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Title: SOME POSSIBLE ROLES FOR SELENIUM IN THE REPRODUCTIVE PHYSIOLOGY OF THE MALE GRAY-TAILED VOLE (MICROTUS CANICAUDUS)

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The testes of the gray-tailed vole (Microtus canicaudus Miller) exhibited a high affinity for selenium relative to other selected tissues. Uptake of selenium-75 in the testis-epididymis complex was delayed relative to uptake by the whole body, kidney, and liver. This pattern of uptake suggested that selenium was metabolically incorporated into developing sperm.

Challenge with unlabeled selenite following ^{75}Se administration reduced subsequent retention by the whole body and testis, but failed to reduce epididymal retention. Challenge with sulfite failed to reduce subsequent retention in any of these compartments. These results suggested that observed ^{75}Se retention patterns in voles were not simply a reflection of metabolic substitution of selenium for sulfur.

Investigation of the intracellular distribution of ^{75}Se in sperm revealed that most of the incorporated selenium was localized

in the nucleus and mitochondria. This observation was consistent with sperm autoradiographs. These latter results indicated that the nuclear selenium was associated with the nuclear envelope and the nucleus proper, where a dense, but patchy distribution was evidenced. The mitochondrial selenium appeared to be mainly associated with the outer mitochondrial membrane.

In the absence of dietary vitamin E, the addition of sufficient selenium to a torula yeast ration significantly reduced the incidence of sperm abnormalities. Body weight and testes weight were significantly increased under these conditions. When no selenium was added to the diet, 60 ppm vitamin E in the ration significantly increased testes weight relative to body weight. With 60 ppm vitamin E in the diet, the addition of selenium produced no significant changes in measured physiological parameters.

Males placed on a vitamin E-supplemented low selenium diet for four weeks or eight weeks did not exhibit impaired reproductive performance relative to selenium-supplemented males.

Some Possible Roles for Selenium in the Reproductive Physiology of the Male Gray-Tailed Vole (Microtus canicaudus)

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INTRODUCTION

The interaction of selenium with biological systems has been the subject of extensive research for over four decades. While the potential toxicity of selenium compounds to mammalian organisms became widely recognized in the 1930's (Rosenfeld and Beath, 1964), it was not until 1957 that selenium was implicated as an essential micronutrient for some vertebrate organisms.

Schwartz and Foltz (1957) discovered that liver necrosis in rats could be prevented by the sulfur amino acids, vitamin E, and a naturally-occurring, but as yet uncharacterized, form of organically incorporated selenium. These findings were followed shortly by the discovery that selenium could, under some circumstances, prevent other vitamin E-related deficiency defects, including white muscle disease in sheep, swine, and cattle, and exudative diathesis in chicks (NRC, 1976). Rosenfeld and Beath (1964), Muth *et al.* (1967), and more recently Pond (1977) have provided comprehensive reviews of selenium nutrition and deficiency syndromes in a variety of species. It is now recognized that selenium is an essential micronutrient for sheep, rats, chicks, and quail. There is also much evidence that it is essential for cattle, swine, and squirrel monkeys (NRC, 1976).

Considerable investigative effort has focused on elucidation of specific biochemical roles for selenium. In 1972 selenium was

found to be an integral component of glutathione peroxidase (GSH-Px). This enzyme has recently been postulated to act as an intracellular antioxidant through degradation of hydrogen peroxide and lipid hydroperoxides in mammals (antioxidant properties have also been attributed to vitamin E). Whanger et al. (1973) isolated selenium-containing protein from heart and semitendinosus muscles of lambs. The catalytic function of this seleno-enzyme has yet to be elucidated. Two additional microbial seleno-enzyme systems have been described, the glycine reductase enzyme of clostridia and the bacterial formate dehydrogenase system (Stadtman, 1974).

It has often been suggested that selenium may simply act as a sulfur analog in biological systems. Levander (1976) has reviewed the recent work in this area, and summarized the chemical and physical similarities of sulfur and selenium. The two elements have similar electronic configurations in their outermost valence shells. In addition, the size, bond energies, ionization potentials and electron affinities of selenium and sulfur are quite similar. Levander has concluded, however, that despite "superficial" similarities selenium and sulfur do not follow identical metabolic pathways.

The role of seleno-enzymes and other selenium compounds in such basic metabolic processes as reproduction is largely open to speculation. The fact that vitamin E-deficient diets are known to adversely affect the reproductive performance of some species of mammals, coupled with the apparent metabolic interrelationships of vitamin E and selenium has aroused interest in the possibility of

specific reproductive roles for selenium.

Vitamin E was first implicated by Evans and Bishop (1922) as the dietary factor responsible for prevention of fetal resorption in white rats. Urner (1931) ascribed this reproductive failure in the absence of vitamin E to arrested development of the placental mesenchyme tissue. Adams (1965) found that vitamin E also prevented fetal resorption in golden hamsters (Mesocricetus auratus). Females on deficient diets exhibited arrested development of the splanchnic mesoderm and malformation of allantoic blood vessels.

Mason (1926) found that male white rats raised as weanlings on a vitamin E deficient diet maintained normal growth, but exhibited reproductive failure characterized by a slow, progressive desquamation and degeneration of the testicular epithelium. Subsequent work has shown that the degeneration is irreversible, involving the most differentiated germ cells first, progressing to more primitive cell types, and in severe cases involving total destruction of the spermatogonia (Shettles, 1960). Sertoli and Leydig cells do not appear to undergo degeneration (Leathem, 1970). While the rat, guinea pig, and hamster exhibit a similar response with respect to vitamin E deficiency, little or no effect has been noted in the rabbit, mouse, or human testes (Leathem, 1970).

Hartley and Grant (1961) reviewed the reproductive aspects of selenium-responsive diseases of New Zealand livestock. Impaired reproductive performance associated with a high incidence of white muscle disease or "unthriftiness" was noted frequently in sheep and

cattle foraging in low selenium areas. While estrus, ovulation, fertilization and early embryonic development proceeded in a normal fashion in affected flocks, a significant increase in embryonic mortality in ewes could be reduced by a single injection of 1-25 μg of selenium prior to mating. Buchanan-Smith et al. (1969) found that ewes required both selenium and vitamin E for satisfactory reproductive performance. However, rams placed on a purified diet very low in selenium for 140 days showed no pathological changes in the testes or in semen quality, as measured by percent live sperm and abnormal sperm. Hill et al. (1969) found that while fecundity (as measured by incidence of twinning) was significantly improved by selenium therapy, fertility was unaffected. The greater incidence of twinning among ewes treated with selenium was possibly attributable to the improved health of treated ewes rather than being a direct effect on reproductive physiology. The prevention of reproductive failure in ewes with selenium has been confirmed by subsequent work (Godwin et al, 1971; Scales, 1974).

In contrast to these data, investigation of the effects of low selenium diets on reproduction in rats indicates that the deleterious reproductive consequences may be mediated chiefly through the male. McCoy and Weswig (1969) found that second generation rats placed on a low selenium diet exhibited reduced growth, loss of hair, and reproductive failure. While supplementation of the diet with sulfur amino acids, sodium sulfate, or increased vitamins was without effect the addition of 0.1 ppm selenium to the diet restored growth, haircoat, and reproductive

capability. Wu et al. (1969) investigated the effects of low dietary selenium on reproduction in rats. Second generation males had a greatly reduced epididymal concentration of sperm. Most of these sperm exhibited impaired motility and morphological abnormalities, including breakage of the axial filaments and/or breakage at the principal piece of the tail. In addition, selenium-deficient males exhibited reduced testicular size and impairment of the epididymis, although spermatogenesis seemed to progress normally in some of the seminiferous tubules.

While addition of selenium to the diet restored reproductive integrity to the males, the addition of vitamin E had no effect, suggesting a possible specific role for selenium in the male reproductive physiology. These findings were confirmed by Wu et al. (1973) who found that vitamin E, ascorbic acid, methylene blue, butylated hydroxytoluene, and N,N'-diphenyl-D-Phenylene diamine (all known intracellular antioxidants) failed to improve reproductive integrity in selenium-depleted male rats. Only selenium was effective as a prophylaxis against this syndrome. Adams (1965) found that selenium seemed to have a sparing effect on vitamin E-deficient golden hamsters with respect to fertility. Hurt et al. (1971) noted that third generation female rats on low selenium diets were able to successfully reproduce, despite other overt symptoms of selenium deficiency. This would seem to further implicate the male of this species as more sensitive with respect to reproductive failure.

Although neither vitamin E nor selenium has been shown to be essential for reproduction in humans, some recent work by Cowgill (1974) suggests a possible correlation between dietary selenium and human reproductive performance. In cross-comparing data on regional distribution of selenium levels in forage crops in the continental United States with corresponding regional birth rates, it was found that low selenium regions had significantly lower birth rates than did high selenium areas. In this light, Schroeder et al. (1970) found that adult human testes ranked third in selenium concentration behind kidney and liver, and the testes of a nine-month-old male contained only half the adult concentration. This seems consistent with the contention that selenium has a specific role in male reproductive physiology.

In the assessment of possible reproductive roles for selenium, the use of a radionuclide of selenium (^{75}Se) has done much to clarify the dynamics of uptake and distribution in the reproductive system, and suggest avenues for further investigation. In mammals investigated to date, the female gonad does not appear to approach the capacity of the male gonad to concentrate administered ^{75}Se (Brown and Burk, 1973). Until recently, the short-term nature of most ^{75}Se tissue distribution studies precluded discovery of the ability of mammalian testes to concentrate selenium. However, Rosenfeld (1964) found that when daily tracer doses of inorganic ^{75}Se were given to rats, the testes showed the greatest concentration of ^{75}Se after 96 hours for organs examined. Anghileri and Marques (1965) observed that

^{75}Se-selenomethionine and ^{75}Se-selenocysteine levels in mice decreased throughout a seven-day period following injection in most organs, while liver activity remained relatively steady, and testicular levels showed a progressive increase. Utilizing whole body autoradiography, Hansson and Jacobsson (1965) found significant levels of injected ^{75}Se-selenomethionine in the epididymis and seminal vesicles of mice in a 16-day uptake study. In 1967, Gunn and co-workers found that after a single subcutaneous injection of a tracer dose of inorganic ^{75}Se to mice, all tissues assayed had attained maximal concentrations between one hour and one day, exclusive of the testes-epididymis complex. Peak ^{75}Se concentrations were attained in the testes at one week, and the subsequent loss of ^{75}Se from the testes was accompanied by a precipitous rise in the ^{75}Se content of the epididymis, culminating in an epididymal peak between two and three weeks following injection. This unique dynamic pattern of ^{75}Se uptake in the testis-epididymis complex suggested that selenium may be metabolically incorporated into developing spermatozoa, which subsequently are transported to the epididymis. Gunn and Gould (1970) showed that ^{75}Se levels in the epididymis of the rat peak first in the caput epididymis followed by sequential peaks in the corpus and cauda epididymis, again indicative of spermatogenic incorporation and subsequent transport to the epididymis. It was further shown that interruption of the sperm transport pathway by surgical severance of the vasa efferentia resulted in a failure of the epididymis to accumulate ^{75}Se. Brown and Burk (1973) performed autoradiography on

epididymal sperm of rats injected with ^{75}Se. Primary localization was seen to be in the midpiece of the sperm, near the vicinity of the mitochondria. In cocks administered ^{75}Se, Patrick et al. (1965) found that sperm were heavily labeled, and paper chromatography revealed that the label was mainly in the protein fraction.

That the dynamics of selenium uptake in male reproductive organs bears a striking similarity to zinc uptake patterns has aroused further interest, since zinc is known to be essential for spermatogenesis in mammals (Gunn and Gould, 1970). Furthermore, both zinc and selenium seem to protect the testes against cadmium-induced vascular injury (Gunn et al., 1968). It has been speculated that selenium binds cadmium at the site of potential injury (possibly the vascular endothelium) and subsequently transports it to an innocuous site. Gunn and Gould's (1970) data indicated that selenium may be incorporated into an earlier phase of spermiogenesis than zinc. (Spermatozoa carrying labeled selenium do not reach the epididymis until one week after spermatozoa transporting labeled zinc.) Furthermore, labeled selenium seems to become "concentrated" in the cauda epididymis with respect to the caput and corpa epididymis, while labeled zinc seems to be "depleted" at this stage.

Gould (1970) has stressed the selectivity of the spermatogenic pathway toward essential micronutrients and physicochemically related elements. His data indicated that while zinc, selenium, and manganese gain entry to the developing spermatozoa within the seminiferous tubules, other micronutrients such as iron, cobalt, molybdenum and iodine show no evidence of accumulation in the

testes or epididymis. It has been speculated that selective barriers exist at the vascular endothelium and/or seminiferous tubular membrane which control access of minerals to developing sperm.

In recent work with the seleno-enzyme glutathione peroxidase, Brown and Senger (1976) found a significant level associated with ejaculate from bulls. Further investigation revealed that the enzyme level in seminal plasma was significantly higher than in epididymal sperm.

The documentation of adverse reproductive performance in some domestic and laboratory species subjected to low selenium dietary regimes has raised questions concerning the role(s) of selenium in the reproductive ecology of wild mammals. Rosenfeld and Beath (1964), NRC (1976), and Pond (1977) have summarized the available data regarding the geographical distribution of selenium in forage in relation to soil geochemistry and other environmental factors. While much of the earth's terrestrial flora contains prophylactic (and in some areas toxic) concentrations of selenium, geographic areas in which very low forage levels of selenium prevail are not uncommon. Kubota et al. (1967) and Allaway (1972) reported that forage levels of selenium in the Pacific Northwest, New England, and Eastern Seaboard regions of the continental United States are particularly low.

It would seem to follow logically that mammalian populations able to survive and reproduce in low selenium regions, either have no physiological requirement for selenium or have to some extent

evolved the capacity to subsist on what is presently considered to be a selenium-deficient diet. To date, the only available data for an outbred wild species regarding the effect of dietary selenium on reproduction is that of Pond (1977), who assessed the effects of dietary selenium and vitamin E on the gray-tailed vole (Microtus canicaudus). The geographic distribution of this species is restricted to an area of the Pacific Northwest characterized by Carter et al. (1968) as extremely deficient in available selenium. In a sampling of alfalfa, grasses, and legumes, it was found that 80% of the samples contained less than 0.1 ppm of selenium, and that the median concentration was less than 0.05 ppm. Pond's data indicated that the gray-tailed vole has a requirement for both selenium and vitamin E. Animals placed on diets deficient in both nutrients exhibited liver necrosis and general "unthriftiness." While either nutrient appeared effective as a prophylaxis, selenium seemed to be more effective than vitamin E. Regarding reproductive parameters, dietary selenium was concentrated in the male reproductive tract. Both selenium and vitamin E seemed to assure the functional integrity of sperm (in contradistinction to rats), and to prevent fetal resorption and increase the fertilization rate in females. While addition of selenium to the diet improved the percentage of fertile matings, it was not established whether the effect was mediated through the female or the male.

Statement of Purpose

The preliminary data for M. canicaudus again suggest the possibility in mammals of a species-specific response to selenium and vitamin E. While the functional integrity of sperm in laboratory rats is dependent upon adequate dietary selenium and appears to be independent of the vitamin E requirement, Pond's (1977) data suggest that male voles may have no specific requirement for selenium in the reproductive tract. This raises questions concerning the possibility of a species-specific reproductive physiology with respect to entry of selenium into the spermatogenic pathway and subsequent incorporation into developing sperm.

The data for M. canicaudus also pose some serious ecological problems. If this species does have a specific requirement for selenium with respect to reproduction, the possibility exists that selenium is in some way a limiting factor in its reproductive potential.

In this context, the present study is an attempt to further clarify the significance of selenium in the reproductive physiology of male gray-tailed voles. The following approaches were used to characterize the gross metabolism and assess the functional significance of selenium in the male reproductive tract:

(1) Whole body and tissue retention studies (utilizing ^{75}Se) in male voles under "normal" and "deficient" dietary regimes of selenium to characterize gross metabolic patterns in the reproductive system relative to other tissues and to patterns in related

species.

(2) Subcellular fractionation of labeled spermatozoa to assess relative selenium uptake in subcellular compartments.

(3) Autoradiographic studies focused on the testes and spermatozoa to elucidate specific patterns of concentration and implicate possible physiological roles for selenium.

(4) An examination of sperm viability, motility, and morphology under differing dietary regimens of selenium and vitamin E (including voles live-trapped from a natural setting) to delineate some possible relationships between dietary levels of selenium, concentration in the reproductive tract, testicular development, and semen quality.

(5) An examination of reproductive success in males as a function of dietary selenium.

