{12} (0.1% triturated)	10.000 Gm.
Vitamin D_2#	0.044 Gm.
Menadione sodium bisulfite	0.400 Gm.
Water soluble vitamin A##	1.000 Gm.
Absolute ethyl alcohol	250 ml.
Distilled water	q.s. 2500 ml.

\# Water dispersible vitamin D_2 (500,000 I.U./Gm.)
Rovimix A-250-W, 294,000 U.S.P. units vitamin A/Gm., Hoffman La Roche, Nutley, N. J.

TABLE 2. 20% protein ration, Experiment III

Torula yeast	40%
Cerelose	44%
Stripped lard	5%
Mineral mix*	6%
Cellulose	5%
Vitamin mix**	25 ml./kg.

* See TABLE 1.
** See TABLE 1.

TABLE 3. Standard Michigan State University pig grower ration.

Ground shelled corn	1554 lb.
Soybean oil meal (50% protein)	300 lb.
Meat and bone scraps	60 lb.
Alfalfa meal (17% protein)	50 lb.
Limestone	12 lb.
Dicalcium phosphate	4 lb.
High Zn trace mineral salts	10 lb.
Vitamin, antibiotic, trace mineral premix*	10 lb.
	2000 lb.

* Vitamin A and D premix#	6
Merck 1231 Mixture (vitamin B complex)	3
Dawes B_{12}	10
Prostrep 20 ##	20
Zn oxide (74% Zn)	1
Ground shell corn	60
	100

\# Vitamin A and D premix:
Vitamin A - 3,628,720 I.U./lb.
Vitamin D - 800,000 I.U./lb.

Procaine penicillin and streptomycin

TABLE 4. Alpha-tocopherol content of feeds, Experiment III.

Ration	Alpha-tocopherol (mg.%)
Basal (6% protein)	0.38
Basal (6% protein) + ethoxyquin	0.43
Basal (20% protein)	0.24
Standard pig grower ration	1.13

21.8 I.U., 3 times weekly (hereinafter designated "low level E") in Experiments I, II, and III and at the rate of 150 I.U., 2 times weekly (hereinafter designated "high level E") in Experiment III.

Selenium was furnished as sodium selenite, 0.2 mg., 3 times weekly.

At the beginning of Experiment I the vitamin E and selenium supplements were both added to the ration at feeding time. After 29 days, when feed consumption by some animals began to decline, these supplements were administered to such animals orally with a syringe. This means of administration was employed exclusively for vitamin E and selenium in Experiments II and III.

Supplemental methionine was furnished as DL methionine. This was mixed with the basal ration at the rate of 1.0 Gm. per 1000 Gm. of feed. This seemingly low level of supplementation was used in order to keep the sulfur-amino acids in proper relation to the other amino acids in this low protein ration. Thus, the ration contained 0.21% total sulfur-amino acids after the addition of methionine. The basal ration (6% protein) contained 0.12% sulfur-amino acids.

For those pigs whose basal ration was supplemented with ethoxyquin, the antioxidant was added to the ration at the level of 0.1% (1 Gm. per 1000 Gm. feed) at the time of mixing.

Hematology

Blood samples were collected from the anterior vena cava. Blood was collected from the brachial artery after electrocution at the time of necropsy. The disodium salt of ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant. Hemoglobin determinations were made by the cyanmethemoglobin method. Packed cell volumes were determined using microhematocrit tubes.

Serum Ornithine Carbamyl Transferase (OCT) Determinations

Blood for serum enzyme activity studies was routinely collected from the anterior vena cava. Blood was collected from the brachial artery at the time of euthanasia. Samples were allowed to clot and were then centrifuged. Serum was removed with a pipette and promptly frozen and stored at -70° C until tested for OCT activity. The determination was made using the technic of Reichard (1957).*

Pathology

The number of days that each pig was maintained on an experimental regimen is given in TABLES 5, 6 and 7. The pigs were necropsied at the ends of the indicated periods.

Pigs in all experiments were killed by electrocution. The carcasses were immediately weighed and the brachial arteries severed for blood collection for laboratory procedures. The livers were removed at necropsy, trimmed of the lymphoid tissue and extrahepatic biliary structures, and weighed on a Mettler balance. Tissues collected for histopathologic studies included sections from the: esophagus, esophageal region of the stomach, glandular region of the stomach, duodenum, jejunum, ileum, colon, mesenteric lymph node, right and left kidneys and adrenals, right and left lateral lobes of the liver, left central lobe of the liver, thymus, pancreas, right quadriceps femoris, skin from the medial aspect of the right thigh, spleen, left ventricle, aorta, cardiac lobe of the right lung, and others where indicated.

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* Sigma kit 108AC, Sigma Chemical Co., St. Louis, Mo.

TABLE 5. Number of days each pig was maintained on experimental regimen, Experiment I.

Pig	Number of days on experiment before necropsy
A-1	53
A-2	53
A-3	53
A-4	53
A-5	43*
A-6	59
A-7	53
A-8	36**
A-9	53
A-10	53
A-11	36*
A-12	53

* Died during course of experiment.

** Killed

TABLE 6. Number of days each pig was maintained on experimental regimen, Experiment II.

Pig	Number of days on experiment before necropsy
B-1	43
B-2	36
B-3	36
B-4	43
B-5	43
B-6	36
B-7	43
B-8	43
B-9	10*
B-10	43
B-11	36
B-12	36

* Killed when found comatose.

TABLE 7. Number of days each pig was maintained on experimental regimen, Experiment III.

Pig	Number of days on experiment before necropsy
C-1	47
C-2	46
C-3	46
C-4	47
C-5	52
C-6	47
C-7	46
C-8	47
C-9	47
C-10	47
C-11	52
C-12	46
C-13	43*
C-14	46
C-15	52
C-16	52
C-17	52
C-18	10**
C-19	38
C-20	38
C-21	38
C-22	38
C-23	52
C-24	52

* Died during course of experiment.

**Killed early to provide examples of tissues at beginning of experiment.

Tissue sections were routinely fixed in 10% buffered formaldehyde solution. They were processed in an automatic processing machine,* sectioned at 6 microns, and stained with hematoxylin and eosin. Separate sections of the livers were fixed with Carnoy's solution and stained with Best's carmine for glycogen content. Formalin-fixed sections were frozen and stained with oil-red-O for fat content. Where indicated, other special stains were employed.

Smears were made of bone marrow from the 5th left rib immediately after euthanasia. They were fixed in methyl alcohol and stained with the May-Grünwald-Giemsa stain.

Analyses of Tissues and Rations for Vitamin E Content

Because of the difficulties inherent in analytical technics for vitamin E, the specimens for analysis were sent to the laboratories of Hoffman La Roche where these procedures are conducted routinely. The technic of Dicks and Matterson (1961) was used for liver analysis. Vitamin E content of rations in Experiment III was determined by a procedure employing solvent extraction, saponification, Florex and secondary magnesium phosphate column chromatography, and colorimetric determination with ferric chloride and alpha, alpha' dipyridyl.

Experimental Designs

1. Experiment I. The various experimental diets for Experiment I and the pigs assigned to each are given in TABLE 8.

2. Experiment II. In TABLE 9 the experimental design of Experiment II is outlined.

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* Autotechnicon, Technicon Co., Chauncey, New York.

TABLE 8. Experimental design, Experiment I

Ration	Pig Numbers
Basal	A-3, A-6, A-8, A-9
Basal + low level E	A-1, A-10
Basal + selenium	A-7, A-12
Basal + methionine	A-5, A-11
Basal + low level E + selenium + methionine	A-2, A-4

TABLE 9. Experimental design, Experiment II

Ration	Pig Numbers
Basal + low level E	B-3, B-7
Basal + selenium	B-5, B-6
Basal + methionine	B-9, B-10
Basal + low level E + selenium	B-1, B-11
Basal + low level E + methionine	B-2, B-8
Basal + selenium + methionine	B-4, B-12

3. Experiment III. In TABLE 10 the experimental design of Experiment III is outlined.

TABLE 10. Experimental design, Experiment III.

Ration	Pig Numbers
Basal	C-1, C-2
Basal + low level E	C-3, C-4
Basal + high level E	C-9, C-10, C-11, C-12
Basal + selenium	C-5, C-6
Basal + ethoxyquin	C-13, C-14, C-15, C-16
Basal + low level E + selenium	C-7, C-8
Basal with 20% protein	C-23, C-24
Basal with 20% protein + low level E	C-21-C-22
Basal with 20% protein + selenium	C-19, C-20
Michigan State University standard grower ration	C-17, C-18

RESULTS

The lesions observed with greatest frequency in the pigs of these 3 experiments were dietary liver necrosis, hydropic degeneration of the hepatocytes, nutritional muscular dystrophy, ulceration and pre-ulcerous lesions of the squamous epithelium of the stomach, and atrophy of the thymus. The pathologic changes were similar in all 3 experiments; therefore a general description is given for each lesion. In addition, there is a section on pathology included with the results of the individual experiments.

Pathology of Dietary Liver Necrosis

Gross lesions. Some of the livers were swollen, and over the surfaces of the organs there were numerous dark red, punctate or slightly larger lesions suggestive of hemorrhage. The livers of pigs A-5 and B-3 are typical of this change (Figures 1 and 2). The surfaces of other livers had irregularly shaped, depressed, roughened areas. This is illustrated in Figures 3 and 4, in photographs of the livers of pigs A-8 and B-7. The livers of many pigs had both lesions (Figure 5).

Histopathology of dietary liver necrosis. The hemorrhage-like lesions observed grossly were sharply demarcated areas of necrosis. This process affected lobules individually or in groups, while the immediately adjoining or surrounding lobules were often not visibly affected (Figure 6). The most distinctive feature of this necrosis was the limitation of

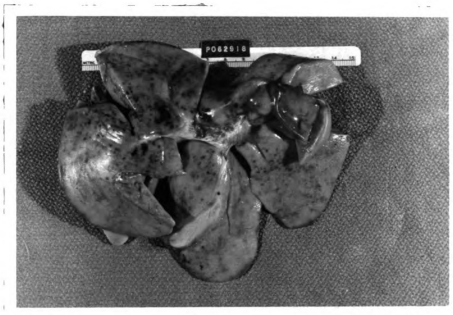

Figure 1. Acute dietary liver necrosis. Pig fed basal ration plus methionine.

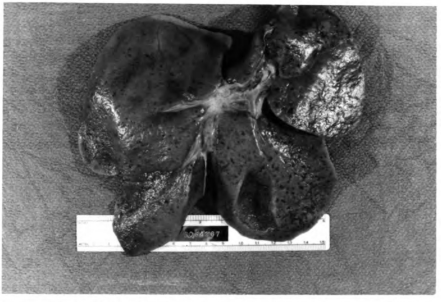

Figure 2. Acute dietary liver necrosis. Pig fed basal ration plus low level vitamin E.

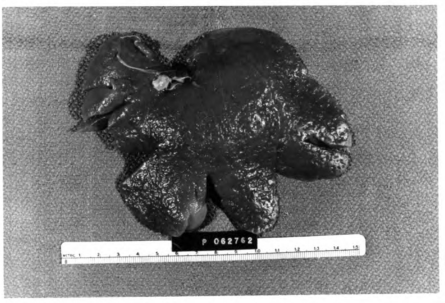

Figure 3. Chronic dietary liver necrosis with fibrosis. Pig fed basal ration.

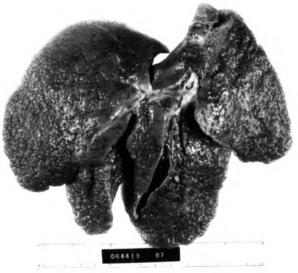

Figure 4. Chronic dietary liver necrosis. Pig fed basal ration plus low level vitamin E.

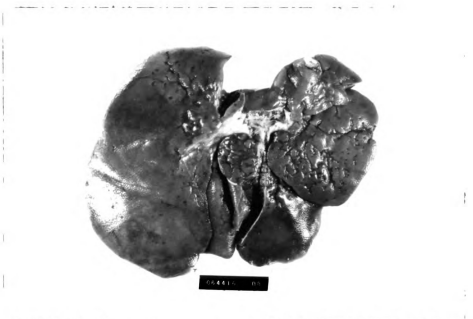

Figure 5. Liver with both acute necrosis and fibrosis. Pig fed basal ration plus methionine and low level vitamin E.