METHODS AND MATERIALS

To delineate possible roles for selenium in the reproductive system of male voles, a series of radioselenium localization experiments were performed. These encompassed whole organ, tissue, cellular and subcellular aspects of selenium localization, as a function of dietary selenium. The interaction of vitamin E and inorganic sulfur on selenium metabolism was also examined. To assess the possible functional significance of selenium in the reproductive physiology of male voles, growth, testicular development, sperm quality, and reproductive success were examined as a function of dietary selenium. In addition, the interaction of vitamin E was considered.

Animal Maintenance and Diets

Experimental animals were from a laboratory colony of M. canicaudus. The colony was initiated from breeding stock live-trapped in Benton County, Oregon from March to August, 1973 (Tyser, 1975) and since maintained in outbred condition. Animals were housed either in fiberglass boxes (60 cm x 15 cm x 15 cm) with wire mesh tops, or standard hanging wire-bottom cages (17 cm x 18 cm x 23 cm) with 0.5 cm mesh hardware cloth floor inserts. Cotton batting was provided for cover and nesting material, and the fiberglass cage bottoms were covered with absorbent wood shavings. All experimental animals were maintained under a photoperiod of 16L:8D, and an ambient temperature of 20-23°C.

The normal laboratory diet of the animals was Purina Rabbit Chow coupled with Purina Rat Chow or Wayne Lab Blox Rat Chow, and tap water ad libitum. The rabbit chow was found to contain approximately 0.13 ppm Se. The two rat chows were found to contain approximately 0.21 ppm and 0.07 ppm Se, respectively. For experiments requiring controlled amounts of dietary selenium, animals were provided with a pelletized torula yeast (TY) ration containing appropriate levels of selenium as Na_2SeO_3 and distilled water ad libitum. The composition of the TY diet is given in Figure 1 in the appendix.

Fluorometric Analysis for Selenium

Where analysis of dietary and tissue selenium levels was required, fluorometry was employed. The procedure is a modification of Olson's (1969) method. A detailed outline of this procedure is given in Figure 2 in the appendix. For analysis of selenium in the TY diets, 10 pellets were randomly selected from a 4-8 kilogram diet stock and homogenized. Duplicate samples weighing 1-3 grams were removed from the homogenate and subjected to fluorometric analysis. Essentially the same sampling procedure was employed for analysis of the standard laboratory ration.

Radionuclide and Radioassay Parameters

The isotope used in this study was ^{75}Se in the selenite form (H_2SeO_3), supplied by New England Nuclear Corporation. Selenium-75 decays by 100% electron capture to ^{75}As, with a 120.4 day half-life.

The gamma and soft x-rays allow efficient detection by liquid scintillation and NaI systems. The low energy Auger and internal conversion electrons make possible high resolution autoradiography. The isotope solution was appropriately diluted in distilled H_2O and administered to experimental animals orally via a 1 ml hypodermic syringe equipped with a blunt-tipped 3.4 cm oral administration needle.

For radioassay of whole body activity, a Packard Armac (Model 1446) liquid scintillation detection system was utilized. Counting geometry was standardized by placing the voles in a 2 ounce plastic vial which was mounted on a plastic holder. This arrangement fit the counting chamber in such a way that the animal was held axially in the center of the counting chamber in a reproducible manner. In radioassay of selected organs, tissues, and cell suspensions, a Packard Auto-Gamma (Model 5017) system was employed. The detector was a 3" x 3" NaI(Tl) well crystal. Samples were placed in the bottom of standard plastic counting tubes which were automatically lowered to a fixed position in the crystal well. In both units the window settings used were 100 to ∞ (arbitrary units). This yielded a counting efficiency of 32% for the Armac unit, and 56% for the Auto-Gamma.

By correcting for the difference in counting efficiencies in the Armac and Auto-Gamma units, tissue activity was related to whole body activity for a given experimental animal. For both counting systems, relative counting standards were used to correct for physical decay and to assess any fluctuation in counting

efficiency over time. Background radiation levels in the Armac with the counting chamber empty compared with levels when a vole was present indicated that the natural background radiation (principally ^{40}K) from the voles was negligible at the aforementioned gain and window settings.

Tissue Selenium Levels in Live-Trapped Voles

A preliminary study was undertaken to gain baseline data on tissue selenium concentration patterns of voles in a specific natural setting. Of particular interest was the possible concentration of selenium in the male reproductive organs, which would hopefully provide some indication of the relative biological activity of these organs with respect to selenium metabolism.

During the month of April, 1977, voles were live-trapped at William L. Finley National Wildlife Refuge, Benton County, Oregon. One hundred collapsible Sherman box traps and 12 Museum Special snap traps were placed near burrow entrances and in runways of M. canicaudus over an area of approximately four acres. Live-trapped males were weighed and sacrificed as captured. Sperm were obtained for analysis of structural and functional integrity, and the carcasses were frozen for later fluorometric analysis of selected tissues. At the termination of the field collection, testes, kidneys, liver, heart, and brain portions for six males were weighed and pooled for fluorometric analysis. To gain some indication of dietary selenium levels, a composite sample of low-lying vegetation (mainly grasses) was collected from the trapping

site. In addition, stomach contents were removed from four animals collected with the snap traps. The 1151 gram (wet weight) vegetation sample and the stomach content sample were desiccated in a drying oven and subjected to fluorometric analysis. The tissue selenium levels were expressed in ppm on a fresh weight basis. Forage and stomach content selenium levels were expressed in ppm dry weight.

Whole Body and Tissue Retention of ^{75}Se

This study was undertaken to determine the generalized retention pattern of a physiologic dose of selenium. Of particular interest was the retention by the testes as compared with other tissues.

Ten animals were weaned at 18-19 days of age and divided into two groups of five. For a period of four weeks, the animals were maintained in fiberglass cages and given Purina Rat Chow, rabbit chow, and tap water ad libitum. At the end of four weeks the animals were weighed and administered a submicrogram dose of radioselenium ($\sim$ 1.7 μCi). Animals were subsequently housed singly in hanging wire-bottom cages and placed on a diet of rat chow and tap water ad libitum. At 1, 3, 5, 7, 12, 17, and 21 days post-administration (PA) the animals were assayed for whole body activity as previously described. At 21 days PA surviving experimental animals were weighed and sacrificed by cervical luxation. Brain, heart, liver, kidneys, and testes were removed, blotted and weighed. Organs were placed in counting tubes and assayed for

activity in the Auto-Gamma unit. Mean percent initial whole body retention per gram (fresh weight basis) was determined for the tissues sampled.

The mean whole body retention value for each assay date was plotted on two-cycle semi-log paper. Regression analysis was performed on the five retention values corresponding to days 5-21 PA. This regression line was taken to represent a rate function describing turnover of selenium in a specific biological compartment. An extrapolated y-intercept value was assumed to represent the total percent body burden of selenium accounted for by this compartment. The time (PA) corresponding to one-half of this value was taken to represent the biological half life (T_b) of selenium in this compartment.

^{75}Se Retention Dynamics in the Testis-Epididymis Complex

This phase of the selenium localization studies was designed to more fully elucidate the dynamics of selenium uptake and retention in the male reproductive organs relative to other selected organs, and relative to whole-body retention. Of particular concern was tracing possible entry of selenium into the spermatogenic pathway in this species.

Twenty-five males were weaned at 17-20 days of age, and group-housed (five animals per cage) in fiberglass boxes, separating siblings whenever possible. The dietary regime was identical to that used in the initial retention study, except that Wayne Lab Blox Rat Chow was used. At the end of four weeks each

animal received, as above, a submicrogram dose of radioselenite corresponding to an activity of approximately 0.3 μCi. Following ^{75}Se administration, all animals were singly housed in standard hanging cages as above. At 1, 4, 7, 14, and 21 days PA, five animals were sacrificed, weighed and assayed for ^{75}Se activity as in the previous study. For each serial sacrifice, mean whole body activity was expressed as percent initial whole body activity, and percent initial whole body activity per gram (fresh weight basis). These data were plotted on semi-log paper for the five sacrifice groups.

Sulfite/Selenite Challenge Following ^{75}Se Administration

This challenge experiment was designed to test the hypothesis that selenium follows a distinct metabolic pathway with respect to sulfur. In this context, 16 voles were weaned at 18-21 days of age, weighed, and placed in fiberglass boxes in groups of four. To increase the sensitivity of the organisms to a challenge dose, a low selenium (0.02 ppm) diet was utilized along with distilled water ad libitum. At the end of four weeks, 12 of the animals were given a submicrogram dose of ^{75}Se (~ 0.19 μCi), assayed for whole body activity, and placed in hanging wire cages as above. Three days following isotope administration, all animals received orally a one micromole challenge dose of sodium selenite or sodium sulfite. Two weeks following ^{75}Se administration, animals were weighed, sacrificed, and assayed for whole body activity. Testes and epididymes were removed and assayed in the Auto-Gamma unit.

For both experimental groups, mean percent initial whole body activity and mean percent initial whole body activity per gram (fresh weight basis) were determined for whole body, testes, and epididymes. The results were compared graphically with similar data generated for voles not given a challenge dose following ^{75}Se administration. The ^{75}Se dose for this baseline group had been ~ 0.27 μCi. The difference in the amount of selenium administered was only on the order of 10^{-2} μg, and was not considered to be significant.

Cellular Fractionation of ^{75}Se-Labeled Spermatozoa

Of concern in this phase of the investigation was the intracellular concentration pattern of administered ^{75}Se in spermatozoa. The effect of vitamin E on these patterns was also investigated.

Ten animals were weaned at 18-22 days of age, weighed, and placed in fiberglass boxes as above, five animals per cage. One group received a TY diet containing approximately 0.02 ppm selenium with no vitamin E supplement. The second group received the same diet supplemented with 60 ppm vitamin E. After four weeks all animals received orally a submicrogram dose of radioselenite ($\sim$ 2.1-4.7 μCi) and were continued on the diets for three additional weeks. At this time, experimental animals were weighed and sacrificed as above. Cauda epididymes and vas deferens were removed and placed on a depression slide in five drops of 0.25 molar sucrose solution containing 0.05 molar sodium phosphate buffer with 10^{-5} molar phenylmethylsulfanylfluoride at a pH of 7.4 Intact

sperm were obtained by making multiple incisions in the above organs. The pooled sperm sample was added to three additional ml of sucrose solution and homogenized for 90 seconds with a Sorvall Omnimixer at maximum rpm. After radioassay of the homogenate using the Auto-Gamma unit, the cell fractions were subjected to differential centrifugation as follows:

	Cell Fraction	Centrifugation
1)	nuclei + debris	1000 g for 10 minutes
2)	mitochondria	10,000 g for 20 minutes
3)	microsome	30,000 g for 30 minutes
4)	cytosol	liquid phase of above centrifugation

For both dietary groups, the four cell fractions were radioassayed in the Auto-Gamma unit, and activity of a cell fraction was expressed as a percentage of the total homogenate activity.

Autoradiographic Analysis of ^{75}Se-Labeled Reproductive Tissue

To more fully investigate the microconcentration patterns of selenium in the reproductive tissues, autoradiography was employed. Four groups of males (four males/group) approximately six weeks of age were housed in fiberglass boxes and placed on a low-selenium TY diet for a period of four weeks to increase retention of administered radioselenium. After the four-week period, each animal received orally a dose of approximately 75 μCi of ^{75}Se. Four animals each were sacrificed at four days, one week, two weeks,

and three weeks following administration.

Small sections of testis, epididymis and vas deferens were removed from each animal and fixed in 2.5% glutaraldehyde in cacodylate buffer at 5°C for approximately 24 hours. At this time, the specimens were washed with cacodylate buffer and post-fixed in 1% OsO_4 buffered with s-collidine for 60 minutes at 5°C (Bennett and Luft, 1959). Following fixation, the tissue sections were dehydrated in ascending concentrations of acetone (Hayat, 1969) and embedded in Epon 812 according to the method of Luft (1961). Thin sections, obtained by cutting with a diamond knife and Porter-Blom MT-2 ultramicrotome, were placed on copper grids, stained with lead citrate (Reynolds, 1963), and coated with a thin film of carbon. They were subsequently coated with Kodak NTE emulsion, allowed to air dry, and placed in a light-tight container at 4°C for two weeks.

Immediately prior to development, a gold latensification procedure was employed to increase the sensitivity of the emulsion (Salpeter and Bachmann, 1964). For development, the specimens were dipped in distilled H_2O for 30 seconds and placed in the developing solution for 8 minutes at 24°C. The developing solution consisted of 0.045% Elon, 0.3% ascorbic acid, 0.5% borax, and 0.1% potassium bromide. At the end of 8 minutes, specimens were washed for 20 seconds in distilled H_2O followed by a 10-second rinse in the stop bath, and a 10-second rinse in distilled H_2O. At this time, the specimens were fixed for 1 minute in Kodak fixer, washed for 5 minutes in distilled H_2O and air dried. The developed sections were examined with a Philips EM-300 electron

microscope* and selected areas were photographed.

Effects of Dietary Selenium and Vitamin E on Sperm Structure and Function

This study was designed to examine the effects of dietary selenium and vitamin E on the structural and functional integrity of spermatozoa. The parameters evaluated included percent live sperm, percent abnormal sperm, and sperm motility. Growth, testicular development, and testicular selenium levels were also examined. In all, nine different physiological parameters were measured. A total of 10 different dietary regimes were employed in this study. The eight TY diets contained the following approximate amounts of selenium and vitamin E.

	Selenium	Vitamin E
1)	0.02 ppm (no Se added)	0.00 ppm (no vitamin E added)
2)	0.28 ppm	0.00 ppm (no vitamin E added)
3)	0.58 ppm	0.00 ppm (no vitamin E added)
4)	0.02 ppm (no Se added)	60.00 ppm
5)	0.04 ppm	60.00 ppm
6)	0.09 ppm	60.00 ppm
7)	0.26 ppm	60.00 ppm
8)	0.64 ppm	60.00 ppm

The TY diet was found to contain 0.02 ppm selenium without selenium supplementation. The inherent vitamin E level in the TY

*Philips Electronic Instruments, Mt. Vernon, NY.

diet was not determined, but this diet is known to be low in vitamin E content (Schwarz, 1961).

For these 8 experimental groups, 12 animals per group were weaned at 17-20 days of age, weighed, and placed in fiberglass boxes, 3 animals per cage. The diets were continued for 56 days at which time all groups were sacrificed for analysis of spermatozoan integrity. Due to high mortalities in some groups, additional experimental animals were added as required to insure adequate numbers for statistical purposes.

Two additional groups were employed in this study for comparative purposes. For the first group, seven adult males (11-12 weeks of age) were randomly selected from the laboratory breeding colony. The second group included six of the adult males live-trapped at William L. Finley National Wildlife Refuge.

At sacrifice, all animals were weighed, and testes were removed, weighed, and frozen for later fluorometric analysis. The vas deferens was removed and placed on a depression slide in two drops of Ringers No. 2 mammalian physiological saline solution. This was subsequently minced with forceps to obtain sufficient sperm. The sperm suspension was appropriately diluted in Ringers solution, and placed on a second depression slide which was transferred to a thermostatically controlled microscope stage heated to 37°C. For each sample, 200 sperm were examined microscopically at 100 x magnification and rated for motility according to the following scheme:

0 - no apparent movement

1 - some movement, but no sustained forward progress

2 - sustained forward progress

The motility value for a given sperm sample was derived by adding the individual sperm motility ratings, and dividing this total by the total number of sperm sampled (200).

A second drop of the sperm suspension was placed on a microscope slide and stained with fast green eosin. For live-dead determination (fast green eosin stains only dead cells) and morphological evaluation, 400 sperm were examined for each experimental animal at 430 x magnification. In addition, selected sperm were photographed.

Where applicable, mean and standard error values were calculated for group physiological responses. This allowed assessment of statistical significance for each parameter measured in regard to levels of dietary selenium. In addition, standard regression analyses were performed on the raw data to assess other possible interrelationships among the measured parameters.

Reproductive Experiments

That selenium may be essential for specific reproductive processes in voles may be of no ecological consequence. That is, voles suffering from a deficiency of dietary selenium may exhibit an impaired reproductive performance secondary to a generalized debilitation. In this context, two reproductive experiments were designed to examine the reproductive performance of voles placed on

a low selenium diet for a relatively short period of time. For the periods tested, it was assumed that overt symptoms of selenium deficiency would not be manifested. In both experiments the diets contained adequate levels of vitamin E.

For the first breeding experiment, 40 males were weaned at 17-19 days of age, weighed, and placed in groups of five in fiberglass boxes, separating siblings whenever possible. Twenty males were placed on a selenium-supplemented diet containing approximately 0.26 ppm of selenium. The remaining males were placed on an unsupplemented TY diet containing an inherent level of approximately 0.02 ppm of selenium. Both diets were supplemented with 60 ppm vitamin E. After 30 days on the TY ration, males were randomly mated to virgin females of the same age group, selected from the breeding colony. Both of the respective TY rations as well as the laboratory ration (rabbit chow plus rat chow) were made available in the breeding cages. The breeding pairs were allowed to remain together for 35 days. Beginning on day 21, all parturitions were recorded. Cumulative percent successful matings (as parturitions) were recorded as a function of time for both groups, between days 21 and 35 following pairing.

For the second experiment, 40 male voles 18-22 days of age were weighed and placed into groups of four in fiberglass boxes, separating siblings when possible. Twenty animals were placed on a TY diet containing approximately 0.26 ppm selenium, and the remaining animals were placed on a TY diet containing an inherent level of approximately 0.02 ppm selenium. As above, both diets

were supplemented with 60 ppm vitamin E. After 52 days on the diets, surviving males were placed singly in partitioned fiberglass boxes opposite virgin females of the same group, selected from the breeding colony. The 0.5 cm wire mesh partitions allowed visual, olfactory, and very limited tactile communication between the breeding pairs.

After two days of the partition treatment, the cotton bedding was switched between male and female for a given breeding pair to ensure adequate olfactory stimulus, and hopefully facilitate the onset of estrus in the females. After two additional days of partition treatment, the partitions were removed. The breeding pairs were allowed to remain together for eight days, at which time the partitions were re-inserted. During the time the breeding pairs were together both the TY diet and the standard laboratory ration were made available. It was assumed that the eight-day exposure of the selenium-depleted males to the laboratory ration would have a minimal impact on reproductive performance. Twenty-five days after the partitions were re-inserted the number of parturitions for both groups were determined and the experiment was terminated.

RESULTS

Tissue Selenium Levels in Live-Trapped Voles

Results of the fluorometric analysis of selected tissues, forage, and stomach contents of voles are reported in Table 1. Of the five organs sampled, the composite testes sample exhibited the highest concentration of selenium, followed by kidney, liver, and brain. The selenium concentration in the heart sample was below the detection limit of the fluorometer. A large difference (a factor of 7.33) was found in selenium concentration between stomach contents and the composite vegetation sample.

Whole Body Retention of Selenium-75

Results of the three-week whole body retention study are reported in Figure 1 and appendix Table 1. In general, whole body retention of ^{75}Se was characterized by a rapid initial elimination followed by a slower, constant rate of elimination. The regression line calculated for the last five data points exhibited a high correlation coefficient ($r^2 = .996$). The biological half-life (T_b) for this slow component was calculated to be approximately 7.0 days. By extrapolating this line to the Y axis, it was determined that the slow component accounted for 27.4% of the initial body burden.

The 24-hour retention value created a shoulder on the elimination curve, rendering calculation of a rate constant for days 0 to 3 uncertain. The shoulder probably reflects the time

Table 1. Selenium levels in selected tissues of male voles from William L. Finley National Wildlife Refuge (stomach contents and low-lying vegetation were also sampled*).

Tissue sample	Mean selenium content - ppm fresh weight (N = 6)
Testis	0.24
Kidney	0.22
Liver	0.11
Brain	0.03
Heart	Not detectable
Vegetation	0.03 (Dry weight)
Stomach contents	0.22 (Dry weight)

*Stomach contents were from 3 males and 1 female.

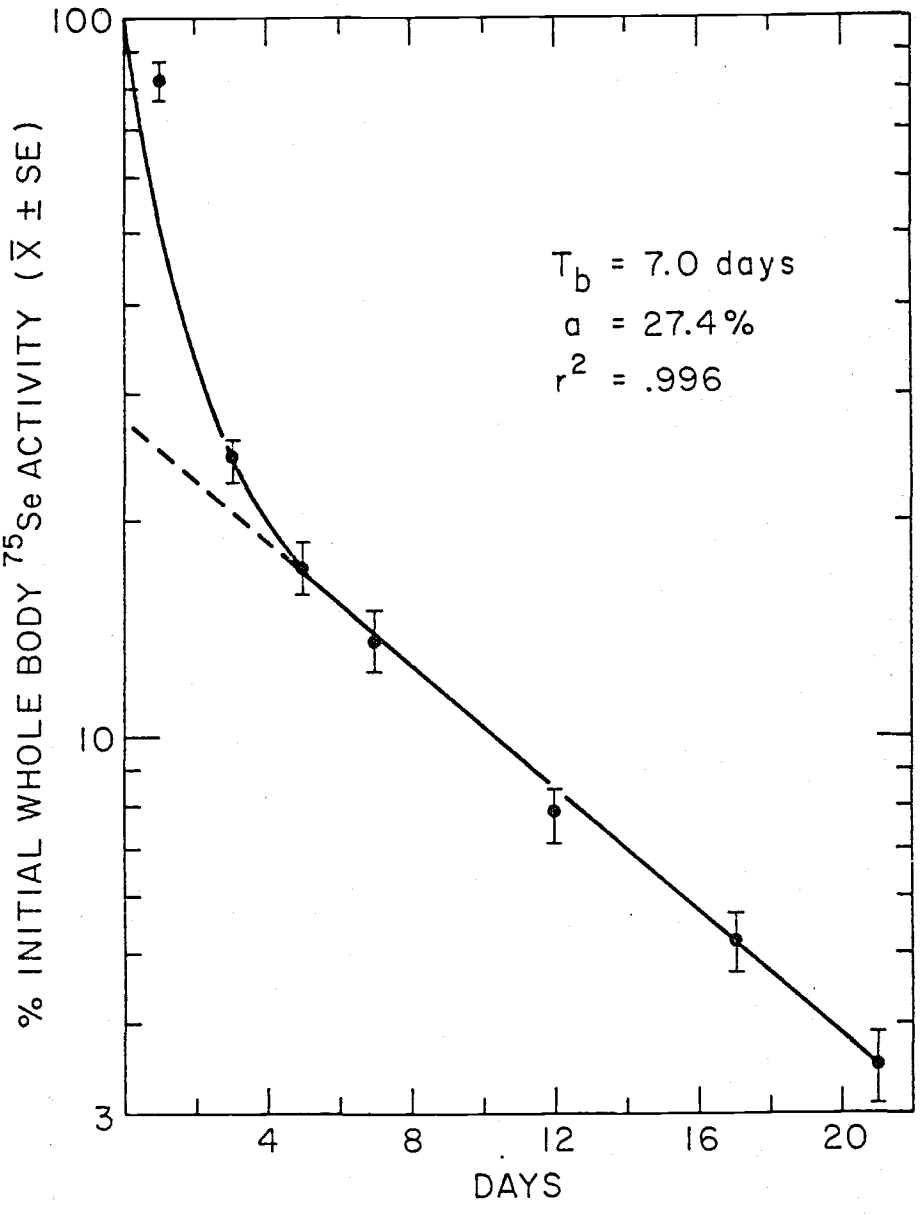

Figure 1. Percent initial whole body ^{75}Se activity in male voles for 21 days following oral administration (N = 5).

lag between oral administration of the isotope and maximum absorption by the gut. By tracing a smooth curve from the 100% value on the Y axis to the day 3 retention value, the biological half-life for this first compartment can be seen to approximate 1 day.

Three-Week Tissue Retention of Selenium-75

Results of the three-week radioassay of selected tissues are reported in Table 2. Testes exhibited the greatest mean concentration of administered ^{75}Se. Testicular retention per gram was not significantly different from kidney retention. A significant difference ($P < .05$) was found between testicular retention and mean retention per gram of the other selected tissues. With the exception of the brain, all tissues assayed showed a statistically significant concentration of radioselenite ($P < .05$) with respect to the whole body. The rank order of tissue concentration (highest to lowest) of radioselenium agreed well with tissue selenium concentration of live-trapped males (Table 1) with the exception of the heart and brain in which the order was reversed.

Three-Week Tissue Retention of Selenium-75: Serial Sacrifice

The tissue retention values are reported in Figures 2 and 3, and appendix Table 2. In general, whole body retention, and retention by the liver and kidneys exhibited peak values at day 1, followed by a persistent decline at later sacrifice dates. The

Table 2. Selenium-75 retention for whole body and selected tissues of male voles at 3 weeks post-administration (N = 5).

Organ	% Initial whole-body retention per gram fresh weight $\bar{x} \pm SE$
Testes	0.62 ± .07
Kidneys	0.48 ± .03
Liver	0.34 ± .06
Heart	0.24 ± .03
Brain	0.12 ± .04
Whole body	0.14 ± .02

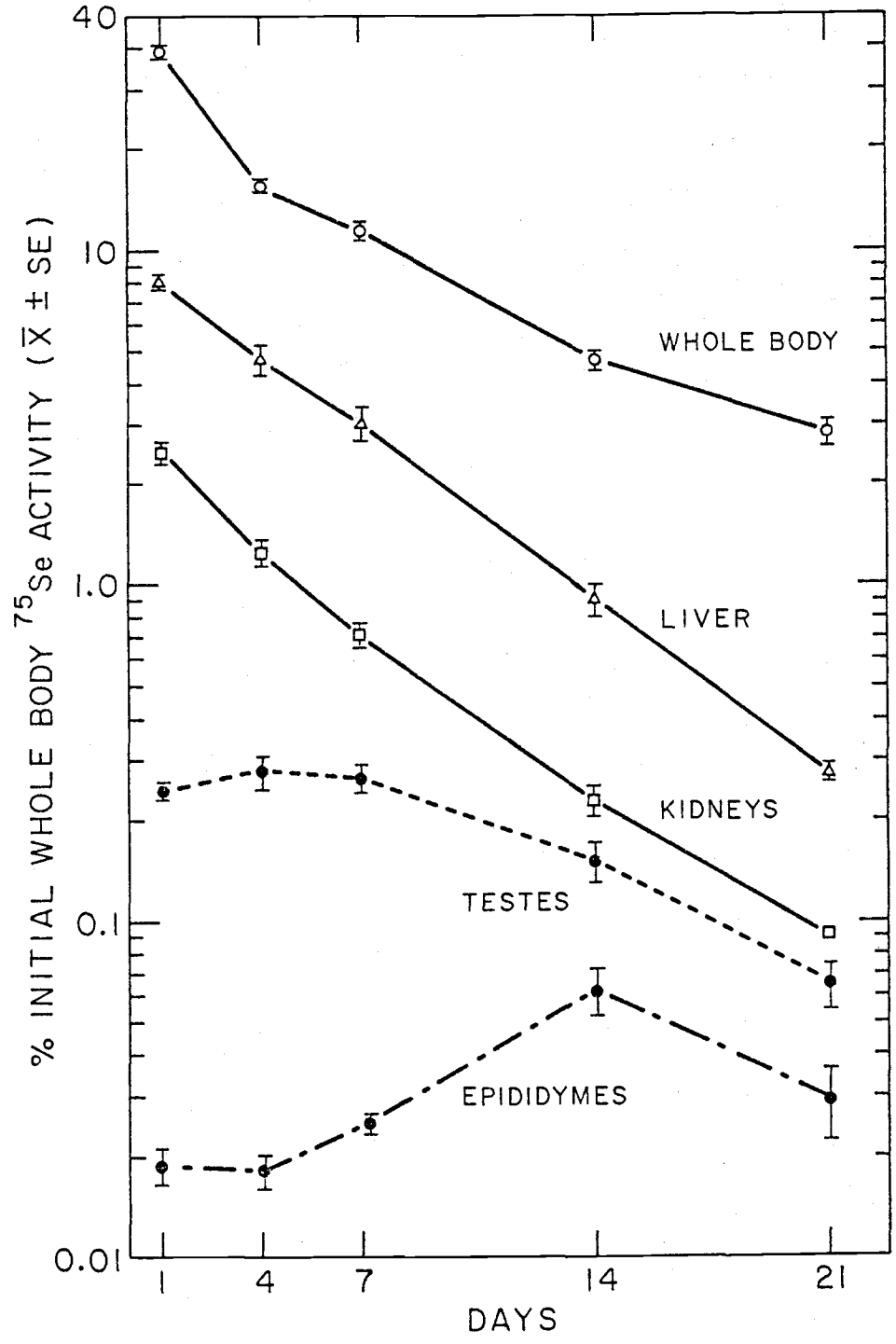

Figure 2. Percent initial whole body ^{75}Se activity for whole body and selected tissues of male voles for 21 days following oral administration: serial sacrifice (N = 5).

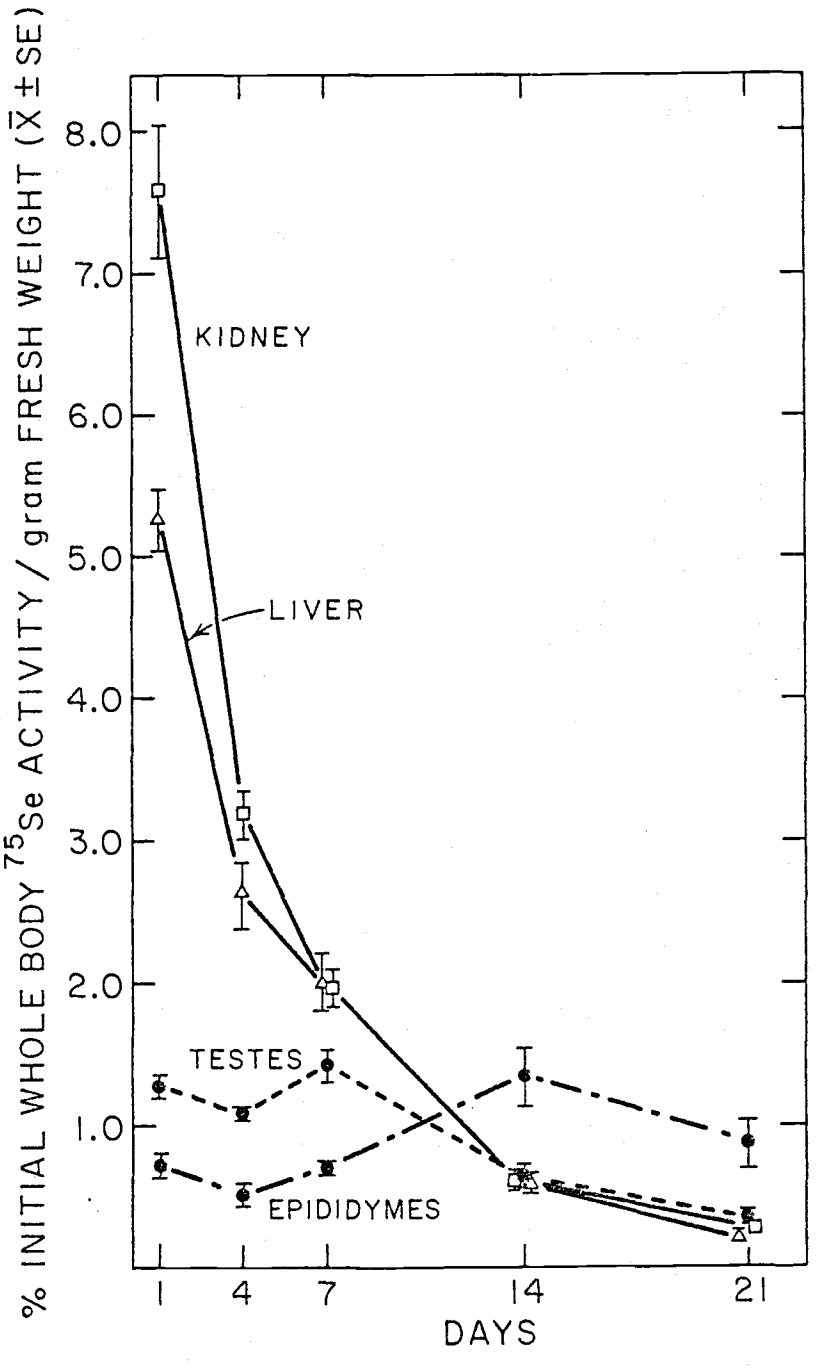

Figure 3. Percent initial whole body ^{75}Se activity per gram fresh weight for selected tissues of male voles for 21 days following oral administration: serial sacrifice (N = 5).

peak retention value for the testes and epididymes was delayed. Eventual decline in ^{75}Se content in the testes was accompanied by a significant rise in epididymal concentration of ^{75}Se. The tissue retention patterns on a per gram basis are similar but not identical to retention patterns based simply on mean percent of initial whole body activity. For the testes, the peak value is at 4 days PA on a whole body basis. On a per gram basis, the testicular levels of ^{75}Se declined from day 1 to day 4, and are at a miximum on day 7 PA. It should be noted, however, that consideration of retention on either basis resulted in no statistically significant differences between ^{75}Se retention for the first three sacrifice dates.