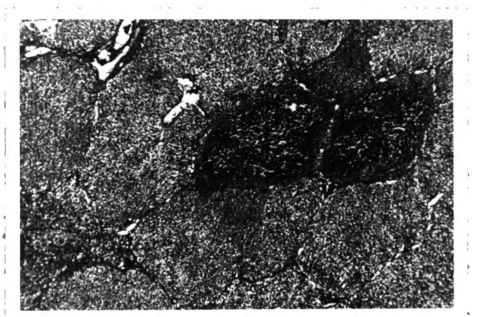

Figure 6. Dietary liver necrosis illustrating characteristic pattern of necrosis. Pig fed basal ration. Hematoxylin and eosin. x 30.

the necrosis by the interlobular connective tissue. There was an absence of hepatic cells in most necrotic lobules. In many cases, karyorrhectic or pyknotic nuclei were the only remaining traces of the necrotic cells (Figure 7). Infiltration of necrotic lobules by polymorphonuclear leukocytes was observed in some sections (Figure 8). In a few instances there was dystrophic mineralization of the degenerative cells (Figure 9).

A typical necrotic hepatic lobule consisted of a blood-filled reticulum with few surviving hepatocytes. However, occasionally foci of necrosis occurred within lobules in which most of the cells were not visibly affected (Figure 10). These foci were located in any region of the lobule. In some sections there were collapsed lobules with an increase of interstitial connective tissue and bile ducts (Figure 11). Often a few surviving parenchymal cells formed gland-like patterns. These cells were frequently very large (Figure 12). Most sections contained combinations of the above changes (Figure 13).

The structures of the portal triads were usually spared during the acute necrotic process (Figure 14).

Hydropic Degeneration of the Liver

A common observation in all 3 experiments was an almost complete failure of the cytoplasm of the hepatocytes in many of the livers to stain with hematoxylin and eosin (Figures 15 and 16). The lesion was not due to fat content, as may be seen in Figure 17, a photomicrograph of a section stained with oil-red-O. Best's carmine stains for glycogen (Figure 18) also failed to explain this change. Therefore, it was concluded that this cytoplasmic change was hydropic degeneration. It was not apparent by gross examination. In such livers the sinusoids were

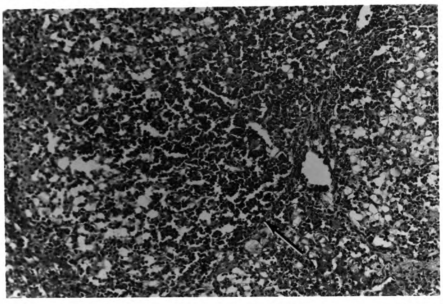

Figure 7. Dietary liver necrosis. Pig fed basal ration. Hematoxylin and eosin. x 120.

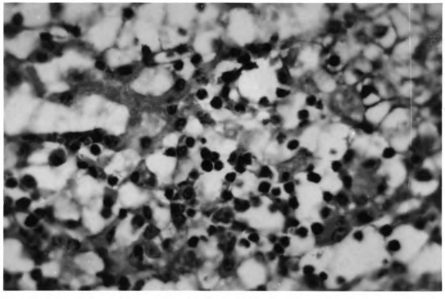

Figure 8. Dietary liver necrosis. Infiltration of necrotic lobule by polymorphonuclear leukocytes. Pig fed basal ration plus low level vitamin E. Hematoxylin and eosin. x 480.

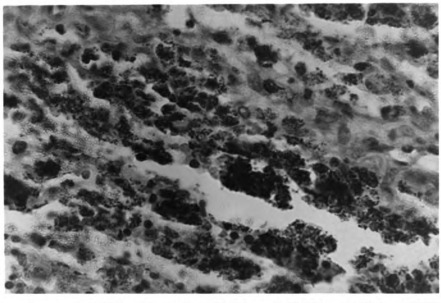

Figure 9. Dietary liver necrosis. Von Kossa-positive material in necrotic area. Pig fed basal ration plus methionine. Hematoxylin and eosin. x 480.

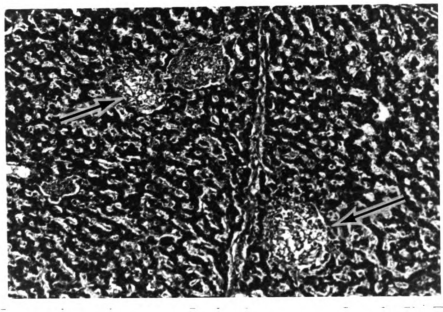

Figure 10. Focal intralobular necrosis. Pig fed basal ration plus methionine and low level vitamin E. Hematoxylin and eosin. x 120.

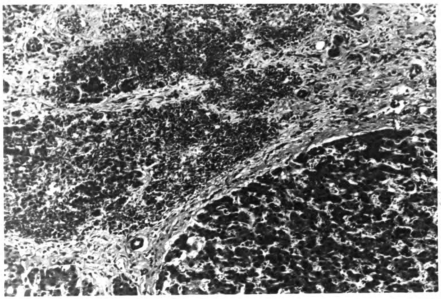

Figure 11. Dietary liver necrosis with desmoplasia and bile duct proliferation. Pig fed basal ration plus methionine. Hematoxylin and eosin. x 120.

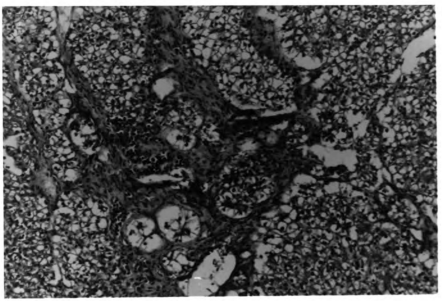

Figure 12. Chronic dietary liver necrosis with increased connective tissue and liver cells forming gland-like nests. Pig fed basal ration. Hematoxylin and eosin. x 120.

Figure 13. Acute necrosis and fibrosis. Pig fed basal ration plus low level vitamin E. Hematoxylin and eosin. x 30.

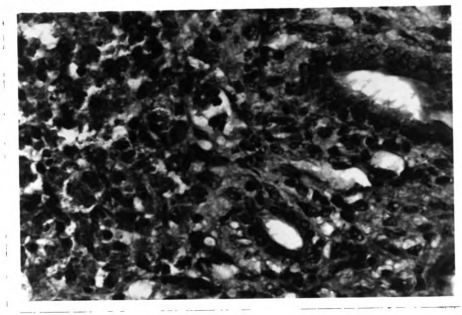

Figure 14. Massive dietary liver necrosis. Bile duct adjacent to necrotic tissue unaffected. Pig fed basal ration plus methionine. Hematoxylin and eosin. x 480.

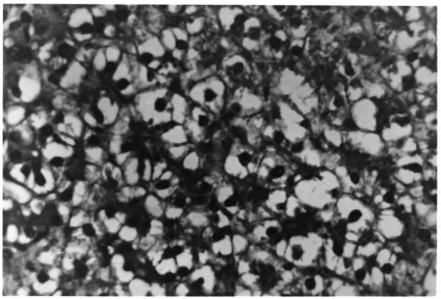

Figure 15. Hydropic degeneration of the liver. Pig fed basal ration plus low level vitamin E. Hematoxylin and eosin. x 480.

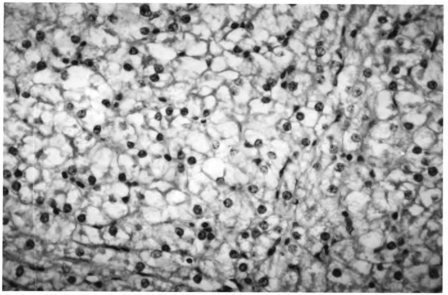

Figure 16. Hydropic degeneration. Pig fed basal ration plus selenium plus low level vitamin E. Hematoxylin and eosin. x 300.

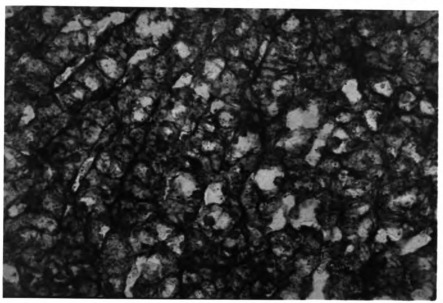

Figure 17. Hydropic degeneration. Same liver as Figure 16. Fat stain which is essentially negative. Oil-red-O stain. x 300.

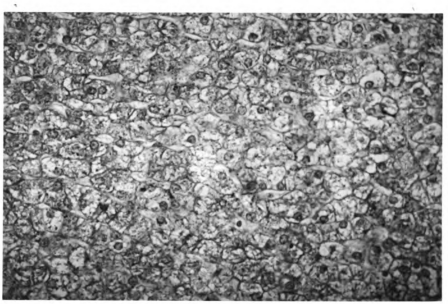

Figure 18. Hydropic degeneration. Same liver as Figure 16. Glycogen stain which is essentially negative. Best's carmine stain. x 300.

compressed to the degree that their lumina often appeared obliterated by the bordering hepatocytes.

The parenchymal cells of the livers of pigs fed 20% protein rations stained normally (Figure 19).

Nutritional Muscular Dystrophy

Nutritional muscular dystrophy (NMD) was observed in some pigs in all 3 experiments (Figures 20, 21, and 22). This disease may occur in varying degrees of severity and is often not detected during gross examination despite fairly extensive microscopic lesions. No NMD was observed grossly in any of the pigs in these 3 trials.

Histopathology of NMD. Nutritional muscular dystrophy was characterized by swelling of the muscle fibers with loss of cross striations. Often there was fragmentation of the fibers (Figures 23 and 24) and the appearance of increased numbers of mesenchymal nuclei in affected areas (Figure 25). These cells frequently formed elongated groupings in and around degenerating muscle fibers. Their exact identity is not clear. They are commonly termed "sarcolemmal cells", but this term is now regarded as a misnomer. Many of them are probably scavenger-type reticuloendothelial phagocytes which have infiltrated in response to the destruction of tissue. These groups of nuclei, often arranged in linear patterns, were the most striking microscopic feature in many cases.

Mineralization was observed in one case of NMD (Figure 26). This material stained well by von Kossa's method (Figure 27). Fibers in an advanced state of degeneration and fragmentation usually had an increased affinity for eosin. Occasionally, centrally located nuclei were observed in degenerated fibers which had been cut in cross section (Figure 28).

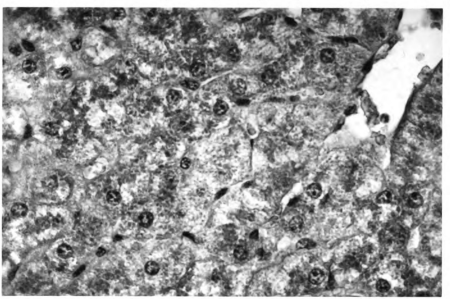

Figure 19. Normally staining hepatocytes in the liver of a pig fed 20% protein ration. Hematoxylin and eosin. x 480.

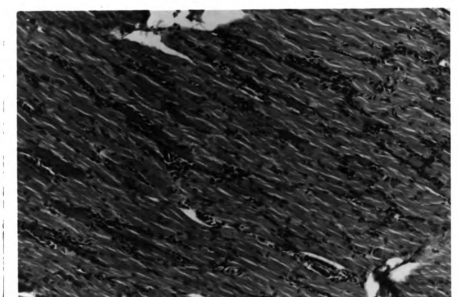

Figure 20. Nutritional muscular dystrophy in a pig fed the basal ration plus selenium. Hematoxylin and eosin. x 120.