Whether or not retention was considered on a per gram basis, the peak retention value for the epididymis was at the two-week sacrifice point. On a per gram basis, the two-week retention value was significantly higher ($P < .05$) than the four-day retention value, but not significantly different from the one-day retention value. When not considered on a per gram basis, both the day 1 and day 4 retention values were significantly lower ($P < .05$) than the two-week retention value. With one exception, whole body retention, as well as tissue retention values, showed statistically significant progressive declines ($P < .05$) from the day 1 sacrifice retention values. This was the case whether or not retention was considered on a per gram basis. The liver showed no significant decline between day 4 and day 7 when considered on a per gram basis.

Figure 3 illustrates that the highest tissue concentration at three-weeks PA was in the epididymes. This concentration was

significantly higher ($P < .05$) than the other three tissues assayed. The second highest concentration of ^{75}Se at three weeks PA was in the testes. In contrast to the previous three-week retention study, testicular levels were significantly greater ($P < .05$) than both the liver and the kidneys.

Sulfite/Selenite Challenge Following ^{75}Se Administration

The results of the selenite/sulfite challenge study are reported in Figure 4 and appendix Table 3. In terms of whole body retention, there was a statistically significant decrease ($P < .05$) in retention when a sodium selenite challenge was given at three days PA. There was no significant decrease in whole body retention when a sodium sulfite challenge was given following ^{75}Se administration. This was the case whether or not retention was considered on a per gram basis.

For the testes, the same pattern of response with respect to the sulfite/selenite challenge was observed. Selenite significantly ($P < .05$) reduced the subsequent retention of previously administered radioselenite, while sulfite had no significant effect.

The epididymes exhibited a unique response to the sulfite/selenite challenge. Neither challenge resulted in a statistically significant decline in subsequent retention of previously administered radioselenite.

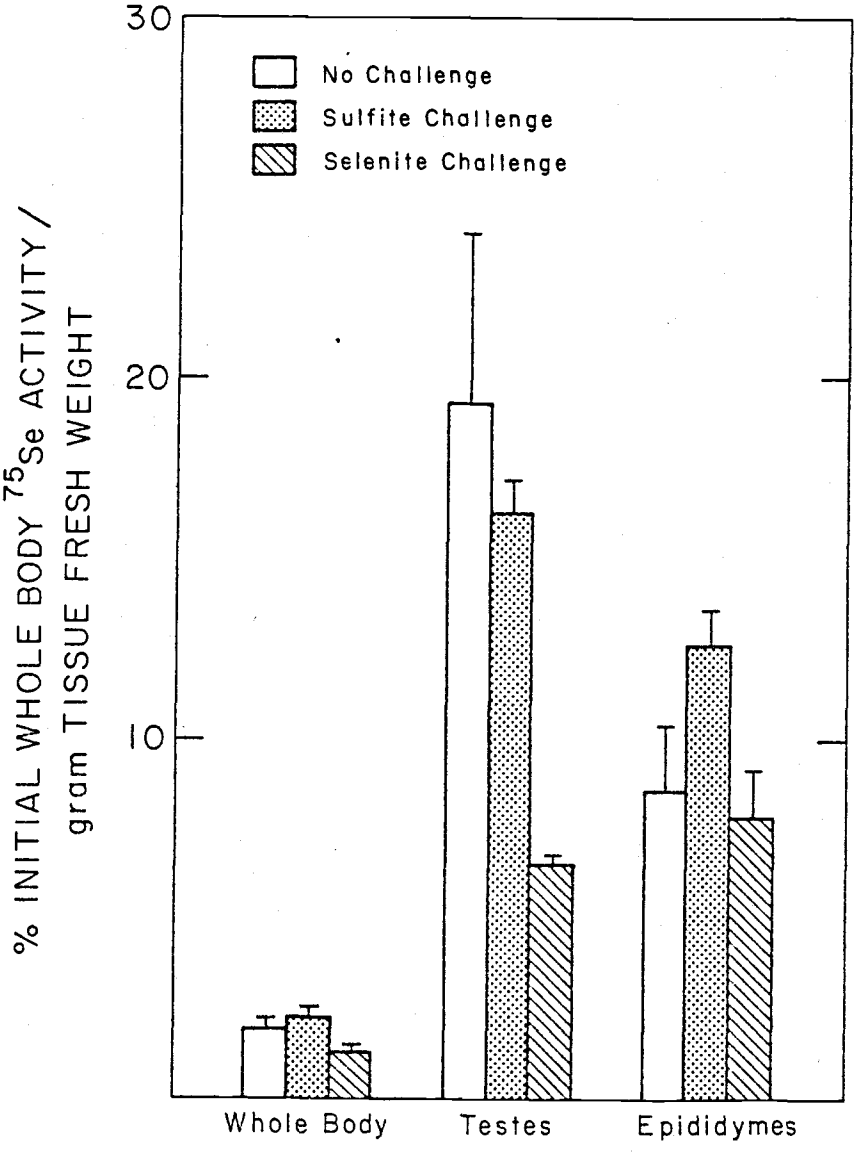

Figure 4. Effect of sulfite and selenite challenge on 2-week retention of previously administered ^{75}Se (N = 5).

Intracellular Distribution of Selenium-75 in Sperm

The intracellular distribution of ^{75}Se in sperm is reported in Figure 5 and appendix Table 4. Whether or not vitamin E was included in the diet, the relative order of intracellular concentration remained the same. The combined nuclear and mitochondrial compartments accounted for over 90% of the total ^{75}Se activity in both cases.

The vitamin E supplemented group exhibited relatively less selenium in the nucleus, and more in the mitochondria than did the unsupplemented group. Also, the fraction of selenium in the cytosol of the unsupplemented group was greater by a factor of 1.4 than in the supplemented group. Little difference between the two groups was observed with respect to the microsomal fraction.

Autoradiography

Sperm autoradiographs are shown in Figures 6-10. Figure 6 shows a longitudinal section through a developing spermatid. The dense, patchy distribution of grains indicates the concentration pattern of ^{75}Se in the nucleus. There appears to be no regular pattern of deposition within the nucleus. Very little concentration of grains is seen at other locations in the tissue sample.

In Figure 7, relatively heavy selenium deposition is again seen in the sperm nuclear region. Unlike the testicular spermatid nucleus, however, a significant deposition of grains is associated with the nuclear membrane (arrow b) in epididymal sperm.

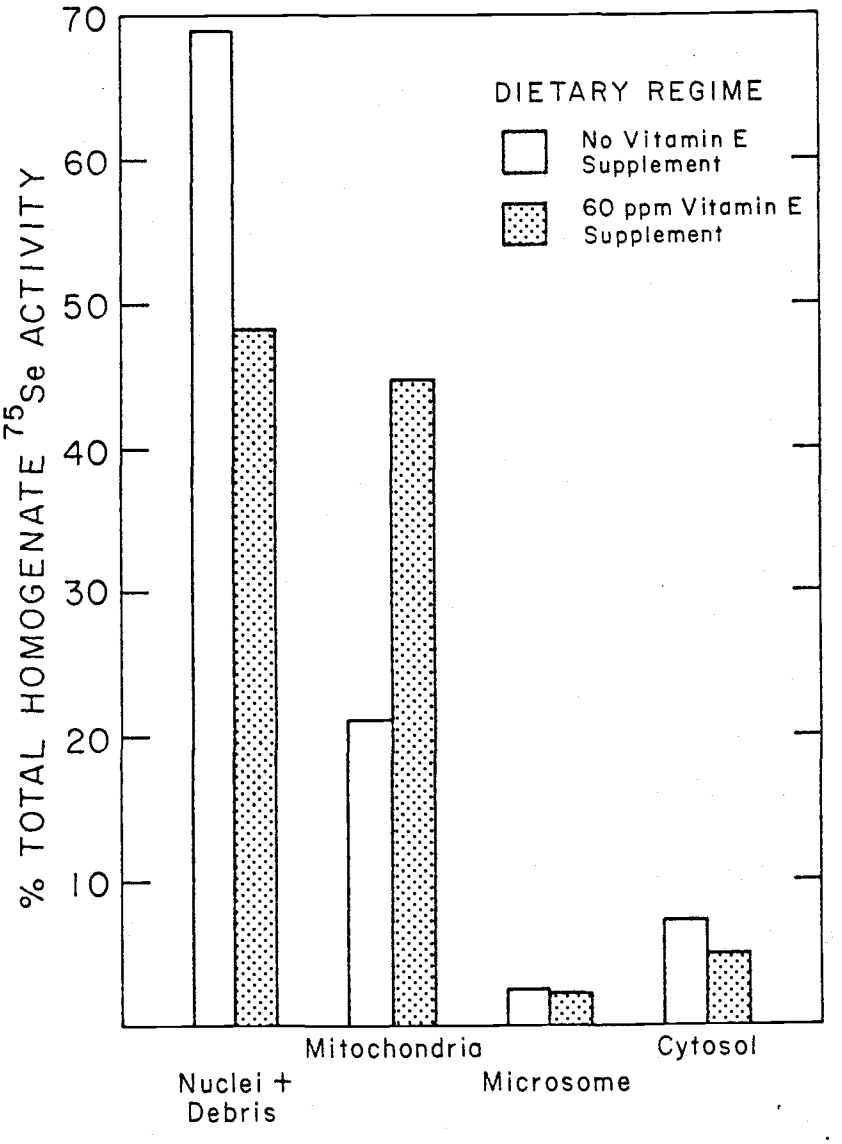

Figure 5. Intracellular distribution of orally-administered ^{75}Se in spermatozoa of voles under two different dietary regimes of vitamin E (composite sample from four animals).

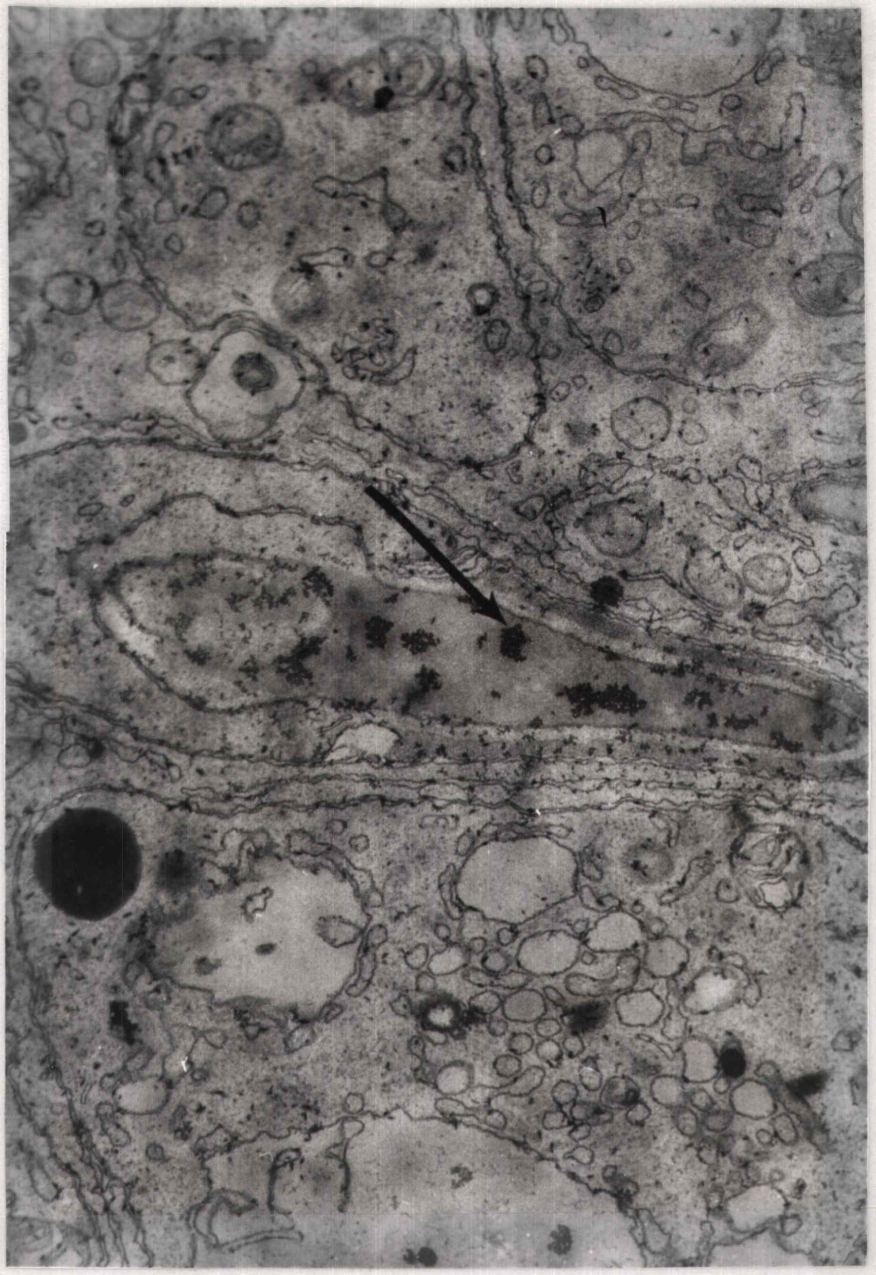

Figure 6. Tissue sample from testis--longitudinal section through a spermatid (28,800 X).

Figure 7. Tissue sample from epididymis showing longitudinal section through sperm heads (28,800 X).

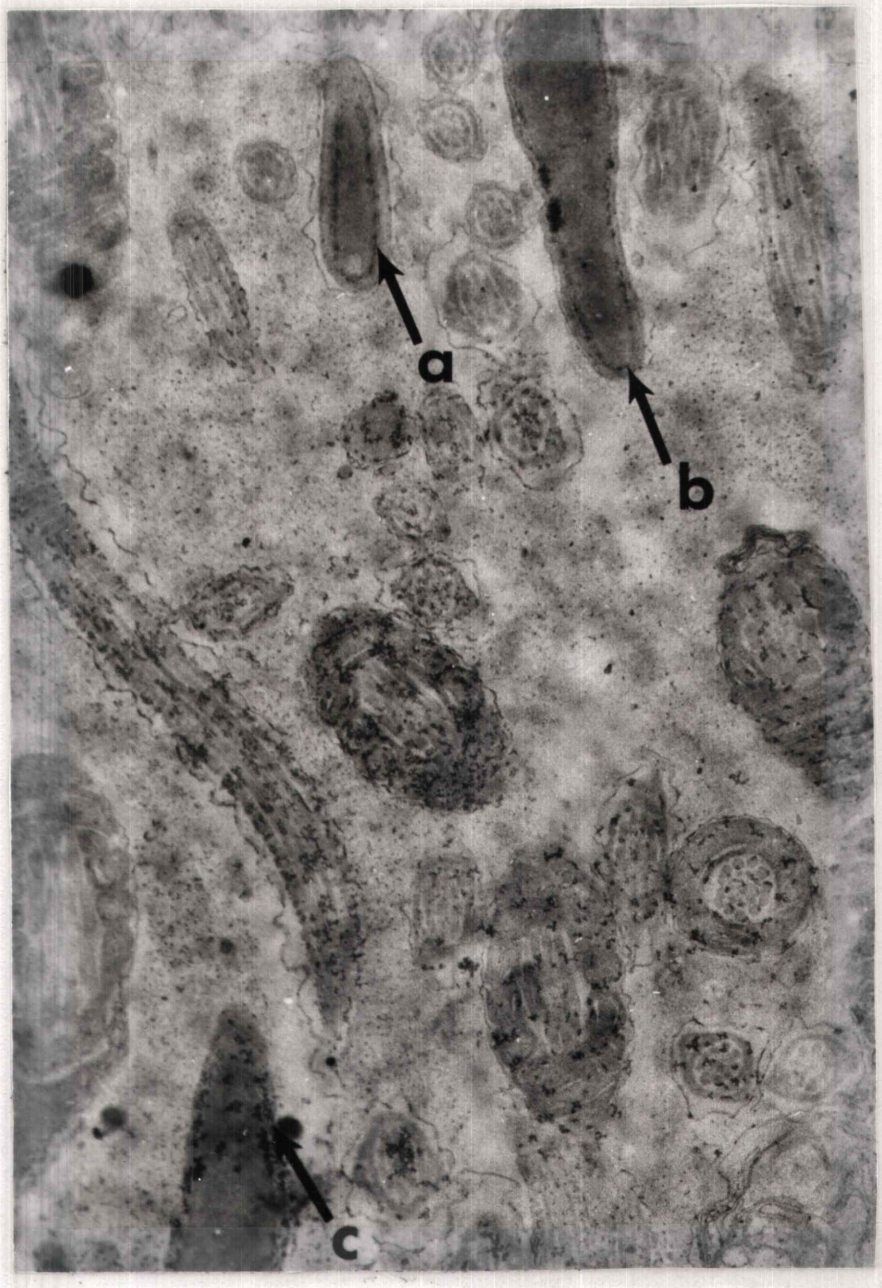

Figure 8. Tissue sample from epididymis showing longitudinal section through sperm heads (22,200 X).