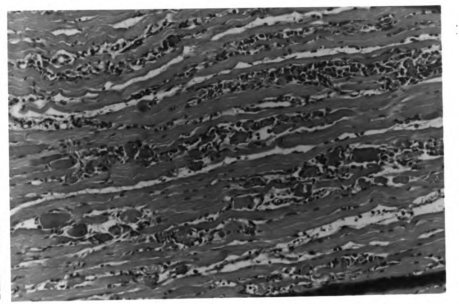

Figure 21. Nutritional muscular dystrophy in a pig fed the basal ration plus methionine. Hematoxylin and eosin. x 120.

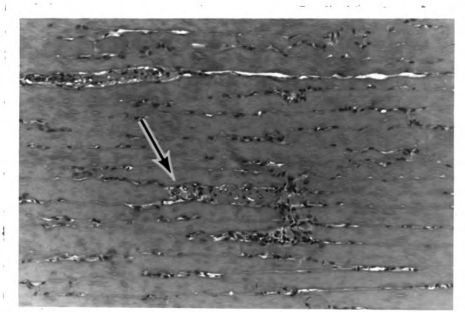

Figure 22. Nutritional muscular dystrophy in a pig fed a 20% protein ration. Hematoxylin and eosin. x 120.

Figure 23. Fragmentation and loss of cross striation in the quadriceps femoris muscle of a pig fed the basal ration plus selenium and low level vitamin E. Hematoxylin and eosin. x 300.

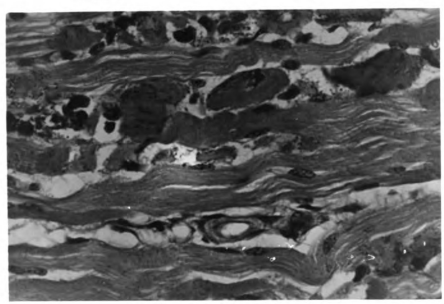

Figure 24. Fragmentation of fibers and loss of cross striations in the quadriceps femoris muscle of a pig fed the basal ration plus methionine. Hematoxylin and eosin. x 480.

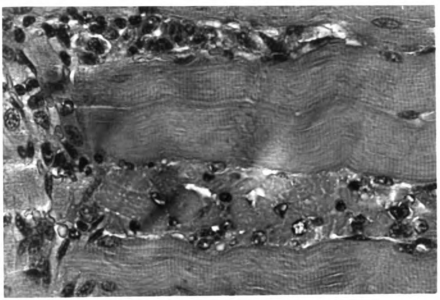

Figure 25. Increased numbers of nuclei in the quadriceps femoris muscle of a pig fed a 20% protein ration. Hematoxylin and eosin. x 480.

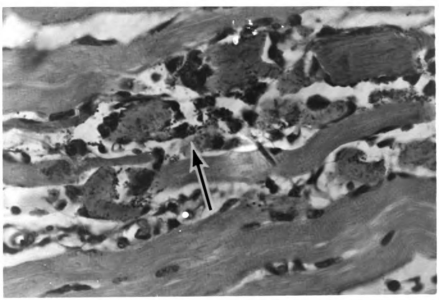

Figure 26. Mineral deposits in the quadriceps femoris muscle of a pig fed the basal ration plus methionine. Hematoxylin and eosin. x 480.

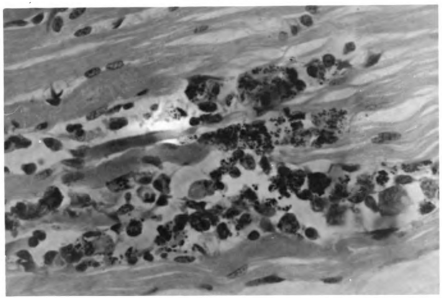

Figure 27. Section from the same muscle as Figure 26. Von Kossa's stain. x 480.

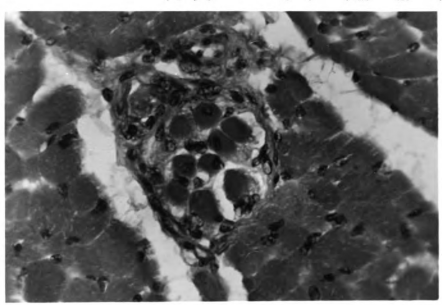

Figure 28. Cross section of a focal area of nutritional muscular dystrophy illustrating fibers with centrally located nuclei. Pig fed basal ration plus selenium plus low level vitamin E. Hematoxylin and eosin. x 480.

Mitotic figures occurred rarely (Figure 29).

Degeneration and Ulceration of the Esophageal Region of the Stomach

Gross lesions. There was a yellowish-white, granular, pseudomembranous material on the squamous epithelium of the stomachs of many of the pigs in these 3 experiments, irrespective of diet. This was often present in the esophagus also. Ulcers and erosions of various sizes were also observed in the esophageal region of the stomach of a large percentage of the animals in all trials. These usually began near the junction with the glandular epithelium. At times the ulcer involved the entire esophageal region of the stomach.

Histopathology of gastric lesions. The yellowish pseudomembranes observed in the esophagi and stomachs during gross examinations represented degenerated, hyperkeratotic and parakeratotic epithelium (Figures 30 and 31) which usually harbored a profuse growth of a fungus. The organism in question occurred as hyphae and as budding yeast forms and stained well with Gomori's methenamine silver (Figure 32). It was identified as Candida albicans. The epithelial cells in these areas were tremendously swollen and vacuolated (Figure 33) and frequently there were intra-epithelial pustules (Figure 34). The underlying papillary layer was edematous.

Ulcerations and/or erosions often occurred alone or in association with the degenerative changes of the squamous epithelium. The denuded areas and underlying structure of these ulcers were often invaded by large numbers of Candida organisms and bacteria (Figure 35). There were extensive inflammatory changes, including polymorphonuclear infiltration

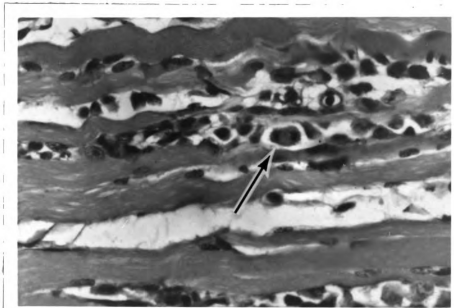

Figure 29. Mitotic division in a cell in an area of nutritional muscular dystrophy. Pig fed basal ration plus methionine. Hematoxylin and eosin. x 480.

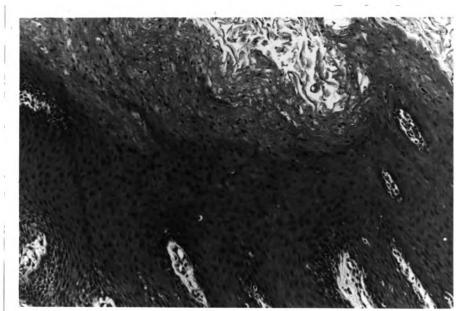

Figure 30. Hyperkeratosis and parakeratosis of squamous epithelium of the stomach of a pig fed the basal ration. Hematoxylin and eosin. x 120.

Figure 31. Hyperkeratosis and parakeratosis in esophageal region of stomach of a pig fed a 20% protein ration. Hematoxylin and eosin. x 30.

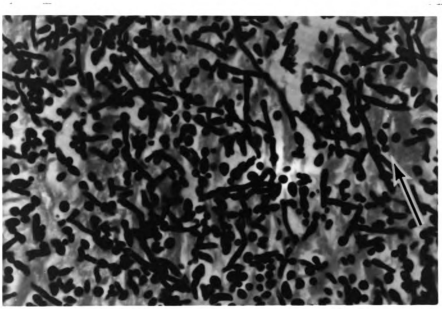

Figure 32. Fungus stain of section from the esophageal region of the stomach of a pig fed the basal ration. Note the budding yeast forms and hyphae. Gomori's methenamine silver stain. x 480.

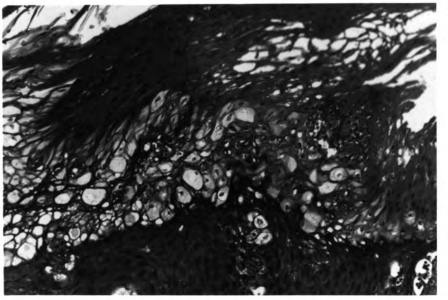

Figure 33. Vacuolation and ballooning degeneration of the squamous epithelium in the stomach of a pig fed a 20% protein ration. Hematoxylin and eosin. x 120.

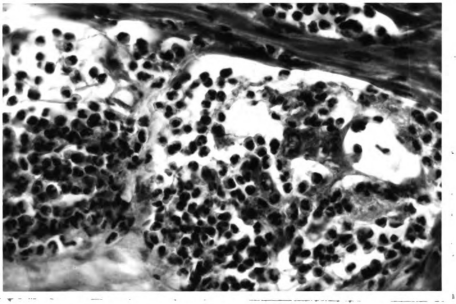

Figure 34. Intra-epithelial pustule in the esophageal region of the stomach of a pig fed a 20% protein ration. Hematoxylin and eosin. x 480.

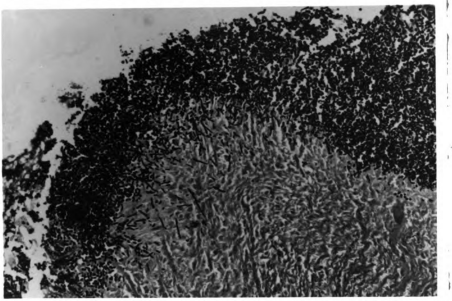

Figure 35. Candida albicans in ulcerated area in the esophageal region of the stomach of a pig fed the basal ration. Gomori's methenamine silver stain. x 120.

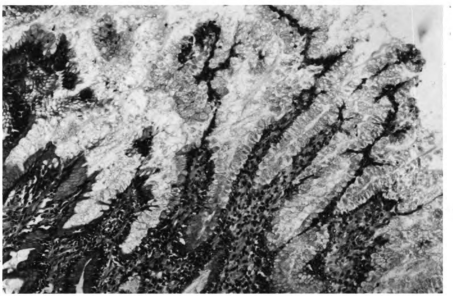

Figure 36. Mucinous degeneration in glandular epithelium adjacent to an ulcer of the squamous epithelium in the stomach of a pig fed the basal ration. Hematoxylin and eosin. x 120.

and fibroplasia. Mucinous degeneration of nearby glandular epithelium was common (Figure 36). Occasionally degenerative changes were observed in vessels of the muscularis or serosa near ulcerated areas (Figures 37, 38, and 39). It is to be re-emphasized that these lesions occurred to some degree in most of the animals in the 3 experiments, regardless of diet.

Atrophy of the Thymus

There was extreme atrophy of the thymus among many of the pigs fed 6% protein diets. In some cases, no thymic tissue was detected at necropsy.

Experiment I

Clinical signs and mortality. There was relatively little clinical evidence of disease referable to the diet among the experimental animals, other than general unthriftiness and poor appetite. Pig A-11 had a fever and vomited blood before dying as a result of a perforated gastric ulcer. Pig A-5 appeared normal the night before it was found in a coma and dying apparently from liver necrosis.

Feed consumption. The 2 pigs (A-2 and A-4) which were fed all 3 supplements had better appetites than the others. They were each eating approximately 380 Gm. of ration daily by the end of the trial, and undoubtedly would have eaten more. The group which ate next best included pigs A-7 and A-12, whose rations were supplemented with selenite. They were receiving 200 to 240 Gm. daily and often left feed in their bowls. All other surviving pigs were eating considerably less than 200 Gm. daily at the conclusion of Experiment I.

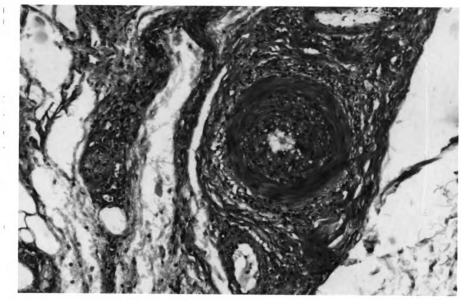

Figure 37. Thickening and vacuolation of the intima of a blood vessel in the serosa of an ulcerated area of the stomach. Pig fed basal ration plus high level vitamin E. Hematoxylin and eosin. x 120.