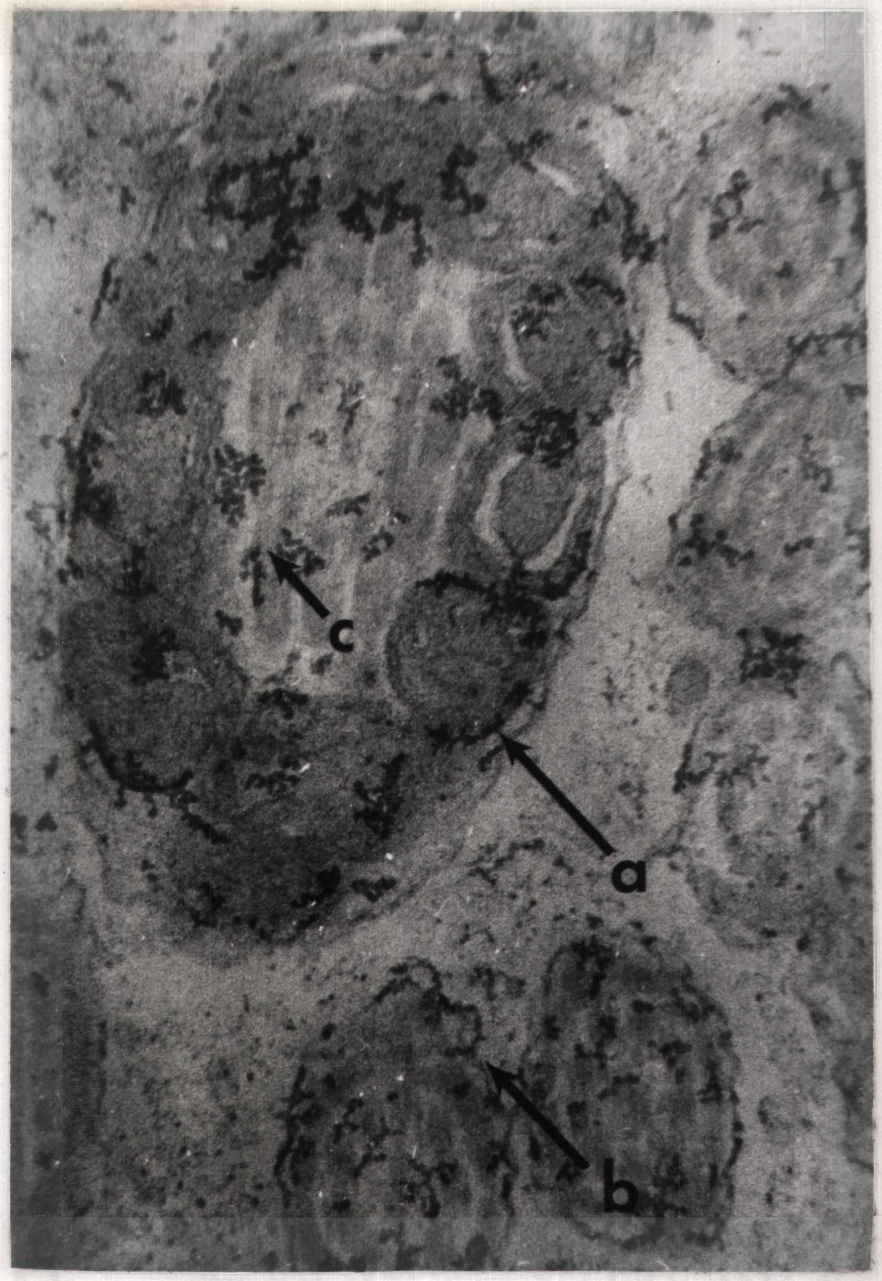

Figure 9. Tissue sample from epididymis showing transverse section through sperm midpiece (114,100 X).

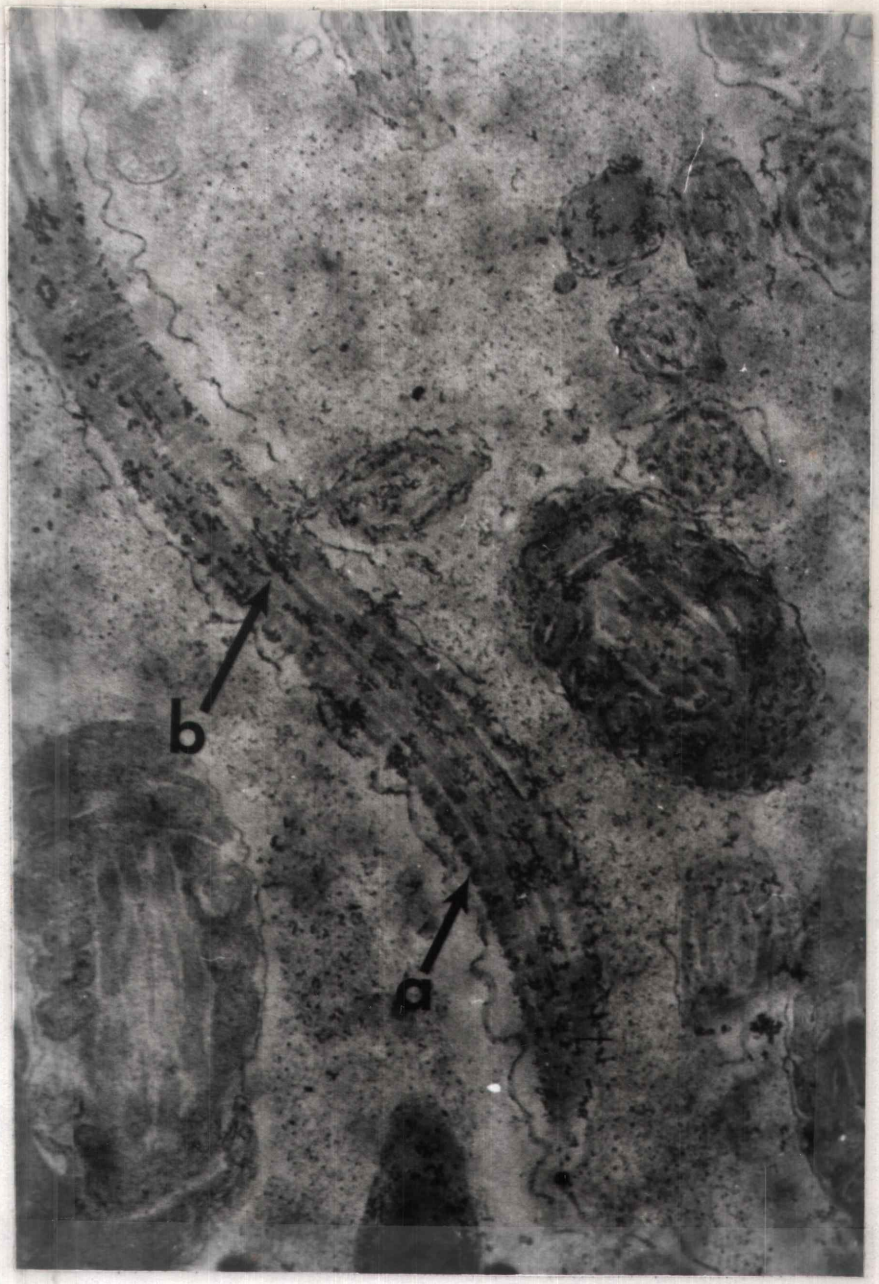

Figure 10. Tissue sample from epididymis showing longitudinal section through sperm tail (83,100 X).

The three arrows in Figure 8 point to additional longitudinal sections through sperm heads. Note the very discrete deposition of grains around the nuclear membrane (arrows a and b). No deposition of grains is apparent within the nucleus of these two specimens. There does appear to be some patchy nuclear distribution in specimen c. Again, a very significant association of grains with the nuclear membrane is evident.

Figure 9 shows a number of transverse sections of sperm midpieces. Arrows a and b point to the preponderant association of grains with the outer mitochondrial membranes. Arrow c points to the less conspicuous deposition of grains apparently associated with the dense fibers surrounding the axial filament complex.

Figure 10 shows a longitudinal section through a sperm tail. Arrow a points to the significant association of grains with the fibrous sheath (a membrane surrounding the dense fibers in the tail). Arrow b points to the association of grains with the dense fibers and axial filament complex.

Effects of Selenium and Vitamin E on Sperm Structure and Function

The effects of selenium and vitamin E on sperm function and other physiological parameters are summarized in Table 3. Generally, the laboratory controls and the live-trapped animals were very similar with respect to the physiological parameters measured. This was somewhat unexpected, since the ages, diets and environments of the two groups were different. The laboratory

Table 3. The effect of selenium and vitamin E on growth, testicular development and function, and sperm integrity.

		A	B	C	D	E	F	G	H	I
Baseline groups vitamin E unknown	LA 0.10 ppm (N = 7)	43.48 ± 2.79	---	0.27 ± .01	0.63 ± .04	0.26	100	45.4 ± 4.1	0.94 ± .07	4.00 ± .94
	FA 0.03 ppm (N = 6)	35.56 ± 0.84	---	0.29 ± .01	0.79 ± .03	0.24	100	45.9 ± 8.8	0.93 ± .05	4.00 ± .70
Dietary selenium gradient no vitamin E supplement	TY 0.02 ppm (N = 5)	26.61 ± 1.38	0.18 ± .02	0.12 ± .03	0.42 ± .08	0.24	100	29.6 ± 9.6	0.66 ± .13	13.90 ± 2.70
	TY 0.28 ppm (N = 6)	28.31 ± 2.59	0.17 ± .02	0.18 ± .07	0.60 ± .12	0.32	83.3	29.6 ± 9.3	0.77 ± .20	9.70 ± 1.50
	TY 0.58 ppm (N = 8)	34.65 ± 1.98	0.33 ± .03	0.25 ± .01	0.73 ± .04	0.33	100	42.7 ± 5.7	0.86 ± .05	5.20 ± 1.20
Dietary selenium gradient 60 ppm vitamin E	TY 0.02 ppm (N = 6)	28.07 ± 2.59	0.18 ± .04	0.24 ± .04	0.78 ± .09	0.17	100	41.7 ± 4.5	0.84 ± .09	10.10 ± 1.50
	TY 0.04 ppm (N = 5)	30.33 ± 1.09	0.23 ± .03	0.22 ± .03	0.73 ± .09	0.42	100	51.5 ± 4.3	1.02 ± .08	6.20 ± 1.00
	TY 0.09 ppm (N = 6)	29.00 ± 1.06	0.22 ± .03	0.12 ± .03	0.39 ± .08	0.30	66.7	34.8 ± 5.7*	0.98 ± .06*	5.20 ± 0.40*
	TY 0.26 ppm (N = 6)	25.30 ± 0.70	0.10 ± .03	0.10 ± .03	0.26 ± .09	0.27	33.3	33.3 ± 26.3**	1.04 ± .18**	6.40 ± 0.70**
	TY 0.64 ppm (N = 8)	37.00 ± 2.60	0.34 ± .04	0.23 ± .02	0.64 ± .05	0.36	100	48.8 ± 3.0	0.91 ± .05	5.90 ± 0.80

Physiological Parameters
A) sacrifice weight $\bar{x}$ ± SE
B) daily weight gain $\bar{x}$ ± SE
C) testes weight at sacrifice (fresh weight) $\bar{x}$ ± SE
D) percent testes weight of body weight $\bar{x}$ ± SE
E) ppm selenium in the testes from composite sample (fresh weight)
F) percent of animals producing sperm
G) percent live sperm $\bar{x}$ ± SE
H) relative sperm motility $\bar{x}$ ± SE
I) percent abnormal sperm $\bar{x}$ ± SE

Dietary Parameters
A) LA - laboratory animals (rat chow + rabbit chow)
B) FA - field animals (natural dietary regime)
C) TY - torula yeast diet

* N = 4
** N = 2

controls were significantly larger ($P < .05$) than the wild-caught voles, and they exhibited significantly smaller testes size ($P < .05$) in relation to body weight.

With no vitamin E supplement, the addition of 0.56 ppm selenium to the diet resulted in a significant increase ($P < .05$) in body weight, daily weight gain, testes weight, and testes weight relative to body weight. There was a substantial increase in testicular selenium (a factor of 1.33) when 0.26 ppm was added, but a further increase in dietary selenium seemed to have little effect. There seemed to be no relation between dietary selenium and percent of the animals producing sperm. The addition of 0.26 ppm selenium seemed to have no effect on percent live sperm. The addition of 0.56 ppm selenium to the diet increased the percentage of live sperm, but the increase was not significant. There appeared to be a trend of increasing sperm motility with increases in dietary selenium, but again, no statistical significance was associated with these increases. With respect to the low selenium group, the addition of 0.56 ppm selenium resulted in a significant decrease ($P < .05$) in the percentage of morphologically abnormal sperm.

In general, dietary increases in selenium produced less dramatic results when a supplement of 60 ppm vitamin E was included in the diet. There seemed to be no general trend in sacrifice weights; however, there was a significant increase ($P < .05$) in body weight between the 0.26 ppm group and the 0.64 ppm group. A significant increase ($P < .05$) was also found between these two groups with respect to daily weight gain. No other major trends were noted

concerning this parameter. There appeared to be no well-established trends in testes weight or percent testes weight relative to body weight with respect to increasing dietary selenium. The addition of 0.02 ppm selenium to the diet increased the testicular concentration of selenium by a factor of 2.5. This appeared to reflect the shoulder of a plateau, however, as further increases in dietary selenium did not increase testicular selenium.

As in the vitamin E unsupplemented groups, dietary selenium appeared to bear no relation to the percentage of animals producing sperm. No significant trends were exhibited either in percent live sperm, or sperm motility, as a function of dietary selenium. Although the addition of 0.02 ppm selenium to the diet reduced the percentage of abnormal sperm the difference was not significant. Further increases in dietary selenium appeared to have no appreciable effect.

Several notable contrasts are seen between the TY group with no added selenium or vitamin E, and the TY group with only vitamin E added. No significant differences were found in sacrifice weight or daily weight gain between the two groups. While the testes in the vitamin E-supplemented group were larger by a factor of two, variability within the respective groups rendered this difference statistically insignificant. There was, however, a significant increase ($P < .05$) in testes weight relative to body weight when vitamin E was added. Interestingly, the addition of vitamin E appeared to reduce by a factor of one-third the testicular concentration of selenium. While the addition of vitamin E seemed to

increase the percentage of live sperm, increase sperm motility, and reduce the percentage of morphological abnormalities, none of these differences were significant.

It is worthwhile to note that the two TY groups fed the highest level of selenium (with and without the vitamin E supplement) were similar to the baseline groups with respect to the parameters. These groups would seem to represent voles in relative good health.

Some additional insight can be gained into these dietary and physiological interrelationships by examining the respective correlation coefficients. The r^2 values for the measured physiological parameters are reported in Table 4. In general, most of the correlation coefficients were low. This was somewhat expected for two major reasons. First, if voles are adapted to a low dietary regime of selenium, additional selenium in the diet may have only a marginal effect. Secondly, the nature of biological systems is such that the performance of any one subsystem (e.g., reproductive system) is some function of the interactive performances of a number of other subsystems. That is, the variability in one parameter is never totally attributable to variation in any other one measured parameter. In this context, low correlation coefficients can be dealt with in a comparative fashion to elucidate the relative importance of relationships among parameters.

With respect to dietary selenium, by far the highest correlation with sperm was with percent abnormal sperm. Dietary selenium could account for 46.0% of the variation in this parameter.

Table 4. Correlation coefficient (r^2) values for measured dietary and physiological parameters.

		1	2	3	4	5	6	7	8	9
0		A .364 B .280	A .471 B .318	A .382 B .023	A .225 B .004	A .787 B .174	A .004 B .007	A .102 B .029	A .114 B .000	A .460 B .069
1			A .780 B .845	A .626 B .293	A .210 B .024	A .231 B .162	A .179 B .116	A .022 B .043	A .000 B .005	A .229 B .056
2				A .552 B .180	A .218 B .004	A .235 B .203	A .138 B .064	A .051 B .090	A .089 B .000	A .176 B .054
3					A .806 B .819	A .298 B .036	A .294 B .491	A .069 B .106	A .378 B .010	A .308 B .006
4						A .187 B .003	A .356 B .493	A .076 B .054	A .058 B .001	A .224 B .005
5							A B	A .049 B .084	A .052 B .048	A .378 B .207
6								--	--	--
7									A .336 B .135	A .483 B .279
8										A .051 B .325
9										

0 - Dietary Se
1 - Sacrifice weight
2 - Daily weight gain
3 - Testes weight
4 - % Testes weight of body weight
5 - ppm Se in testes
6 - % animals producing sperm
7 - % live sperm
8 - Sperm motility
9 - Percent abnormal sperm

A - No vitamin E supplement
B - 60 ppm vitamin E supplement

Interestingly, this was reduced to 6.9% in the presence of vitamin E. There seemed to be essentially no relationship between dietary selenium and the other sperm parameters. With respect to testicular function, dietary selenium could account for 78.7% of the variation in testicular selenium levels. This was reduced to 17.4% in the presence of vitamin E. Dietary selenium accounted for 38.2% of the variation in testes weight. This was reduced to 2.3% in the presence of vitamin E. There seemed to be some correlation between selenium and body weight. This correlation was also reduced when vitamin E was present.

Testes weight was more strongly correlated with body weight (.626) than with either dietary selenium (.382) or ppm selenium in the testes (.298). When vitamin E was added to the diet, all of these correlations were substantially reduced.

Next to its correlation with dietary selenium (.787), selenium concentration in the testes was most strongly correlated with percent abnormal sperm (.378). Again, when vitamin E was added to the diet, the correlations were reduced.

The percentage of animals producing sperm was most strongly related to percent testes weight of body weight (.493), and testes weight (.492), when vitamin E was added to the diet. When vitamin E was removed, these correlations were reduced to .356 and .294, respectively.

The percentage of live sperm could account for 48.3% of the variability in percentage of abnormal sperm. This was reduced to 29.3% in the presence of vitamin E.

Relative sperm motility was slightly correlated with percent abnormal sperm (.325) in the presence of vitamin E. This was reduced to .051 when vitamin E was absent from the diet. With no vitamin E added to the diet, testes size accounted for 37.8% of the variability in sperm motility. This was reduced to 1.0% in the presence of vitamin E.

Selected photographs of some of the observed sperm abnormalities are exhibited in Figures 11-13. Figure 11 shows two of the most commonly observed abnormalities. By far the most commonly observed abnormality was a missing head. These seemed to bear no relationship to selenium or vitamin E. The unusual conformation seen in the lower photograph predominated in animals with no selenium or vitamin E supplement. Some of the specimens were observed swimming in this conformation.

Figure 12 illustrates less commonly observed abnormalities of the principal piece of the tail. Although these photographs were from unsupplemented animals, abnormalities such as these occurred in all dietary groups.

Figure 13 illustrates two very uncommonly observed abnormalities. The upper photo shows apparent dissolution of the sperm head. The lower photo shows extensive fibrous protrusion, analogous to the abnormalities observed in selenium-deficient rats.

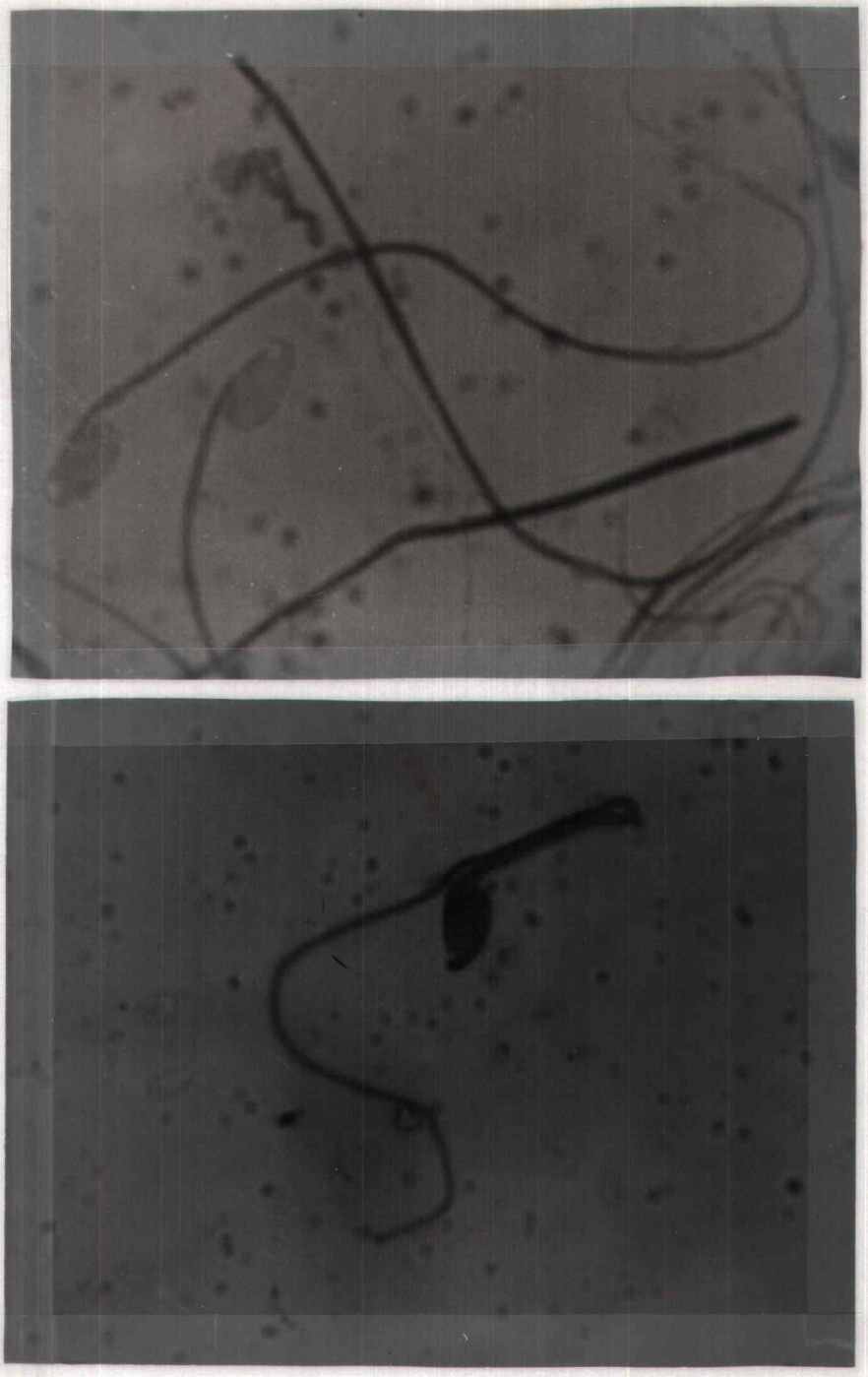

Figure 11. Vole sperm abnormalities. The upper photograph is from an animal supplemented with 0.26 ppm Se and no vitamin E. Note the two normal appearing sperm and the two sperm without heads. The lower photograph is from an animal with no Se or vitamin E supplement. Note that the head and midpiece are bent 180° in the direction of the tail.

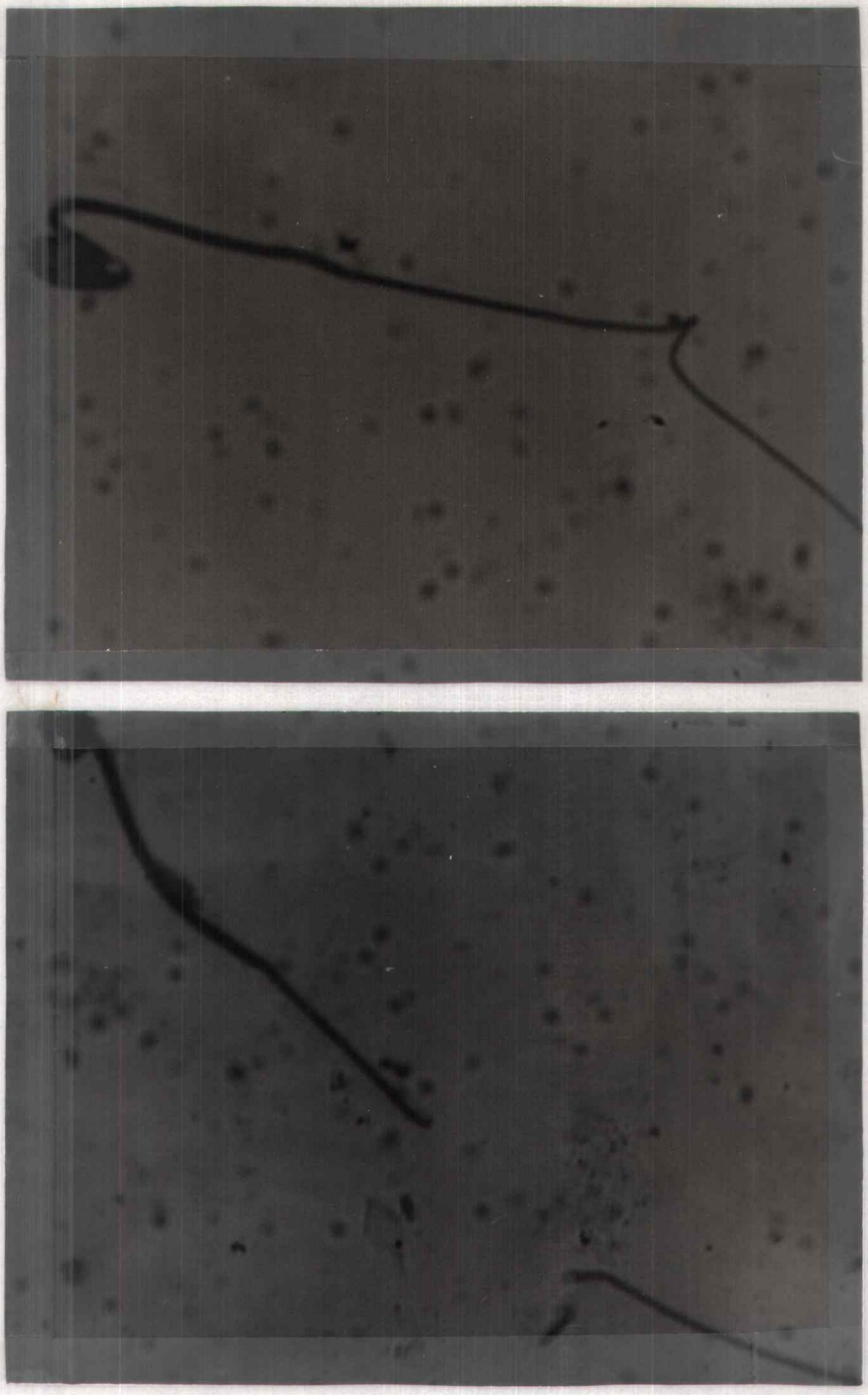

Figure 12. Vole sperm abnormalities. These two photographs depict typically observed abnormalities associated with the principal piece of the tail. Neither animal received a vitamin E or selenium supplement.

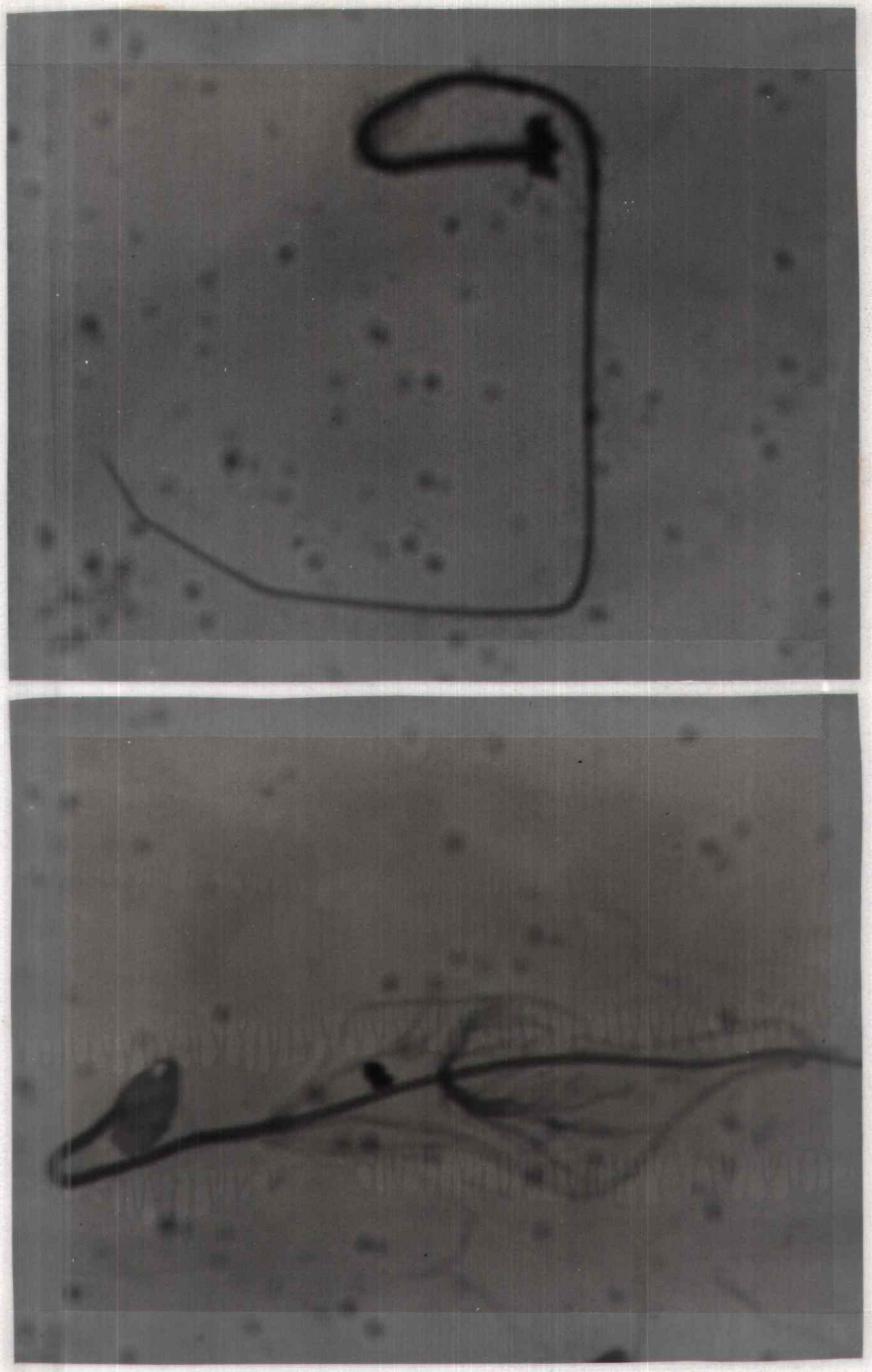

Figure 13. Vole sperm abnormalities. Two of the more uncommonly observed abnormalities are represented in these two photographs. The upper photograph is a specimen with part of the head missing. The lower photograph shows extensive protrusion of dense fibers and/or axial filaments from the main body of the tail. Neither received selenium or vitamin E supplements in the diet.

Effects of Selenium on Reproductive Performance in Male Voles

The results of the first male fertility experiment is reported in Table 5. In general, there appeared to be no significant difference in reproductive performance between the two groups. A greater percentage of females were pregnant in the high selenium group at the termination of the experiment. Females mated to males on the low selenium diet, however, gave birth one day earlier than the first females in the high selenium group. Up to day 30 the cumulative percent parturitions in the low selenium group was greater than or equal to the high selenium group.

For the second reproductive experiment, there were only nine intact breeding pairs at the end of the experimental period for the high selenium group. None of the females were pregnant in this group at the termination of the experiment. There were 11 surviving breeding pairs in the low selenium group. One female gave birth to four offspring in this group. Again, the results do not point to a well-defined difference in reproductive performance between the two greatment groups of males.

Table 5. Cumulative percent breeding success of male voles as a function of dietary selenium from 4 weeks prior to mating

Days post mating	0.02 ppm selenium (Basal level) (N = 15)	0.26 ppm selenium (N = 13)
20	0	0
21	0	0
22	0	0
23	7.7	0
24	15.4	13.3
25	15.4	13.3
26	23.1	13.3
27	23.1	13.3
28	30.1	13.3
29	30.1	26.7
30	30.1	26.7
31	30.1	33.3
32	30.1	33.3
33	30.1	33.3
34	30.1	33.3
35	30.1	33.3

DISCUSSION

Tissue Selenium Levels in Field-Collected Male Voles

The rank order of tissue concentration of selenium for the field-collected animals agreed fairly well with Pond's (1977) data. Pond's experimental animals, reared on TY diets containing variable concentrations of selenium and vitamin E, exhibited the following declining order of tissue concentration of selenium: kidney, liver, testes, brain, and heart. This is similar, but not identical, to the field-collected animals in the present study, where testicular selenium concentrations were higher than other tissues sampled. It should be noted, however, that the testes ranked first or second in order of concentration in Pond's animals where selenium concentrations in the diet were 0.1 ppm or less.