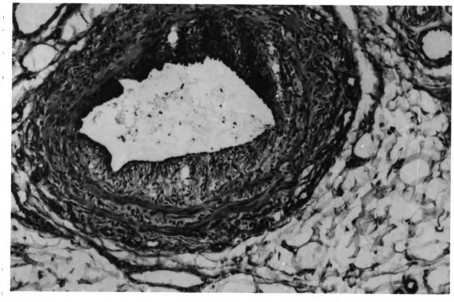

Figure 38. Degenerative changes in the wall of a blood vessel in the serosa near a gastric ulcer. Pig fed basal ration plus low level vitamin E. Hematoxylin and eosin. x 120.

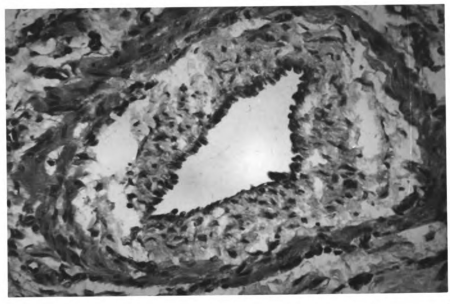

Figure 39. Intimal swelling in the wall of a vein in the serosa near an ulcer of the esophageal region of the stomach. Pig fed basal ration. Hematoxylin and eosin. x 300.

Figure 40. Appearance of some of the pigs of Experiment I: pig 4 fed basal ration plus selenium, low level E, and methionine; pig 7 fed basal ration plus selenium; pig 10 fed basal ration plus low level vitamin E; pig 6 fed basal ration.

Growth. The weights of pigs during the course of Experiment I are presented in TABLE 11. Total weight gain, number of days on experiment, and average daily gain are given in TABLE 12. In Figure 40 it is possible to compare the physical appearance of pigs on the complete supplement (pig 4), selenium (pig 7), low level E (pig 10), and the basal ration (pig 6). There was a great difference between pig 6 and the others, but relatively little difference among the other 3 pigs.

All pigs made poor gains on the 6% protein ration. Those receiving methionine, alone or in combination with other supplements, grew significantly better than the others. Pig A-11, affected with severe gastric ulceration, was an exception.

Liver weights. The weights of the livers of the pigs of Experiment I at necropsy and the ratio of this weight to body weight are presented in TABLE 13. Although there was great variation among these figures, there was no significant pattern of variation.

Hematology. Packed cell volume and hemoglobin values for the pigs of Experiment I are presented in TABLES 14 and 15. There was a fairly uniform decline in both values among all groups throughout the course of the experiment. Hemoglobin values are omitted for day 54 because of technical problems with the determination.

OCT activity. Serum ornithine carbamyl transferase activities for the pigs of Experiment I are presented in TABLE 16. Although the highest values occurred in pigs with lesions of dietary liver necrosis (A-3 and A-6), none exceeded the values reported by Wretlind et al. (1959) as normal for pigs.

TABLE 11. Weights of pigs, Experiment I (Gm.)

Pig No.	Supplement	Days of Age 16	22	28	35	42	49	52	56	59	63	69	74	75
A-1	Low E	2580	2835	3204	3034	3402	3686		3742		3714	3680		
A-2	Se + meth + low E	2743	3232	3686	4167	4876	5670		6322		6549	6010		
A-3	Basal	2892	3147	3374	3572	3856	3997		4338		4224	3970		
A-4	Se + meth + low E	2963	3430	3572	4168	5018	5557		5925		6095	5950		
A-5	Meth	2991	3289	3657	4309	4990	5840		6662	6500				
A-6	Basal	2849	3147	3402	3572	3600	4054		3997		3856		3969	3770
A-7	Se	3041	3402	3657	3941	4309	4848		5188		5301	5315		
A-8	Basal	2892	3147	3600	3629	3827	4026	4000						
A-9	Basal	2821	3204	3487	3714	3827	4139		4253		4366	4470		
A-10	Low E	2800	3119	3289	3827	3856	4253		4167		4309	4220		
A-11	Meth	3377	3799	4167	4678	4933	5188	4600						
A-12	Se	3055	3289	3742	3969	4196	4593		4848		5075	5012		

TABLE 12. Total weight gains and average daily gains, Experiment I (Gm.)

Pig No.	Supplement	Total weight gain	No. days on experiment	Approximate average daily gain
A-1	Low E	1100	53	21
A-2	Se + meth + low E	3267	53	62
A-3	Basal	1078	53	20
A-4	Se + meth + low E	2987	53	56
A-5	Meth	3509	43*	82
A-6	Basal	921	59	16
A-7	Se	2274	53	43
A-8	Basal	1108	36**	31
A-9	Basal	1649	53	31
A-10	Low E	1420	53	27
A-11	Meth	1223	36*	34
A-12	Se	1957	53	37

* Died

** Killed

TABLE 13. Liver weights and ratios of body weights to liver weights, Experiment I

Pig No.	Supplement	Age at necropsy (days)	No. days on experiment	Liver wt. (Gm.)	Ratio of body wt. to liver wt.
A-1	Low E	69	53	145.0	25.0
A-2	Se + low E + meth	69	53	191.0	31.4
A-3	Basal	69	53	111.5	36.0
A-4	Se + low E + meth	69	53	138.0	43.1
A-5	Meth	59	43	320.0	20.0
A-6	Basal	75	59	73.0	52.0
A-7	Se	69	53	162.2	33.0
A-8	Basal	52	36	130.0	31.0
A-9	Basal	69	53	105.0	43.0
A-10	Low E	69	53	---	---
A-11	Meth	52	36	200.0	23.0
A-12	Se	69	53	155.0	32.0

TABLE 14. Hematocrit values (packed cell volume per cent), Experiment I

Pig No.	Supplement	Day of Experiment 8	15	24	30	37	44	52	54	58
A-1	Low E	38	32	30	32	29	29	---	30	---
A-2	Low E + se + meth	35	31	clot-ted	30	28	28	---	33	---
A-3	Basal	36	28	32	33	27	20	---	28	---
A-4	Low E + se + meth	36	34	28	30	26	26	---	29	---
A-5	Meth	35	28	31	34	29	---	---	---	---
A-6	Basal	clot-ted	30	38	38	37	33	32	---	30
A-7	Se	33	30	33	28	29	30	---	31	---
A-8	Basal	37	32	37	36	34	---	---	---	---
A-9	Basal	34	32	33	29	29	27	---	28	---
A-10	Low E	37	32	34	34	36	33	---	35	---
A-11	Meth	35	37	33	28	---	---	---	---	---
A-12	Se	37	32	32	30	29	29	---	34	---

TABLE 15. Hemoglobin values (Gm./100 ml.), Experiment I

Pig No.	Supplement	Day of Experiment 8	15	24	30	37	44	52	54	58
A-1	Low E	12.2	10.6	10.0	10.0	9.2	9.2			
A-2	Low E + se + meth	11.4	10.3	clot-ted	9.7	8.7	9.5			
A-3	Basal	11.8	9.2	10.0	10.5	8.8	6.2			
A-4	Low E + se + meth	11.8	11.2	9.5	9.7	8.1	8.5			
A-5	Meth	11.2	9.2	10.0	11.0	9.2				
A-6	Basal	clot-ted	10.0	12.5	12.2	12.2	11.0	9.6	---	9.5
A-7	Se	10.6	10.0	10.5	9.2	9.2	9.8			
A-8	Basal	12.0	10.4	12.5	11.7	11.2				
A-9	Basal	10.9	10.6	11.0	9.8	9.5	9.0			
A-10	Low E	12.2	10.3	11.5	11.0	11.5	11.2			
A-11	Meth	11.2	11.8	11.0	9.2					
A-12	Se	11.5	10.6	10.3	9.7	9.3	9.5			

TABLE 16. Serum ornithine carbamyl transferase activity, Experiment I

Pig No.	Supplement	Serum OCT activity on day of experiment indicated (Reichard units)		
		Day 44	Day 52	Day 59
A-1	Low E	0.29	0.23	
A-2	Se + low E + meth	0.31	0.27	
A-3	Basal	0.29	0.43	
A-4	Se + low E + meth	0.35	0.31	
A-5	Meth	0.39	---	
A-6	Basal	0.39	0.45	0.50
A-7	Se	0.29	0.30	
A-8	Basal	0.35	---	
A-9	Basal	0.23	0.29	
A-10	Low E	0.26	0.29	
A-11	Meth	0.31	---	
A-12	Se	.25	0.29	

Pathology. The incidences of grossly evident liver disease and extreme atrophy of the thymus among the pigs of Experiment I are summarized in TABLE 17. "Extreme atrophy" in this case indicates that no thymic tissue was detected at necropsy.

Other gross lesions. Subcutaneous edema of the hindquarters was observed in pigs A-3, A-6, A-8, and A-9, all of which had extensively fibrosed livers. A mild fibrinous peritonitis occurred in pigs A-5 and A-9. Pig A-5 also had subserous edema of the intestine and a considerable amount of fluid in the peritoneal cavity. The heart of A-8 was collapsed and flabby. Pig A-11 died following perforation of an ulcer of the glandular region of the stomach near the greater curvature (Figure 41). This pig also had a rent in the left side of the diaphragm through which part of the liver had herniated. There were about 100 ml. of dark brown, watery fluid in the pleural and peritoneal cavities. The liver was a mottled yellowish brown with many small, scattered, dark, depressed areas of irregular shape (Figure 42).

Histopathology. The incidences of microscopic lesions of dietary liver necrosis, hydropic degeneration of the liver, nutritional muscular dystrophy, and gastric epithelial changes are summarized in TABLE 18. Lesions of acute necrosis which had not been observed at necropsy were detected microscopically. Lesions of the squamous epithelium of the stomach occurred in all but one animal of those examined. Selenium did not protect against nutritional muscular dystrophy.

TABLE 17. Gross evidence of liver disease and extreme atrophy of thymus, Experiment I

Pig No.	Supplement	Dietary Liver Necrosis		Extreme atrophy of thymus
		Acute necrosis	Fibrosis	
A-1	Low E	0	0	+
A-2	Se + low E + meth	0	0	+
A-3	Basal	0	+	0
A-4	Se + low E meth	0	0	not examined
A-5	Meth	+	0	0
A-6	Basal	0	+	+
A-7	Se	0	0	+
A-8	Basal	0	+	+
A-9	Basal	0	+	+
A-10	Low E	0	+	+
A-11	Meth	+	0	0
A-12	Se	0	0	+

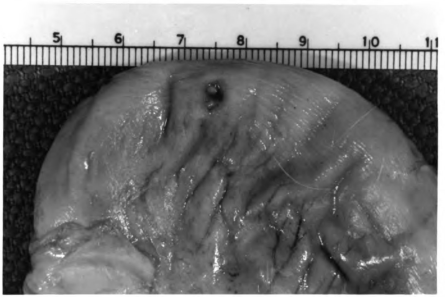

Figure 41. Perforated ulcer of the glandular epithelium of pig A-11. Pig was fed basal ration plus methionine.

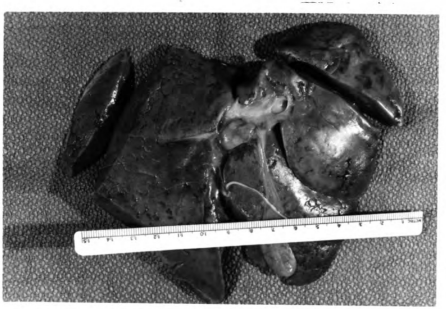

Figure 42. Liver of pig A-11. Swelling typical of acute dietary liver necrosis.

TABLE 18. Incidence of microscopic lesions, Experiment I

Pig No.	Supplement	DLN* Acute necrosis	DLN* Fibrosis	Hydropic degeneration of liver	NMD**	Lesions of epithelium of esophageal region of stomach: Epithelial degeneration	Erosion	Ulceration
A-1	Low E	0	0	+	0			+
A-2	Se + low E + meth	0	0	+	0	0	0	0
A-3	Basal	+	0	+	0			+
A-4	Se + low E + meth	0	0	0	0	not examined		
A-5	Meth	+	0	+	0			+
A-6	Basal	+	+	+	0			+
A-7	Se	0	0	+	+		+	0
A-8	Basal	+	+	+	0			+
A-9	Basal	0	+	+	0			+
A-10	Low E	0	0	+	0	+	0	0
A-11	Meth	+	+	0	0	perforated ulcer of glandular epithelium		
A-12	Se	0	0	+	0			+

* Dietary liver necrosis

** Nutritional muscular dystrophy

Vitamin E analysis of livers. The livers of the pigs of Experiment I were analyzed for alpha-tocopherol content. The results are given in TABLE 19.

Experiment II

Clinical signs and mortality. As was the case in Experiment I, there was little clinical evidence of nutritional disease other than general unthriftiness and poor hair coat. On the 10th day of the experiment pig B-9 was found comatose, although it had appeared normal the previous evening. It was electrocuted, and massive necrosis of the liver was observed at necropsy. On the 25th day of the experiment pig B-7 was depressed, weak in the hindquarters, and vomiting.

Feed consumption. In general, pigs of Experiment II had better appetites than those of Experiment I. At the conclusion of the trial, pigs B-2 and B-8, receiving both methionine and low vitamin E, and pig B-4, receiving methionine and selenite, and pig B-1, receiving selenite and low level vitamin E, were each consuming approximately 700 Gm. of feed daily. This was considerably more than the amount eaten by any of the pigs receiving single supplements. Approximate daily feed consumption shortly before termination of the experiment is given for each animal in TABLE 20. Such figures are somewhat inexact due to spilling and the difficulties of measuring leftovers accurately. Also, there was often wide variation in the daily feed consumption by individual animals.

Growth. Weight gains were considerably better than those of Experiment I. With one exception (pig A-12), the methionine-supplemented pigs grew

TABLE 19. Alpha-tocopherol content of livers, Experiment I

Pig No.	Supplement	Alpha-tocopherol (mg.%)
A-1	Low E	0.26
A-2	Se + low E + meth	0.17
A-3	Basal	0.06
A-4	Se + low E + meth	0.26
A-5	Meth	0.18
A-6	Basal	0.12
A-7	Se	0.24
A-8	Basal	0.08
A-9	Basal	0.14
A-10	Low E	0.23
A-11	Meth	0.61
A-12	Se	0.04

TABLE 20. Feed consumption, Experiment II

Pig No.	Supplement	No. days on experiment	Approximate daily feed consumption at conclusion of experiment (Gm.)
B-1	Se + low E	43	700
B-2	Meth + low E	36	700
B-3	Low E	36	360
B-4	Se + meth	43	700
B-5	Se	43	400
B-6	Se	36	450
B-7	Low E	43	280
B-8	Meth + low E	43	700
B-9	Meth	10	died on 10th day
B-10	Meth	43	380
B-11	Se + low E	36	340
B-12	Se + meth	36	470

at a rate well above the average rates of growth of all other groups. Weights of pigs throughout Experiment II are presented in TABLE 21. Total weight gains and average daily gains are given in TABLE 22. In Figures 43 and 44 it is possible to compare the appearances of the pigs fed doubly supplemented rations (Figure 43, pigs B-1, B-4, and B-8) with the appearances of pigs fed single supplements (Figure 44, pigs B-5, B-7, and B-10).

Liver weights. Weights of livers and ratios of body weights to liver weights are presented in TABLE 23. There was no apparent relationship between dietary liver necrosis and ratio of body weight to liver size.

Hematology. Hemoglobin and packed cell volume values through day 27 of Experiment II are presented in TABLES 24 and 25. These values declined generally throughout the experiment. The few exceptions to this trend were not of experimental significance.

Serum ornithine carbamyl transferase activity. Serum ornithine carbamyl transferase activity values on the days indicated are presented in TABLE 26. As in Experiment I, the highest values occurred in pigs with lesions of dietary liver necrosis (B-3, B-7, B-8, B-10). However, all fell within the range of normal values reported by Wretlind et al. (1959).

Pathology

Gross lesions. The occurrence of grossly visible lesions of dietary liver necrosis and extreme atrophy of the thymus are summarized in TABLE 27. Liver lesions occurred in all pigs which had not received selenium.

TABLE 21. Weights of pigs, Experiment II

Pig No.	Supplement	Days of Age 29	35	39	42	49	56	64	65	70	72
B-1	Se + low E	6308	6464		7088	7938	9044	10660		11737	11895
B-2	Meth + low E	6291	6435		7286	8647	10348	12559	12902		
B-3	Low E	5794	5897		6606	7371	8165	8562	8810		
B-4	Se + meth	5672	5868		6747	7598	8930	11057		12219	12640
B-5	Se	4750	4933		5387	6124	6861	7711		8222	8310
B-6	Se	4053	4224		4423	4791	5415	5585	5550		
B-7	Low E	6246	6350		6747	7569	8023	8987		8363	8947
B-8	Meth + low E	5869	6039		6861	8136	9441	11567		12928	13488
B-9	Meth	4811	5046	4590							
B-10	Meth	4296	4479		5018	5925	6917	7995		9072	8995
B-11	Se + low E	4161	4423		4593	5160	5727	6294	6215		
B-12	Se + meth	3055	3147		3657	4196	4848	5185	5180		

TABLE 22. Total weight gains and average daily gains, Experiment II (Gm.)

Pig No.	Supplement	Total weight gain	No. days on experiment	Approximate average daily gain
B-1	Se + low E	5587	43	130
B-2	Meth + low E	6611	36	184
B-3	Low E	3016	36	84
B-4	Se + meth	6968	43	162
B-5	Se	3560	43	83
B-6	Se	1497	36	42
B-7	Low E	2701	43	63
B-8	Meth + low E	7620	43	177
B-9	Meth	-221	10*	-22
B-10	Meth	4699	43	109
B-11	Se + low E	2054	36	57
B-12	Se + meth	2125	36	59

* Pig B-9 was in a coma on the morning of day 10. It was killed and necropsied at that time.

Figure 43. Appearances of some of the pigs fed double supplements, Experiment II: pig B-1 fed basal ration plus selenium and low level vitamin E; pig B-4 fed basal ration plus selenium and methionine; pig B-8 fed basal ration plus methionine and low level vitamin E.

Figure 44. Appearances of some of the pigs fed single supplements, Experiment II: pig B-10 fed basal ration plus methionine; pig B-7 fed basal ration plus low level vitamin E; pig B-5 fed basal ration plus selenium.

TABLE 23. Liver weights and ratios of body weights to liver weights, Experiment II

Pig No.	Supplement	Age at necropsy (days)	No. days on experiment	Liver wt. (Gm.)	Ratio of body wt. to liver wt.
B-1	Se + low E	72	43	446.0	27
B-2	Meth + low E	65	36	366.0	35
B-3	Low E	65	36	342.0	26
B-4	Se + meth	72	43	357.8	35
B-5	Se	72	43	281.8	30
B-6	Se	65	36	262.1	21
B-7	Low E	72	43	355.8	25
B-8	Meth + low E	72	43	411.5	33
B-9	Meth	39	10	103.0	45
B-10	Meth	72	43	300.5	30
B-11	Se + low E	65	36	192.8	32
B-12	Se + meth	65	36	132.5	39

TABLE 24. Hematocrit values (packed cell volume per cent), Experiment II

Pig No.	Supplement	Day of Experiment -2	6	12	13	20	27
B-1	Se + low E	37	33		36	36	35
B-2	Meth + low E	36	35		36	33	32
B-3	Low E	36	31		34	33	35
B-4	Se + meth	35	33		35	33	32
B-5	Se	39	37		36	40	36
B-6	Se	37	30		39	33	35
B-7	Low E	36	36		39	33	39
B-8	Meth + low E	36	34		35	35	33
B-9	Meth	38	30	40			
B-10	Meth	31	32		33	32	31
B-11	Se + low E	34	29		31	30	30
B-12	Se + meth	31	30		30	29	26

TABLE 25. Hemoglobin values (Gm./100 ml.), Experiment II

Pig No.	Supplement	Day of Experiment -2	6	12	13	20	27
B-1	Se + meth	11.2	10.7		11.5	11.8	11.0
B-2	Meth + low E	11.2	11.5		11.8	11.0	10.3
B-3	Low E	10.8	10.0		10.9	10.8	11.0
B-4	Se + meth	10.6	10.5		10.8	10.6	10.3
B-5	Se	12.4	11.8		11.8	12.5	11.5
B-6	Se	11.2	9.7		12.5	10.5	11.0
B-7	Low E	11.7	11.5		12.3	11.0	12.2
B-8	Meth + low E	11.2	11.2		11.2	11.3	10.9
B-9	Meth	12.0	9.7	11.2			
B-10	Meth	9.7	10.5		11.0	10.3	9.7
B-11	Se + low E	10.5	9.2		10.0	9.5	9.2
B-12	Se + meth	9.5	9.5		9.5	9.2	8.0

TABLE 26. Serum ornithine carbamyl transferase activity, Experiment II

Pig No.	Supplement	Serum OCT activity on day of experiment indicated (Reichard units)			
		Day 6	Day 10	Day 27	Day 35
B-1	Se + low E	0.30		0.28	0.24
B-2	Low E + meth	0.33		0.31	0.26
B-3	Low E	0.28		1.43	0.41
B-4	Se + meth	0.79		0.50	0.23
B-5	Se	0.35		0.35	0.23
B-6	Se	0.90		0.33	0.21
B-7	Low E	0.26		0.69	0.53
B-8	Low E + meth	0.34		1.18	0.20
B-9	Meth	---	2.55	---	---
B-10	Meth	0.29		0.88	0.46
B-11	Se + low E	0.46		0.26	0.21
B-12	Se + meth	0.26		0.25	0.21

TABLE 27. Gross evidence of liver disease and extreme atrophy of thymus, Experiment II

Pig No.	Supplement	Dietary liver necrosis		Extreme atrophy of thymus
		Acute necrosis	Fibrosis	
B-1	Se + low E	0	0	0
B-2	Meth + low E	0	+	+
B-3	Low E	+	0	0
B-4	Se + meth	0	0	0
B-5	Se	0	0	+
B-6	Se	0	0	+
B-7	Low E	0	+	+
B-8	Meth + low E	+	+	+
B-9	Meth	+	0	+
B-10	Meth	+	0	+
B-11	Se + low E	0	0	0
B-12	Se + meth	0	0	+

The lesions observed in pig B-9 were unusual. This pig was electrocuted when apparently near death. In addition to typical acute necrosis, the liver had a mottled appearance due to the contrast of the dark necrotic areas with a yellowish, granular fibrinous membrane which covered the organ in a patchy pattern.

Histopathology. The occurrence of microscopic lesions of dietary liver necrosis, hydropic degeneration of the liver, nutritional muscular dystrophy, and gastric ulcers among pigs of Experiment II is summarized in TABLE 28.

In addition to the lesions indicated in TABLE 28, there was a focal area of degeneration containing von Kossa-positive material in the myocardium of the left ventricle of pig B-10 (Figures 45 and 46). An unusual change was observed in the liver of pig B-9. This was one of the few protein-deficient animals which did not have hydropic degeneration of the liver. However, there were numerous vacuoles in the few lobules which had not undergone necrosis. In contrast to hydropic degeneration, this vacuolation appeared to be extracellular (Figures 47 and 48). It is thought that this lesion represented edema of the space of Disse as described by Obel (1953).

Experiment III

Clinical signs and mortality. Pig C-13 was the only animal to die during the course of the experiment. The carcass was found bloated and in a poor state of preservation on the morning of day 43. C-13 had appeared normal the previous evening and had consumed its feed and water.