In examining testicular selenium levels as a function of dietary selenium, Table 4 bears further scrutiny. Where no selenium supplement was added to the TY diets (with or without vitamin E) testicular selenium levels were at their lowest. While moderate supplementation appeared to increase testicular levels, further supplementation did not increase testicular selenium appreciably. This suggests that once the physiological requirements for selenium in the testes are met, surplus is shunted to other compartments and/or eliminated from the body as required. This might also partially explain the observation that the testes drop in rank order of tissue selenium concentration when diets containing higher

levels of selenium are administered.

Although M. canicaudus is known to breed throughout the year, Pearson (1972) noted seasonal fluctuations in activity (based on trapping success) which were attributed partially to breeding activity. In this regard, a distinct yearly cycle of ^{65}Zn activity in the dorso-lateral prostate gland has been noted in the male laboratory rat (Gunn and Gould, 1958). Further research may well document similar cyclic activity in the testis-epididymis complex associated with reproductive rhythms. If selenium is essential for spermatogenesis (as is zinc), a yearly cycle of selenium activity in vole reproductive tissues may exist. In this context, the high selenium levels (with respect to other organs) noted here may partially reflect a seasonal peak in breeding activity. A more extensive field investigation would be needed to determine if such a yearly cycle exists in this species.

It is perplexing that the stomach contents of field-collected voles exhibited a significantly higher selenium level than the sampled vegetation. Ganther et al. (1976) reported that the stomach mucosa of rats contains a moderate concentration of selenium. It is possible that some of the stomach mucosa were removed along with the stomach contents. Perhaps the most attractive explanation would entail the existence of selective foraging mechanisms in this species to maximize selenium intake. A major problem with this interpretation, however, is that available data (Carter et al., 1968) indicate only a one to twofold difference in selenium concentration in vegetation growing in low selenium areas. A more

thorough investigation is merited to elaborate on these findings.

Whole Body Retention of Selenium-75

The whole body retention pattern for voles is qualitatively similar to data from laboratory rats and mice. Blincoe (1960), Ewan et al. (1967), and Thompson and Stewart (1973) found that two rate constants governed selenium turnover in the rat. In a more recent study, Furchner et al. (1975) determined that long-term selenium turnover in rats and mice could be described by the sum of three rate constants.

Most of the work with rats indicated a longer biological half-life for both components, than was found to be the case for voles. Furchner et al. (1975) determined, however, that within limits the biological turnover rate of ^{75}Se was related to body size. Burk et al. (1972) found that whole body retention in rats was also affected by dietary selenium levels prior to ^{75}Se administration, as well as the magnitude of the ^{75}Se dose. An increase in either of these variables decreased whole body retention time in rats.

Blincoe (1960) has postulated that the two rate constants may reflect the renal clearance of two chemically different forms of selenium. Alternatively, he suggested that the longer component represented turnover of selenium in tissues in which it was metabolically bound. Burk et al. (1972) and Thompson and Stewart (1973) attributed much of the rapid initial loss of selenium to urinary excretion. They also determined that 91-93% of orally-administered ^{75}Se (as selenite) was absorbed by the gut. Imbach

and Sternberg (1967) found that following GI absorption of administered ^{75}Se in the rat, much of it was initially bound to plasma proteins in the blood. It was speculated that these proteins act as initial carriers of the trace element.

Based on these studies, the whole body retention values given here for voles would only seem to be relevant to the specific dietary regime and other experimental conditions employed. In similar work done earlier in this laboratory using low selenium TY diets, over 50% of the administered dose was retained after 14 days. This suggests that this species may possess mechanisms to regulate the retention of dietary selenium based on current physiological need.

As a working hypothesis, it would seem reasonable to assume that the rapid component in vole's retention patterns represent the initial excretion of unbound selenium, while the slow component represents turnover of organically incorporated selenium.

Retention of Selenium-75 in the Testis-Epididymis Complex and Other Selected Tissues

The major reason for inclusion of non-reproductive tissues in the present retention studies was for comparative purposes. Ganther et al. (1976) has reviewed recent data which suggests that the mammalian kidney and liver contain much higher concentrations of GSH-Px than does the testis. Furthermore, the available data for mice and rats (Gunn et al., 1967; Gunn and Gould, 1970) indicate that the testis has a retention pattern significantly different from

the aforementioned two organs. Gunn and Gould (1970) stated that surgical interruption of the sperm-transport pathway in rats results in no observable peak concentrations of ^{75}Se in the epididymis. This would seem to indicate spermatogenic incorporation of selenium by the rat. That the testis-epididymis accumulation pattern in voles is similar to data from mice and rats suggests that selenium is incorporated into the spermatogenic pathway in M. canicaudus.

Figure 3 indicates a decrease (although not statistically significant) in ^{75}Se levels in the testes and epididymes between days 1 and 4 PA. The initial high levels of selenium in these two organs might be accounted for by blood plasma-bound ^{75}Se most of which is rapidly eliminated from the body. It should be noted that the increases in testicular retention between days 1 and 7 (or days 4 and 7) PA are not significant at the 95% confidence level. Testicular retention can, however, be contrasted with the significant decreases between days 1 and 7 for other organs assayed. Most similar studies done with laboratory rodents have employed TY diets to deplete the animals and subsequently accentuate the uptake curves. Earlier studies in this laboratory utilizing TY diets for this purpose revealed that pre-depletion of voles prior to ^{75}Se administration also enhanced the variability between individuals with respect to uptake and retention of selenium. This increased variability, probably a reflection of individual differences between animals, negated the effect of increased testicular uptake relative to statistical significance.

The pattern of uptake and retention in the vole testis-epididymis complex merits attention not only because of its uniqueness but because of its magnitude. In the initial three-week retention study, the testes were found to retain the highest concentration of selenium for the tissues sampled. In the three-week serial sacrifice study which followed, the epididymes were found to contain the highest concentration of ^{75}Se for tissues sampled, followed by the testes. A more dramatic demonstration of concentration of ^{75}Se in the testis-epididymis complex is seen in Brown and Burk's (1973) data for rats. At three weeks PA these organs contained 41.8% of the total body ^{75}Se. Gunn and Gould (1970) have stated that the concentration of a mineral in the testes is not an indication of its importance to the gonad. It would seem, however, that if selenium is an essential micronutrient for M. canicaudus, efficient utilization of available selenium would be an evolutionary imperative. In this context, it would be expected that the generous allocation of selenium to the reproductive tissues has some adaptive significance.

Sulfite/Selenite Challenge Following Selenium-75 Administration

The challenge dose of selenium reduced subsequent whole body retention of ^{75}Se by greater than a factor of two. This suggests that even after three days, a considerable amount of retained ^{75}Se was not in a firmly bound form. The retention of ^{75}Se in the testes was reduced by greater than a factor of four (on a per gram basis).

The finding that the testes exhibited a delayed uptake of selenium may partially explain the apparent large percentage of as yet unbound selenium present in the gonad at this time. It should be noted that when not considered on a per gram basis, the subsequent retention is reduced by a factor of only two. One of the animals in the selenium challenge group had relatively small testes with very little ^{75}Se retention in relation to testes size. This effectively reduced the average retention value for the testes. In general, testes weights are not well-correlated with body weights in this species, promoting discrepancies such as these.

That the epididymes showed no significant decrease in subsequent ^{75}Se retention when challenged with unlabeled selenium is more difficult to explain. Gunn et al. (1970) stated that one week after ^{75}Se administration to rats it is not possible to prevent its incorporation into spermatozoa by subsequent challenge with selenium. The testis-uptake curves for ^{75}Se in voles suggest that the duration of spermatogenesis for this species is shorter than for rats. In this context, a challenge of selenium three days after ^{75}Se administration may be too late to prevent spermatogenic incorporation. Thus, although a substantial portion of the testicular selenium may have been unbound at the time of the challenge, the bulk of the ^{75}Se reflected in the two-week epididymal peak may reflect that fraction of testicular ^{75}Se which was already bound to developing spermatozoa.

That the sulfite challenge was unable to reduce subsequent retention of ^{75}Se is consistent with Levander's (1976) hypothesis.

Levander stated that the following redox equation points to a major difference between sulfur and selenium:

$$H_2SeO_3 + 2H_2SO_3 \rightarrow Se + 2H_2SO_4 + H_2O$$

Thus, the selenium in selenite is reduced, while the sulfur in sulfite is oxidized. According to Levander, the tendency of the former to be reduced and the latter to be oxidized is reflected in the difference in metabolic fate of these two nutrients. Another difference lies in the dissociation behavior of the selenohydryl group of selenocysteine compared to the sulfhydryl group of cysteine. At physiological pH the selenohydryl group is dissociated while the sulfhydryl group is not.

Underwood (1971) has reviewed the work pointing to the metabolic interchangeability of seleno amino acids with their sulfur counterparts in mammals. Much of the supporting evidence stems from apparent chromatographic isolation of the two seleno-amino acids from animals previously administered ^{75}Se. Schwarz and Sweeney (1964), however, found that selenite could bind a variety of sulfur compounds in vitro to give compounds with chromatographic properties similar to the parent sulfur compounds.

In a more recent study, Miller and Sheppard (1973) injected rats with a mixture of ^{75}Se-selenomethionine and ^{35}S-methionine which were subsequently recovered in proteins from kidney and liver fractions. The subsequent tissue distribution of the two labeled isotopes was very dissimilar. It would seem that a full understanding of the differences between these two elements awaits the precise description

of the form(s) of selenium in animal proteins.

Intracellular Distribution of Selenium-75 in Spermatozoa

Cellular fractionation techniques have been used for over 15 years in an attempt to delineate intracellular distribution patterns of administered ^{75}Se. This study represents the first application of the technique to mammalian spermatozoa. The nucleus and the mitochondria of vole sperm appear to be by far the most significant cellular compartment with respect to selenium concentration. Brown and Burk (1973) fractionated the testes of rats administered ^{75}Se. At three weeks PA the intracellular distribution was as follows:

Nuclear Fraction	64.1%
Mitochondrial Fraction	16.3%
Microsomal Fraction	8.6%
Soluble Fraction	11.3%

The rank order of concentration is the same as for the sperm assayed in the present study. Again, the nucleus appears to be the most important selenium repository, followed by the mitochondria. An interesting aspect of Brown and Burk's data concerns the dependency of time PA on the intracellular distribution of ^{75}Se. At three hours PA only 26.9% of the homogenate activity was found in the nucleus, and the difference between this and the three-week value could be attributed to the soluble fraction. A reasonable explanation here would be that selenium is in an unbound soluble state initially, and subsequently becomes incorporated into the

nucleus. The mitochondria, however, showed very little change in concentration during the three-week period. Brown and Burk (1973), as well as earlier workers (McConnell and Roth, 1962), have substantiated that the nucleus and mitochondria of liver fractions concentrate relatively less radioselenium than do the testes.

Sperm Autoradiographs

The sperm autoradiographs are consistent with the results of the intracellular distribution study, in that a large percentage of the labeled selenium was seen in the nuclear and mitochondrial regions. It would be simplistic to attempt to explain the observed distribution patterns in terms of GSH-Px or any other singular seleno-protein. Tripp (personal communication) has stated that unpublished data from her laboratory regarding chromatographic analysis of ^{75}Se-labeled tissues suggest the existence of at least 11 seleno-proteins.

It is more probable that the observed intracellular associations of ^{75}Se in sperm reflect metabolic allocation to a multiplicity of proteins and/or other biomolecules. Based on the observed associations in vole sperm, it would seem reasonable to postulate the existence of at least three different seleno-proteins in this cell.

There would appear to be two major intracellular structural associations of selenium in vole sperm. The sperm nucleus is one major site of deposition. The other major structural association of selenium appears to be with membranes. These include the

nuclear membrane, outer mitochondrial membrane, and the fibrous sheath membrane of the tail. The remainder of selenium appears to be located in the dense fibers, axial filament complex, and possibly a small amount in the mitochondrial inner membrane and matrix.

The apparent high concentration of selenium in the sperm nucleus is difficult to explain. Levander (1976) has pointed out the almost universal association of selenium with sulfur in protein forms. In most somatic cells, the histone protein associated with DNA in the nucleus contains a full complement of amino acids, including cysteine and methionine (Davis and Langford, 1970). In many mammalian species, however, spermatid maturation is associated with a replacement of histones by nucleoprotamines. Protamines are short chain polyamines containing relatively few amino acids. Mann (1964) has stated that none of the protamines have been found to contain sulfur amino acids. Monesi (1973) stated that the sperm head contains other residual proteins including a sulfur-rich keratin-like protein. Although it was speculated that the protein was probably localized in the plasma membrane, no definite proof is yet available.

The absence of sulfur amino acids from protamines does not eliminate the possibility of selenium incorporation into these or other nuclear molecules. Levander (1976) has cautioned against the *a priori* assumption that selenium is bonded to sulfur in proteins, and speculated that further research may uncover hitherto unsuspected roles for the micronutrient. Monesi (1973) speculated

that nucleoprotamines may play a protective role by stabilizing the sperm genome against thermal denaturation or other alterations during storage, and during passage through the male and female genital tracts. Selenium may have an important role in this or other functional means of assuring DNA integrity.

There is some evidence to suggest that selenium may be incorporated into the transfer RNA of microorganisms (Saelinger et al., 1972). In mammalian sperm, however, maturation is accompanied by a genetic inactivation of the nucleus, including elimination of nuclear RNA (Monesi, 1973).

The significant association of selenium with membranes suggests a number of hypotheses to account for its presence in these structures. The present evidence, in conjunction with other related work, points away from the hypothesis that GSH-Px is the predominant selenium-containing protein in sperm, or in the testes. Lawrence et al. (1975) found that rat testes contained low levels of glutathione peroxidase in relation to other tissues and that testicular levels of this enzyme were relatively unresponsive to dietary selenium. In addition, Brown and Senger (1976) found that most of the glutathione peroxidase associated with bovine semen was not localized in the sperm, but in the seminal plasma. Finally, work with liver mitochondria indicated that a substantial portion of the mitochondrial GSH-Px was associated with the mitochondrial matrix (Levander, 1976). In vole sperm, however, virtually 100% of the mitochondrial selenium seems to be associated with the outer membrane.

It is proposed here that the purpose of this membrane-bound selenium is in some way to promote the structural and/or functional integrity of such intracellular membranes. As such, selenium may be an integral component of an as yet uncharacterized structural protein, unrelated to GSH-Px. The implications of this role will be discussed in a later section.

The localization of selenium in the dense fibers and axial filament complex may partially reflect the small amount of GSH-Px localized in sperm. Sperm are known to have an inherently low level of catalase (Tosic and Walton, 1946) (an enzyme which destroys H_2O_2) and may require some small amount of GSH-Px to aid in the reduction of H_2O_2. Alternatively, the localization may reflect other uncharacterized selenoproteins. Based on the intracellular distribution data and autoradiographs, these compartments do not appear to account for a large percentage of the selenium in sperm.

Effect of Selenium on Reproductive Physiology

The most general observation to be made from the physiology data is that selenium and vitamin E appear to have interrelated functions in male reproductive physiology. This is illustrated in several relationships. Either selenium or vitamin E significantly increased ($P < .05$) the testes weight relative to body weight. While 0.58 ppm selenium significantly reduced ($P < .05$) the percent of abnormal sperm in the absence of vitamin E, 0.04 ppm of selenium did so significantly ($P < .05$) when vitamin E was added to the diet.

The correlation coefficients provide additional indication that the functions of selenium and vitamin E in the testes are complementary. The addition of vitamin E to the diet reduced the correlation between dietary selenium and selenium concentration in the testes, as well as between dietary selenium and sperm abnormalities.

In contrast to results with rats, there appeared to be no relation between dietary selenium and sperm motility, with or without vitamin E in the diet. This may reflect species differences in relative sensitivity to selenium deficiency. That is, analogous sperm physiological subsystems related to motility in rat sperm, may not have been sensitive to the extant level of selenium depletion in vole sperm. Furthermore, the rats were maintained on the TY diets for a significantly longer period than were the voles.

Selenium also seemed to have an effect on the general health of the animals, as manifested in weight gains and sacrifice weight. In the absence of vitamin E, the addition of selenium to the diet significantly increased ($P < .05$) daily weight gain and sacrifice weight. That vitamin E function also overlaps here is illustrated in that selenium could not significantly increase the above parameters when vitamin E was present. Selenium may be more important in this regard, since the addition of 60 ppm vitamin E with no selenium supplement failed to result in significant weight gains or sacrifice weight.