TABLE 28. Incidence of microscopic lesions, Experiment II

Pig No.	Supplement	DLN*		Hydropic degeneration of liver	NMD**	Lesions of epithelium of esophageal region of stomach		
		Acute necrosis	Fibrosis			Epithelial degeneration	Erosion	Ulceration
B-1	Se + low E	0	0	+	+	+	0	0
B-2	Meth + low E	+	+	+	0	+	0	0
B-3	Low E	+	+	+	0			+
B-4	Se + meth	0	0	+	0	+	0	0
B-5	Se	0	0	+	+	+	0	0
B-6	Se	0	0	+	0	+	0	0
B-7	Low E	+	+	+	0			+
B-8	Meth + low E	+	+	+	0			+
B-9	Meth	+	0	0	0		+	0
B-10	Meth	+	+	+	+		+	0
B-11	Se + low E	0	0	+	0			+
B-12	Se + meth	0	0	0	0	+	0	0

* Dietary liver necrosis

** Nutritional muscular dystrophy

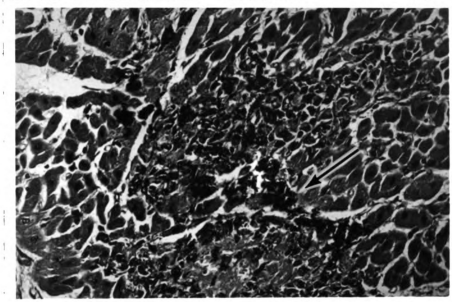

Figure 45. Degeneration in the myocardium of a pig fed basal ration plus methionine. Hematoxylin and eosin. x 300.

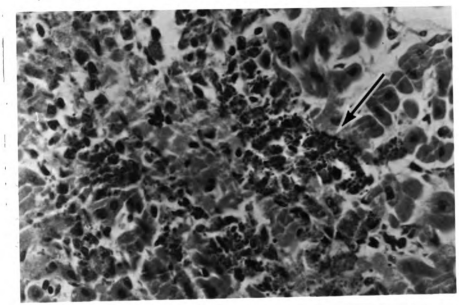

Figure 46. Mineralization in the same area shown in Figure 45. Von Kossa's stain. x 480.

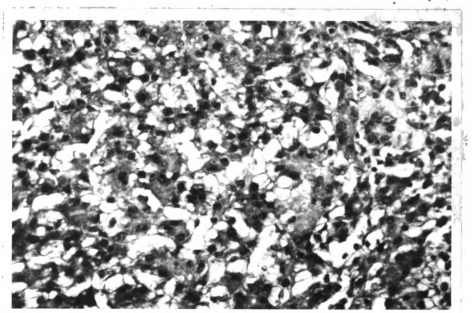

Figure 47. Extracellular vacuolation in the liver. Pig fed basal diet plus methionine. Hematoxylin and eosin. x 300.

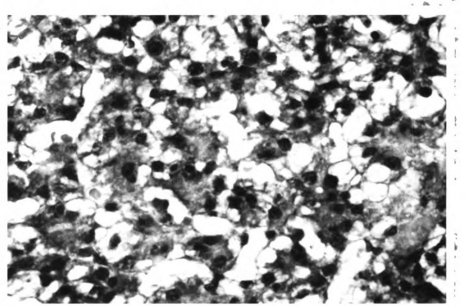

Figure 48. Greater magnification of lesion illustrated in Figure 47. Hematoxylin and eosin. x 480.

Pigs C-19 through C-24, all on 20% protein rations, had frequent attacks of diarrhea, but their appetites were not affected at these times.

Pigs C-19 and C-20 had a febrile illness during the period from day 22 through day 24. During this time they ate poorly, and C-20 was observed vomiting blood.

Pig C-16 was very unthrifty throughout the experiment.

On day 45, C-3 was very depressed and passed dark feces. The next day its eyelids were swollen, and the pig appeared weak. On the same day C-4 was ataxic but ate as usual.

Feed consumption - 6% protein groups. Feed consumption by pigs being fed the 6% protein ration varied considerably even within groups receiving the same supplements. This was at times due to illnesses probably unrelated to diet. The approximate daily feed consumption by pigs on the 6% protein ration at the end of Experiment III is presented in TABLE 29.

Feed consumption - 20% protein groups. Except for a brief period as mentioned under the discussion of clinical signs, pigs in this group ate all their feed and undoubtedly would have eaten more. Pigs C-19 through C-22 were eating 2200 Gm. of feed per pair daily by day 38 when they were killed. Pigs C-23 and C-24 together were eating 3000 Gm. of feed daily when killed on day 52.

Feed consumption - pig fed standard grower. Pig C-17, fed a standard pig ration, was eating approximately 850 Gm. daily when killed on day 52. It would have eaten more, but this amount represented the approximate capacity of the feed bowl.

TABLE 29. Feed consumption, 6% protein group, Experiment III

Pig No.	Supplement	No. days on experiment	Approximate daily feed consumption at conclusion of experiment (Gm.)
C-1	Basal	47	330
C-2	Basal	46	250
C-3	Low E	46	210
C-4	Low E	47	500
C-5	Se	52	650
C-6	Se	47	560
C-7	Se + low E	46	500
C-8	Se + low E	47	500
C-9	High E	47	450
C-10	High E	47	450
C-11	High E	52	650
C-12	High E	46	560
C-13	EQN*	43**	560
C-14	EQN	46	240
C-15	EQN	52	550
C-16	EQN	52	200

* Ethoxyquin

** Died

Growth. The weights of pigs throughout Experiment III are given in TABLE 30. The total gain and average daily gain are given in TABLE 31. Those pigs receiving 20% protein rations grew at a much better rate than those fed 6% rations. Pig C-17, fed a standard grower ration, also made much better gains than those on the 6% protein ration.

Liver weights. Weights of livers and ratios of body weights to liver weights are given in TABLE 32. There is no apparent relationship between this ratio and dietary deficiency or supplementation with any of the various supplements.

Hematology. In TABLES 33 and 34 the packed cell volume and hemoglobin values for the pigs of Experiment III are given. These data do not suggest a relationship between any of the diets and a specific effect on the hemogram.

Serum ornithine carbamyl transferase activity. Serum OCT activities on the days indicated are shown in TABLE 35. Values were somewhat higher among the pigs with lesions of dietary liver necrosis. Unfortunately, some samples were unsatisfactory for analysis.

Pathology

Gross lesions. The incidences of gross lesions of dietary liver necrosis and extreme atrophy of the thymus are summarized in TABLE 36. Neither of these lesions was observed in any of the pigs fed the higher levels of vitamin E or protein. Selenium prevented necrosis of the liver.

TABLE 30. Weights of pigs, Experiment III

Pig No.	Supplement	Day of Experiment 1	9	10	18	30	37	38	43	44	46	47	52
C-1	Basal	5188	5131		5897	12587	7484			8165		8055	
C-2	Basal	4848	5018		5557	6180	6464			6974	6630		
C-3	Low E	3042	4111		4763	4820	5046			5954	5720		
C-4	Low E	4933	5443		6322	7428	8732			9469		9080	
C-5	Se	4621	4763		5273	6124	7258			8165			9110
C-6	Se	4479	4848		5330	6577	7484			8505		8620	
C-7	Se + low E	4224	4593		4933	6010	6974			7881	7960		
C-8	Se + low E	3912	4224		4621	6124	6804			7995		7945	
C-9	High E	4763	5160		5585	7144	8222			8335		8600	
C-10	High E	4423	4111		4564	5103	5840			6521		6750	
C-11	High E	4621	5415		5698	6917	7428			7371			8020
C-12	High E	5103	5585		5897	7144	7881			8505	8350		
C-13	EQN*	5472	5783		6662	8165	9129		10155				
C-14	EQN	3827	3969		4054	4536	4933			5188	5140		
C-15	EQN	4366	4621		4933	6124	6804			7428			7850
C-16	EQN	3515	3572		3686	4196	3969			3969			4080
C-17	MSU grower	3005	2977		4564	6804	8562			10518			11793
C-18	MSU grower	---	---	2475									
C-19	20% protein + Se	9526	12701		14969	19505	22680	21319					
C-20	20% protein + Se	9072	12701		15422	21319	24494	21773					
C-21	20% protein + low E	7711	10433		12701	18598	22226	20412					
C-22	20% protein + low E	9526	13608		14515	20866	24041	21773					
C-23	20% protein	9979	12247		15422	21773	27216			34020			38102
C-24	20% protein	7711	9526		10886	15876	19051			23134			27216

* Ethoxyquin

TABLE 31. Total weight gains and average daily gains, Experiment III (Gm.)

Pig No.	Supplement	Total weight gain	No. days on experiment	Average daily gain
C-1	Basal	2867	47	61
C-2	Basal	1782	46	39
C-3	Low E	2318	46	50
C-4	Low E	4147	47	88
C-5	Se	4489	52	86
C-6	Se	4141	47	88
C-7	Se + low E	3736	46	81
C-8	Se + low E	4034	47	86
C-9	High E	3837	47	82
C-10	High E	2327	47	50
C-11	High E	3399	52	65
C-12	High E	3247	46	71
C-13	EQN*	4683	43	109
C-14	EQN	1313	46	29
C-15	EQN	3484	52	67
C-16	EQN	565	52	11
C-17	MSU grower	8788	52	169
C-18	MSU grower	---	---	---
C-19	20% protein + Se	11794	38	310
C-20	20% protein + Se	12701	38	334
C-21	20% protein + Se	12701	38	334
C-22	20% protein + low E	12247	38	322
C-23	20% protein	28123	52	541
C-24	20% protein	19505	52	375

* Ethoxyquin

TABLE 32. Liver weights and ratios of body weights to liver weights, Experiment III

Pig No.	Supplement	Age at necropsy (days)	Days on experiment	Liver wt. (Gm.)	Ratio b.w. to liver wt.
C-1	Basal	67	47	---	---
C-2	Basal	64	46	238	28
C-3	Low E	66	46	231	25
C-4	Low E	65	47	216	42
C-5	Se	72	52	308	30
C-6	Se	65	47	275	31
C-7	Se + low E	66	46	306	26
C-8	Se + low E	65	47	228	35
C-9	High E	65	47	234	37
C-10	High E	67	47	246	27
C-11	High E	70	52	345	23
C-12	High E	66	46	311	27
C-13	EQN*	61	43	280	36
C-14	EQN	66	46	238	22
C-15	EQN	70	52	238	33
C-16	EQN	72	52	115	36
C-17	MSU grower	72	52	277	43
C-18	MSU grower	30	10	80.2	31
C-19	20% protein + Se	63	38	559	38
C-20	20% protein + Se	63	38	740	29
C-21	20% protein + low E	63	38	648	32
C-22	20% protein + low E	63	38	717	30
C-23	20% protein	77	52	1132	34
C-24	20% protein	77	52	959	28

* Ethoxyquin

TABLE 33. Hematocrit values (packed cell volume per cent), Experiment III

Pig No.	Supplement	Day of Experiment 3	11	30	37	44
C-1	Basal	34.0	---	36.0	39.0	40.0
C-2	Basal	41.5	---	42.0	40.0	41.0
C-3	Low E	23.5	---	39.0	---	29.0
C-4	Low E	42.0	---	40.0	43.0	40.0
C-5	Se	41.0	---	36.0	---	39.0
C-6	Se	35.5	---	37.0	---	37.0
C-7	Se + low E	40.0	---	36.0	41.0	37.0
C-8	Se + low E	38.0	---	36.0	41.0	41.0
C-9	High E	35.0	---	44.0	43.0	42.0
C-10	High E	41.0	---	35.0	27.5	39.0
C-11	High E	42.0	---	35.0	36.0	34.0
C-12	High E	40.0	---	35.5	39.0	38.0
C-13	EQN*	36.0	---	37.0	36.0	---
C-14	EQN	39.0	---	33.0	36.0	37.5
C-15	EQN	34.5	---	34.0	32.0	34.0
C-16	EQN	41.0	---	39.0	33.0	33.0
C-17	MSU grower	41.0	---	34.0	35.0	35.0
C-18	MSU grower	36.0	33.5	---	---	---
C-19	20% protein + Se	36.5	---	38.5	37.0	---
C-20	20% protein + Se	40.0	---	37.0	42.0	---
C-21	20% protein + low E	41.0	---	40.0	40.0	---
C-22	20% protein + low E	37.0	---	40.5	38.0	---
C-23	20% protein	34.0	---	46.5	42.0	42.5
C-24	20% protein	37.0	---	38.0	39.0	39.5

* Ethoxyquin

TABLE 34. Hemoglobin (Gm./100 ml.), Experiment III

Pig No.	Supplement	Day of Experiment 3	11	30	37	44
C-1	Basal	11.0	---	11.9	12.6	12.4
C-2	Basal	12.4	---	13.1	13.1	12.8
C-3	Low E	7.7	---	12.9	---	9.2
C-4	Low E	13.1	---	12.7	14.2	12.7
C-5	Se	13.1	---	12.0	---	12.8
C-6	Se	10.5	---	11.4	---	11.7
C-7	Se + low E	12.7	---	12.0	13.1	12.2
C-8	Se + low E	12.0	---	-1.4	13.1	13.1
C-9	High E	10.5	---	13.4	14.2	13.1
C-10	High E	12.6	---	11.7	9.4	12.2
C-11	High E	12.7	---	11.9	12.0	10.8
C-12	High E	13.1	---	11.9	12.4	12.0
C-13	EQN*	11.1	---	12.0	11.7	---
C-14	EQN	12.2	---	11.1	11.7	11.8
C-15	EQN	10.8	---	10.8	10.5	10.6
C-16	EQN	12.6	---	12.5	10.8	10.9
C-17	MSU grower	13.1	---	11.1	11.1	11.3
C-18	MSU grower	11.4	10.0	---	---	---
C-19	20% protein + Se	11.4	---	12.4	12.4	---
C-20	20% protein + Se	12.7	---	12.0	13.4	---
C-21	20% protein + low E	12.9	---	12.7	13.0	---
C-22	20% protein + low E	11.4	---	12.7	12.6	---
C-23	20% protein	10.2	---	14.6	13.8	13.6
C-24	20% protein	11.4	---	12.4	12.7	12.6

* Ethoxyquin

TABLE 35. Serum ornithine carbamyl transferase activity, Experiment III

Pig No.	Supplement	Day of Experiment 17	44
C-1	Basal	---	0.51
C-2	Basal	---	0.56
C-3	Low E	---	0.48
C-4	Low E	---	0.23
C-5	Se	0.21	0.23
C-6	Se	0.23	0.23
C-7	Se + low E	0.20	0.21
C-8	Se + low E	0.21	0.20
C-9	High E	---	0.24
C-10	High E	0.23	---
C-11	High E	0.28	0.23
C-12	High E	---	0.19
C-13	EQN*	0.30	---
C-14	EQN	---	0.24
C-15	EQN	0.17	0.19
C-16	EQN	0.13	0.16
C-17	Grower ration	0.21	0.19
C-18	Grower ration	---	---
C-19	20% protein + Se	0.25	---
C-20	20% protein + Se	---	---
C-21	20% protein + low E	0.23	---
C-22	20% protein + low E	0.20	---
C-23	20% protein	---	0.19
C-24	20% protein	0.33	0.30

* Ethoxyquin

TABLE 36. Gross evidence of liver disease and extreme atrophy of thymus, Experiment III

Pig No.	Supplement	Dietary liver necrosis		Extreme atrophy of thymus
		Acute necrosis	Fibrosis	
C-1	Basal	+	+	0
C-2	Basal	+	+	+
C-3	Low E	0	+	0
C-4	Low E	0	0	0
C-5	Se	0	0	0
C-6	Se	0	0	0
C-7	Se + low E	0	0	+
C-8	Se + low E	0	0	0
C-9	High E	0	0	0
C-10	High E	0	0	0
C-11	High E	0	0	0
C-12	High E	0	0	0
C-13	EQN*	+	0	0
C-14	EQN	0	0	+
C-15	EQN	0	0	+
C-16	EQN	0	0	+
C-17	Grower ration	0	0	not examined
C-18	Grower ration	0	0	0
C-19	20% protein + Se	0	0	0
C-20	20% protein + Se	0	0	0
C-21	20% protein + low E	0	0	0
C-22	20% protein + low E	0	0	0
C-23	20% protein	0	0	0
C-24	20% protein	0	0	0

* Ethoxyquin

One of 4 pigs (C-13) fed the antioxidant EQN was affected with dietary liver necrosis. This pig died suddenly, although the extent of the liver changes was very limited by gross examination. Other observations made during the necropsy of C-13 included a pronounced gaseous distention of the gastrointestinal tract, a crepitating subcutaneous edema of the inguinal and scrotal regions, and congestion of the brain, thymus, lungs, and skin of the anterior 1/2 of the body. The gross evidence of liver necrosis in pig C-13 consisted of a few superficial reddened spots representing individual lobules or lobules in small groups. Bacteriologic cultures of the edematous subcutaneous tissues yielded Clostridium perfringens.

Pig C-3 was observed at necropsy to have edema of the eyelids, and the carcass was pale. Pig C-16 had severe ulceration of the squamous epithelium of the stomach. The stomach contents were watery and the color of digested blood.

Histopathology. The incidence of microscopic lesions of dietary liver necrosis, hydropic degeneration of the liver cells, nutritional muscular dystrophy, and gastric epithelial defects among the pigs of Experiment III is summarized in TABLE 37. No additional cases of liver necrosis were discovered by microscopic examination. The higher level of vitamin E and the ration containing 20% protein appeared to be fully protective against dietary liver necrosis. There was no hydropic degeneration of the hepatocytes in pigs which were fed 20% protein diets. High level vitamin E apparently completely controlled nutritional muscular dystrophy. Lesions of the gastric squamous epithelium were not as severe among pigs fed the higher protein diets and standard

TABLE 37. Incidence of microscopic lesions, Experiment III

Pig No.	Supplement	DLN* Acute necrosis	DLN* Fibrosis	Hydropic degeneration of liver	NMD**	Lesions of epithelium of esophageal region of stomach: Epithelial degeneration	Erosion	Ulceration
C-1	Basal	+	+	+	0			+
C-2	Basal	+	+	+	+			+
C-3	Low E	+	+	+	0			+
C-4	Low E	0	0	0	0			+
C-5	Se	0	0	+	+			+
C-6	Se	0	0	+	+			+
C-7	Se + low E	0	0	+	+	+	0	0
C-8	Se + low E	0	0	0	0	not examined		
C-9	High E	0	0	0	0	not examined		
C-10	High E	0	0	0	0			+
C-11	High E	0	0	+	0		+	0
C-12	High E	0	0	+	0			+
C-13	EQN***	+	0	0	not examined	not examined		
C-14	EQN	0	0	+	0			+
C-15	EQN	0	0	+	0		+	0
C-16	EQN	0	0	0	not examined			+
C-17	Grower ration	0	0	0	not examined	not examined		
C-18	Grower ration	0	0	0	0	+	0	0
C-19	20% protein + se	0	0	0	0	+	0	0
C-20	20% protein + se	0	0	0	0		+	0
C-21	20% protein + low E	0	0	0	0	+	0	0
C-22	20% protein + low E	0	0	0	0	+	0	0
C-23	20% protein	0	0	0	+	+	0	0
C-24	20% protein	0	0	0	+	+	0	0

* Dietary liver necrosis
** Nutritional muscular dystrophy
*** Ethoxyquin

grower ration. However, the incidence of gastric lesions was similar to the incidence among pigs on 6% protein diets. Hemorrhages were observed in the thymus of pig C-13.

Bone marrow smears. In the bone marrow smears of many of the pigs fed vitamin E-deficient diets, including those fed low level vitamin E, there were numerous cells of the erythroid series which contained 2 nuclei or a single, lobed nucleus (Figure 49). This abnormality was observed in prorubricytes, rubricytes, and metarubricytes. Such forms also occurred in the marrow of pig C-17, which was fed the standard pig ration. Among the 4 pigs fed high level vitamin E, only C-10 had an occasional erythroid cell with these nuclear abnormalities (Figure 50).

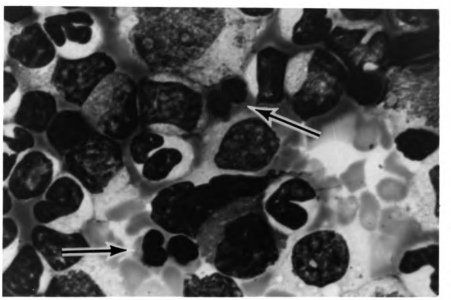

Figure 49. Binucleate cells and lobed nuclei of cells of the erythroid series in bone marrow smear from a pig fed basal ration. May-Grünwald-Giemsa stain. x 1200.

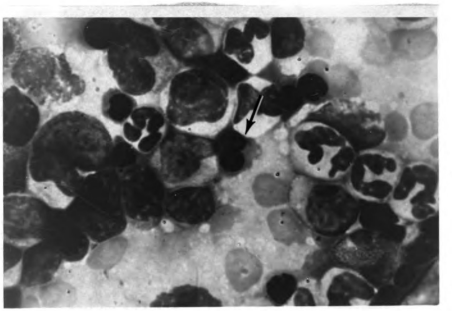

Figure 50. Binucleate erythroid cell in bone marrow smear of a pig fed basal ration supplemented with high level vitamin E. May-Grünwald-Giemsa stain. x 1200.

DISCUSSION

Clinical Signs and Mortality

These experiments support Obel's statement (1953) that clinical signs are not a prominent feature of dietary liver necrosis in swine. Pigs, which died with lesions suggestive of dietary liver necrosis as the cause of death, were sometimes found dead in the morning although they showed no signs of serious illness the previous evening. In one case (B-9), a pig was found comatose in the morning after having refused its meal the night before. Other signs noted occasionally or in individual animals included vomiting, edema of the eyelids, posterior weakness, and dark feces which appeared to contain digested blood. The last mentioned sign was observed in a pig which had a severe hemorrhagic gastric ulcer. Obel mentions the appearance of blood in the feces as one of the signs of hepatosis diaetetica.

It is probable that some of the pigs with nutritional muscular dystrophy had disturbances of locomotion, but no such signs were noticed. This may have been due in part to the close confinement of most of the pigs in small cages. However, no abnormalities of gait were observed in pigs C-23 and C-24, both of which were maintained in a large pen and had microscopic lesions of NMD.

The brief, acute illness of C-19 and C-20 was not thought to be related to the experiment.

Severe, hemorrhagic gastric ulceration was the most likely reason for the poor condition of pig C-16 throughout most of Experiment III.

The watery, dark stomach contents, observed in this pig at necropsy, are in agreement with the observations of Reese et al. (1966a).

The over-all mortality rate among the pigs of the 3 experiments was relatively low, despite the fact that many animals had extensive, chronic lesions of dietary liver necrosis. One may conclude, therefore, that DLN in pigs has a greater tendency toward a chronic course than the same disease in rats. In the latter species, early death is the rule (Schwarz, 1958).

The circumstances under which pig C-13 died and the unusual necropsy findings bear several similarities to some of the features reported by Stowe (1962) in connection with sudden deaths among tocopherol-deficient pigs. Stowe reported several instances in which deaths were associated with rapid, extreme post-mortem tympanites, crepitating effusions in the posterior parts of the body, and the isolation of clostridial organisms. He speculated that deaths in such cases were the result of overwhelming stress due to saprophytic clostridial organisms. In any case, it appears that such deaths represent a distinct syndrome, identifiable by the following 4 characteristics:

1. Sudden death.
2. Rapid post-mortem tympanites.
3. Crepitant subcutaneous swellings.
4. Isolation of bacteria of the genus Clostridium.

It remains to be determined if tocopherol deficiency is an essential factor in the pathogenesis of this condition. It appears that the relationship of this syndrome in swine to tocopherol deficiency warrants further investigation.

Feed Consumption

Feed consumption was uniformly poor among pigs fed the basal 6% protein ration. Supplementation with selenium, high level vitamin E, or combinations of supplements as employed in Experiment II all resulted in enhancement of appetites. Pigs fed a 20% protein ration ate greater amounts than those fed 6% rations, with no appreciable change in appetites when supplements were added to the ration.

Pigs A-5 and C-13, both of which died with lesions of dietary liver necrosis, were eating comparatively well up to the times of death. The appetite of Pig B-9 was slightly reduced for about 24 hours before the pig was found comatose with DLN.

Growth

Weight gains were extremely poor among pigs of Experiment I. Pig A-5, whose diet was supplemented with methionine, was growing at a better rate than the other pigs of Experiment I when it died of dietary liver necrosis. This suggests that the increased growth rate with additional dietary methionine might have acted as a stress factor in precipitating fatal DLN.

In Experiment II, the supplements appeared to act synergistically to promote growth in many cases (e.g., pigs B-1, B-2, B-4, B-8).

In Experiment III, it was obvious that increased protein in the ration was much more effective in promoting growth than any of the supplements, including tocopherol. The data of Experiment III support the findings of Pellegrini (1958) and Stokstad *et al.* (1958), who reported that tocopherol-deficient pigs grew as well as controls. The death of C-13, like that of A-5, was an example of sudden death in an

animal which appeared to be among the thriftiest and healthiest in the trial.

Liver Weights

It was not possible to demonstrate any consistent relationship between liver weight or the ratio of body weight to liver weight and dietary liver disease.

Hematology

The rather uniform reduction in packed cell volume and hemoglobin values in Experiments I and II were probably as much a reflection of protein deficiency as of tocopherol deficiency. In Experiment III, there was little change in the hemograms. Thus, these experiments do not support the findings of Nafstad (1965), who reported a definite anemia in pigs fed vitamin E-deficient diets for 4 weeks. Stokstad et al. (1958) found no changes in packed cell volume or hemoglobin values in vitamin E-deprived pigs.

The hematocrit value of pig C-16 declined from 41% to 33%, probably as a result of gastric hemorrhage.

Serum Ornithine Carbamyl Transferase Activity

Although the highest values for serum OCT activity were observed in pigs with dietary liver necrosis, the figures were all well within the normal ranges reported by Wretlind et al. (1959). It is possible that there was a uniform decline in enzyme activity while the samples were stored in the frozen state or that significant deterioration occurred before freezing. In any case, the values obtained are regarded as a useful index of dietary liver necrosis when viewed in relation to each other.

The sharp rise followed by an abrupt decline in OCT activity in the serums of pigs B-3 and B-8 is puzzling. One possible explanation for this would be a temporary remission of the acute necrotic process and a period during which the proliferative processes were dominant.

Pathology

Dietary liver necrosis. The results of these experiments indicate that either selenium or alpha-tocopherol in adequate amounts in the diet affords pigs complete protection against dietary liver necrosis. One of 4 pigs fed ethoxyquin had lesions of DLN. The absence of necrosis in pigs C-23 and C-24 is contrary to the findings of Eggert et al. (1957), who reported that 4 of 6 pigs fed vitamin E-deficient diets containing 40% Torula yeast died of liver necrosis within 53 days. Pellegrini (1958) observed fatal liver necrosis within 50 to 70 days in pigs fed 32 to 45% Torula yeast diets. It is possible that dietary liver necrosis would have occurred in C-23 and C-24 if the pigs had been fed the experimental ration beyond the 52nd day. Another possible explanation for the fact that the livers of C-23 and C-24 were free of necrosis is that Torula yeast may vary in selenium content. Some rations containing 40% Torula yeast might contain enough selenium to prevent DLN.

Gross and microscopic study of livers in various stages of DLN enables the pathologist to draw some conclusions regarding the sequence by which the described lesions occur. Pre-necrotic changes are probably not a prominent feature of this disease, although mineralization and edema of the space of Disse may occur. The necrosis occurs suddenly, and, in acute, fatal cases, the liver is swollen and scattered with numerous spots resembling petechiae. Microscopically, these are found

to be lobules which have filled with blood after necrosis of the normal cellular elements. The interlobular connective tissue is intact, and characteristically limits the necrosis within individual lobules, although adjacent lobules may be affected simultaneously. Within a necrotic lobule cytoplasmic and nuclear fragments may be seen, but there is apparently prompt lysis of much of this cellular debris, so that the lobule consists of little more than a blood-filled reticulum. Infiltration by polymorphonuclear leukocytes is seen in some lobules. The portal structures are relatively free of lesions. At this point, the necrotic lobule apparently collapses, and is partially replaced by proliferating connective tissue and bile ducts. A few surviving hepatocytes are often seen arranged in small, gland-like groups surrounded by connective tissue. The roughened surface observed grossly in chronic DLN results from the contraction of the new scar tissue. Various combinations of these lesions in the same liver bear testimony to the fact that dietary liver necrosis in the pig is often characterized by repeated attacks of acute necrosis with subsequent fibrosis.

Atrophy of the thymus. Atrophy of the thymus is a common finding in animals fed diets deficient in protein (Follis, 1958). The data of Experiment III suggest that tocopherol as well as protein may have some protective effect against atrophy of the thymus.

Hydropic degeneration of the liver. The occurrence of this lesion indicated that it was associated with protein deficiency. This is in agreement with the reports of Hove and Seibold (1955) and Kosterlitz (1944).

Nutritional muscular dystrophy. The results of these experiments suggest that adequate dietary vitamin E affords pigs complete protection against NMD. Due to the fact that the muscles of only 2 of the 4 pigs fed ethoxyquin were examined, no inferences were drawn regarding the effect of a synthetic antioxidant on the occurrence of this lesion. The results of these trials support the findings of Grant and Thafvelin (1958) and Swahn and Thafvelin (1962), who reported that selenium did not completely protect vitamin E-deficient pigs from nutritional muscular dystrophy. This is in contrast to the effectiveness of selenium in the prevention of NMD in lambs and calves reported by Schubert et al. (1961) and Hartley and Grant (1961).

Lesions of the epithelium of the esophageal region of the stomach. The stomach lesions observed were classified as: (1) epithelial degeneration characterized by hyperkeratosis, parakeratosis, and ballooning degeneration; (2) erosions; and (3) ulcers. These were regarded as representing different degrees of severity of a single disease process, with degenerative changes appearing as a precurser to actual desquamation. Candida albicans was considered an opportunist in the diseased tissues. Obel (1953), in her comprehensive treatise on dietary liver necrosis (hepatosis diaetetica), considered ulceration of the squamous epithelium of the stomach an important and fairly constant feature of the disease. The results of these experiments do not support this view. The incidence of these lesions approached 100% among the pigs of these 3 experiments, irrespective of diet or the occurrence of other lesions. Although the number of animals involved does not permit absolute conclusions, the data suggest that the lesions are less severe in pigs

whose diets contain adequate protein. In this connection, Nafstad (1967a) reported that high protein rations reduced the severity but not the incidence of gastric ulcers in pigs. However, these experiments do not support Nafstad's view that vitamin E exerts a partial protective effect against ulcers (Nafstad, 1967a,b). The results of these experiments are in agreement with Nuwer et al. (1965), who found no protective effect when tocopherol was added to an ulcerogenic swine ration.

The degenerative lesions observed in blood vessels in tissues underlying ulcerated areas of gastric mucosa were also reported by Bicknell (1965). These unusual changes are probably the result of ulceration of the nearby mucosa. However, one cannot ignore the possibility that the reverse is true: that vascular lesions play an important role in the pathogenesis of gastric ulcers in swine.

There were several lesions which Obel (1953) reported as frequently associated with hepatosis diaetetica which were not observed in these studies. Some of the more important of these were: acute nephrosis, purulent cholangitis, yellow fat, and fibrinoid degeneration of small arteries in the mesentery, heart, liver, and kidneys. No explanation can be given for the failure of these changes to occur in the pigs used in this research.

Bone Marrow Smears

Study of the bone marrow smears of the pigs of Experiment III revealed nuclear abnormalities in cells of the erythroid series of many pigs similar to those described by Nafstad (1965). Such cells were somewhat less numerous in the marrows of pigs fed selenium or ethoxyquin than in the marrows of those fed the basal or low level vitamin E diets.

However, in pigs fed high level vitamin E, abnormal erythroid elements were not seen except in one animal (C-10), and then only rarely.

These results are in general agreement with the report of Nafstad (1965). However, she observed no reduction in the number of abnormal cells in the bone marrow of pigs fed selenium supplements. She regarded these lesions as evidence of a block in red cell maturation in tocopherol-deficient pigs.

In the examination and interpretation of the marrow smears of pigs in Experiment III, it was often difficult to determine whether an apparently binucleated erythroid cell was indeed such a cell or merely the result of an artifact of preparation.

Feed and Liver Analyses for Alpha-Tocopherol

The slightly higher level of alpha-tocopherol in the basal ration with added ethoxyquin is probably due to a protective effect of the antioxidant upon tocopherol. Until further data are available, the significance of the tocopherol content of the livers of pigs of Experiment I is not clear.

SUMMARY

Three experiments were conducted to study the pathology and pathogenesis of dietary liver necrosis (DLN) in the pig and to clarify the roles of vitamin E, selenium, the sulfur amino acids, and total dietary protein in the prevention of this disease. A total of 48 pigs was used in the 3 trials. The basal 6% protein diet contained Torula yeast as a source of protein and vitamin E-free lard as a source of fat. The various supplements employed included vitamin E at 2 levels, selenium, and methionine, each alone, and in several combinations. Other pigs were fed a diet containing the antioxidant ethoxyquin, and 6 pigs were fed rations containing sufficient Torula yeast to provide a 20% protein diet.

Dietary liver necrosis occurred consistently in pigs fed the basal 6% protein ration, or the basal 6% protein ration supplemented with low levels of vitamin E or with methionine. Supplementation with selenium, high levels of vitamin E, or additional protein completely prevented dietary liver necrosis during the periods of these trials. Lesions of DLN were observed in 1 of 4 pigs fed ethoxyquin.

Acute dietary liver necrosis appeared grossly as scattered red spots on the surfaces and in the parenchyma of affected livers. Chronic changes were represented by irregularly shaped, roughened areas resulting from the contraction of scar tissue.

Microscopically the most striking features were necrosis of hepatocytes, pooling of blood in the necrotic lobules, and a characteristic limitation of the necrosis by the interlobular septa. Connective tissue and bile duct proliferation occurred as a sequel to necrosis.

Microscopic lesions of nutritional muscular dystrophy (NMD) were observed in some pigs of all groups except those fed the higher level of vitamin E or ethoxyquin. Muscle tissue of only 2 of 4 pigs fed ethoxyquin was examined for this lesion.

Ulcers or pre-ulcerous changes of the squamous epithelium of the stomach were observed in virtually all the pigs in these experiments. These lesions were somewhat less severe among pigs fed 20% protein rations.

Hydropic degeneration of hepatocytes and extreme atrophy of the thymus occurred consistently in pigs fed 6% protein diets.

Serum ornithine carbamyl transferase activity of pigs with dietary liver necrosis was generally higher than that of pigs free of DLN. However, none exceeded values reported as normal for pigs by Wretlind et al. (1959).

Nuclear abnormalities in erythroid cells in bone marrow smears were relatively common among pigs maintained on vitamin E-deficient diets, whereas such lesions were extremely rare among the group fed a high level of vitamin E.

Growth rates were very poor in pigs fed 6% protein diets, but tocopherol deficiency appeared to have little effect on growth.

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VITA

Robert L. Michel was born in Philadelphia, Pennsylvania, on July 20, 1924. He attended the public schools of that city and had completed the preveterinary course and the first year of veterinary school at the University of Pennsylvania when his education was interrupted by a period of service in the U.S. Army during World War II. Returning to veterinary school after the war, the writer completed his training, receiving the V.M.D. degree in 1949.

Following his graduation from veterinary school, the author practiced for one year with Dr. H. B. Prothero in Johnstown, Pennsylvania. For the next 13 years, the writer conducted a general veterinary practice in the rural Pennsylvania community of Troy, Bradford County. In the fall of 1963, he entered the graduate school of Michigan State University, majoring in veterinary pathology.

The author married Marjorie R. Kephart of Philadelphia and Ocean City, New Jersey, in 1948. They have 4 children: Virginia, 15, Janice 13, Andrea, 11, and Richard, 9.