Finally, it should be recognized that the effects of the two nutrients on reproductive physiology may be both direct and indirect. The indirect effects on reproductive performance are mediated through the effect of vitamin E and selenium on the general health of the animal. The direct effects pertain to specific requirements for the two nutrients in the reproductive tissues. A useful way of conceptualizing these nutritional effects on reproduction is schematically illustrated in Figure 14.

Some indication of the relative importance of direct/indirect effects of selenium on reproduction can be gained by examination of the physiology correlation coefficients. For example, a greater correlation exists between dietary selenium and sperm abnormalities than between body weight or weight gain and sperm abnormalities. Conversely, the size of the testes are more highly correlated with body weight than with dietary selenium. These relationships will be additionally discussed in a later section.

Effect of Selenium on Reproductive Performance of Male Voles

The 30-day exposure of males to the two TY diets (selenium-supplemented and unsupplemented) undoubtedly resulted in significant differences in tissue selenium levels between the two groups. Pilot studies conducted earlier in this laboratory indicated that males similarly exposed to differences in dietary selenium for one month showed dramatic differences in retention of administered ^{75}Se. If the 30-day tissue depletion had created suboptimal physiological

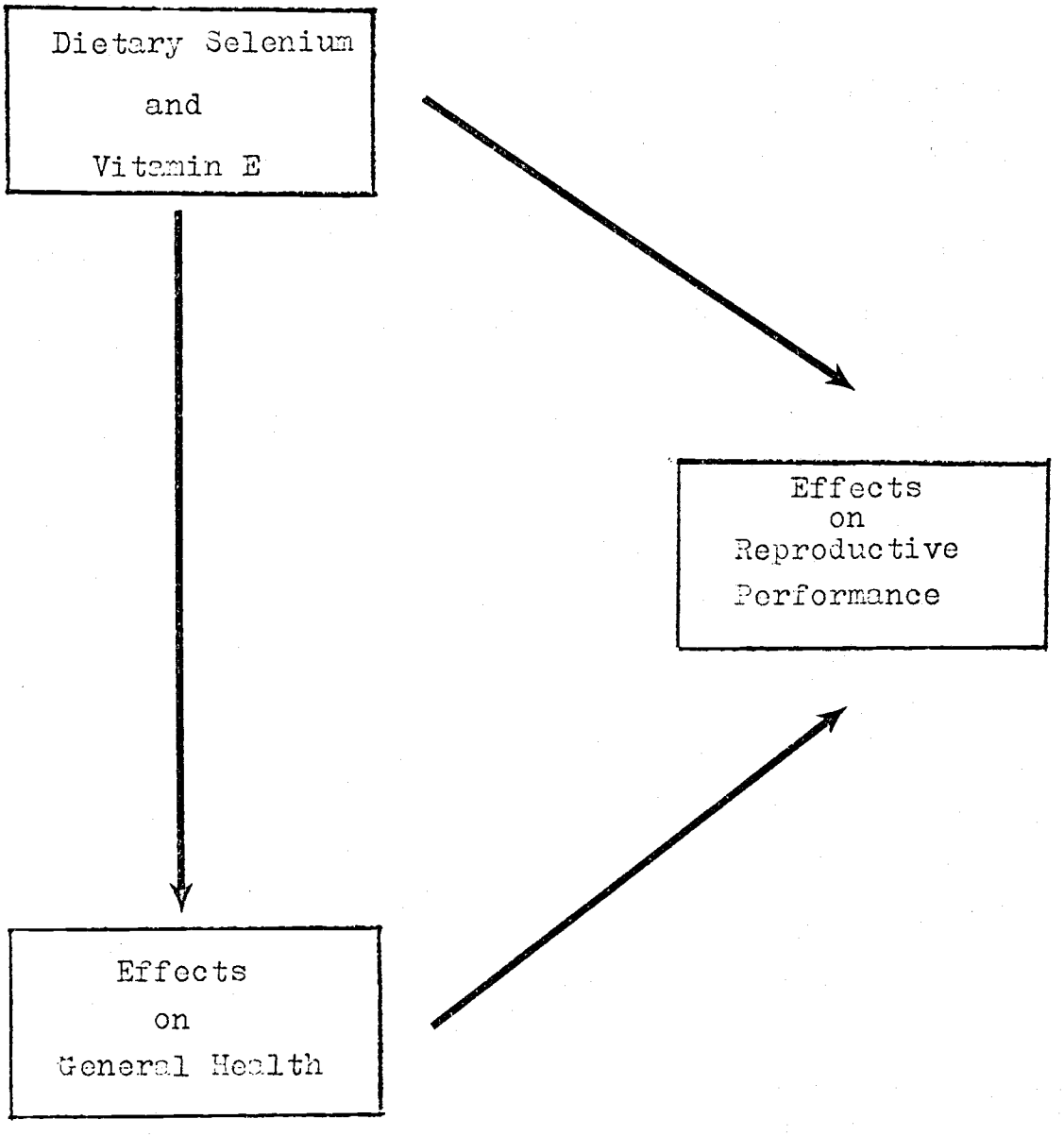

Figure 14. Proposed mode of effect of selenium and vitamin E on male reproductive performance.

conditions with respect to reproduction, an initial difference in reproductive performance between the two groups would be expected. This was not, however, the case.

In general, cumulative percent parturitions were much lower than was expected for the control group. This arouses suspicion concerning the possible nutritional inadequacy of the TY diet for voles. As such, the gradual increase in percent cumulative parturitions may have been partially due to availability of the standard laboratory ration to the males. The inadequacy of the diet is further suspect when considering the high mortality rate of males in both groups.

The results of the second reproductive study support the contention that the inadequacy of the diet was a major factor in the reproductive status of the males. There was an average mortality rate of greater than 50% for males between the time of weaning and the termination of the breeding experiment. That none of the control males were able to impregnate females further points to the overshadowing influence of the TY diet on male reproductive performance.

Finally, it should be recognized that the attainment of a "selenium deficient" condition in M. canicaudus may be problematic, as this species is exclusively restricted to a low selenium natural environment. It would therefore be expected that M. canicaudus is relatively well-adapted to a low dietary intake of selenium. The level of selenium in the composite vegetation sample was comparable to the basal level in the TY diet. In this light, the more important

question concerning reproduction, is whether the normal intake of selenium in wild M. canicaudus is limiting, in terms of reproductive performance.

General Discussion

The results of these studies in conjunction with other recent work on the subject suggest a general hypothesis to account for the interaction of selenium and vitamin E in testicular and spermatozoan physiology. As will be seen, the hypothesis is consistent with a considerable body of data, and draws on major aspects of two dominant hypotheses regarding selenium-vitamin E interactions in mammals.

Hoekstra (1975) has succinctly presented one of these explanations for vitamin E-selenium interaction. Appendix Figure 3 illustrates the essential features of this hypothesis. According to this view, vitamin E functions as a general protector of lipoproteins and oxidizable lipid components of enzymes. This is accomplished through the prevention of lipid hydroperoxide formation from unsaturated lipids, and subsequent autocatalytic lipid peroxidation resulting in cell damage. Selenium, as a component of glutathione peroxidase, has the dual role of converting lipid hydroperoxides to alcohol and water, as well as reducing H_2O_2 to H_2O.

The Diplock-Lucy hypothesis comprises a second major viewpoint regarding vitamin E-selenium interaction (Diplock and Lucy, 1973). According to this view (appendix Figure 4) the role of vitamin E

is not to prevent the autoxidation of unsaturated lipids, but to protect a reduced form of membrane-bound selenium from oxidation. In this role, vitamin E is membrane-bound also, and functions "by virtue of a specific physico-chemical interaction between its phytyl side chain and the fatty acyl chains of polyunsaturated phospholipids . . . ." (p. 208). This hypothesis was put forth to account for the observation that the maintenance of selenium in the reduced state in rat liver mitochondria and microsomes seemed to depend upon the presence of vitamin E.

As a working hypothesis, it is submitted that vitamin E functions as a general lipid antioxidant in the testes. One postulated function of selenium, as mentioned earlier, is to promote intracellular membrane integrity. It is proposed that this specific function of selenium in sperm is critical with respect to possible impairment of reproductive performance. In this view, the functions of vitamin E and selenium are complementary, but in a different sense than postulated by Hoekstra (1975). Thus, while the presence of membrane-bound selenoproteins may enhance the resistance of membranes to gross oxidative destruction, the presence of vitamin E may reduce the incidence of oxidative insult.

This proposed vitamin E-selenium interaction would predict the following hypothetical sequence of intracellular events. The absence of vitamin E would precipitate an increase in the rate of chemical insult to membranes by oxidant stressors such as hydrogen peroxide and lipid hydroperoxides. The oxidation of lipid constituents of membranes may expose seleno-proteins close to the

membrane surface to H_2O_2 or molecular oxygen. If, as Diplock and Lucy (1973) have suggested, the protein-bound selenium is in a reduced state, exposure of these selenides to oxidants may result in a change in the selenium oxidation state, protein denaturation, and subsequent localized membrane destabilization.

Conversely, in the absence of dietary selenium, the presence of vitamin E would be expected to spare membrane-bound selenium from oxidation. Eventually, however, a failure to offset natural membrane turnover of selenium would result in membrane destabilization and ultimate dissolution. Considered singularly, then, vitamin E and selenium are seen as necessary, but not sufficient conditions for long-term reproductive integrity in males.

The physiological responses of male rats to vitamin E and selenium deficiencies can be consistently explained in terms of the above general hypothesis. For example, vitamin E deficiency results in a general necrosis of the testicular germinal epithelium (Leathem, 1970). In vitamin E-depleted rats, the degenerate testes are low in lipid and phosolipid content, and the degeneration process is ameliorated by the addition of saturated fats.

By contrast, in selenium-deficient rats active spermatogenesis still occurs in some of the seminiferous tubules. Selenium deficiency appears to be more specifically manifested in spermatozoan pathology. Wu et al. (1969, 1973) found the epididymis of selenium-deficient rats to contain cellular debris. Furthermore, very few sperm were collected from the cauda epididymis, and most of those collected exhibited reduced motility and/or morphological

abnormalities. The observed morphological abnormalities included protrusion of the dense fibers from the tail. Wu et al. (1973) speculated that this abnormality may have been a result of membrane destruction. More specifically, this may have resulted from destruction of the fibrous sheath membrane surrounding the dense fibers of the tail.

In terms of the general hypothesis, the data of Wu et al. (1973) might be interpreted as follows: long-term tissue depletion of selenium prevented normal replacement of membrane-bound selenium lost from natural turnover. Eventual destabilization of outer mitochondrial membranes inhibited mitochondrial function as manifested in reduced sperm motility. Continued depletion of membrane-bound selenium resulted in general membrane dissolution, one consequence of which was an increase in observed sperm abnormalities.

Many of the data for voles are also consistent with the working hypothesis. As was found to be the case with rats, selenium seemed to be more critical with respect to sperm abnormalities. In the absence of vitamin E, dietary selenium increases were able to significantly reduce sperm abnormalities. Vitamin E alone, however, was unable to significantly reduce the incidence of sperm abnormalities. Consistent with the hypothesis, vitamin E appeared to reduce the level of dietary selenium necessary to significantly abate sperm abnormalities. Congruent with this notion, the addition of vitamin E to the diet reduced the correlation between dietary selenium and percent abnormal sperm.

While vitamin E could not significantly reduce sperm abnormalities, it could significantly increase the testes weight relative to body weight. This is in concordance with the notion of vitamin E being more important as a general testicular antioxidant. It should be noted that dietary selenium also significantly increased testes size in relation to body weight. This does not conflict with the hypothesis. Selenium may have had a temporary ameliorating effect on vitamin E deficiency in the vole.

The results of the intracellular ^{75}Se distribution studies are also consistent with the hypothesis. The absence of vitamin E from the diet would be expected to increase the rate of oxidative insult to sperm intracellular membranes. The fact that the diet was also low in selenium would tend to accelerate membrane destabilization. If intracellular membranes, e.g., mitochondrial membranes, were in a destabilized state when the sperm were homogenized, some of the membrane-bound selenium could have been released. This would result in an increase in cytosolic ^{75}Se as was found to be the case here. This can be seen more clearly if the nuclear fraction is ignored (because of variability caused by unfractionated cells) and intracellular distribution is based on the remaining three compartments. The results of such a consideration are as follows:

	Vitamin E Supplement	No Vitamin E Supplement
Mitochondria	86.4%	69.2%
Microsome	4.2%	7.5%
Cytosol	9.4%	23.3%

Presented in this fashion, the data are very consistent with the working hypothesis.

Leathem (1970) has stated that while the rat, guinea pig, and hamster exhibit vitamin E deficient testicular degeneration, this is not manifested in the rabbit, mouse, or goat. The findings for the latter three species do not necessarily preclude the essentiality of vitamin in the testes. It is possible that much less vitamin E is required in the testes of these species, and that they were not sufficiently depleted to observe a response. In this same context, voles may have not been sufficiently depleted of selenium to observe a high incidence of sperm fibrous protrusions and/or significantly reduced sperm motility. The possibility of genuine species differences with respect to a specific testicular requirement for selenium and/or vitamin E merits more extensive investigation. Such information would determine the scope of applicability of the hypothesis presented here.

Although the present hypothesis may prove to be incorrect, it provides a consistent framework within which many of the data can be explained. More importantly, many of the physiological responses predicted by the hypothesis can be tested. For example, if selenium and vitamin E are mutually supportive, but not interchangeable in their functions in testes, then long-term vitamin E degeneration of the testicular epithelium in rats (and voles) should be delayed but not prevented by increases in dietary selenium. Also, ^{75}Se-labeled sperm can be fractionated and assayed for total GSH-Px activity to determine what proportion of the label

can be accounted for by GSH-Px in various subcellular compartments.

Finally, the sperm autoradiographs provide guidance in terms of where to begin searching for new selenium-containing proteins in attempts to isolate and characterize such proteins. Much additional work remains before sufficient numbers of seleno-molecules can be characterized to consistently explain the totality of observed physiological responses to selenium deficiency in mammals.

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APPENDIX

TORULA YEAST RATION

Constituents	Amount/Kg
Torula yeast	400 g
Sucrose	415 g
Corn oil	50 g
Vitamin mix[1]	10 g
HMW salt mixture[2]	50 g
BW 100 Solka-Floc[3]	75 g
Vitamin A	10 mg
Vitamin D	100 µg
Vitamin E	Added according to group requirement
Selenium (Na_2SeO_3)	

[1] Vitamin Mix

Constituents	Amoung/100 g
Thiamin HCL	40 mg
Riboflavin	25 mg
B_6-pyridoxine HCL	20 mg
Ca D-pantothenate	200 mg
Choline-chloride	10 g
Niacin	1 g
Menadione	10 mg
Folic acid	20 mg
Biotin	10 mg
B_{12}	1 g
Lactose carrier	87.675 g

[2] Hubbell et al. (1937).

[3] BW 100 Solka-Floc purified cellulose, Brown Company, New York, N.Y.

Figure 1. Composition of torula yeast ration (Pond, 1977).

1. Add sample to 50 ml Erlenmeyer flask. Add 10 ml of concentrated nitric acid and allow to digest at room temperature for at least 4 hours.

2. Heat slowly on a hot plate until the nitric acid boils then increase the heat until the dark brown fumes become light brown.

3. Remove flask from heat and add 2 ml 70% perchloric acid and swirl.

4. Continue heating to distill off nitric acid. After the appearance of white perchloric fumes, continue heating for 15 minutes. Then remove from heat and cool.

   Note: While HNO_3 is distilling out of flask, the bubbles formed will be large. As the distillation approaches completion, small bubbles are formed. This happens just prior to the evolution of $HClO_4$ fumes. If heated too much at this point charring may occur. If the sample starts to develop a dark color, remove it from the heat, cool under cold water, then add 1.0 ml concentrated HNO_3 and finish digesting the sample.

5. Cool the flask and add 1.0 ml H_2O. Reheat until perchloric acid fumes then remove the flask and cool after approximately 1 minute.

6. Add approximately 1 ml H_2O and 1 ml 2.5 N HCl.

7. Cool to room temperature and add 5.0 ml of stabilizing solution (Hydroxylamine-ethylendiaminetetraacetic acid) and 2 drops of cresol red indicator.

8. Add 5 N NH_4OH until the pink solution turns straw yellow. Then add 6 N HCl until the solution turns a peach color.

9. Dim lights or use yellow light.

10. Transfer sample to separatory funnel (adjusting volume of aqueous phase in separatory funnel to 50 ml) and add 5 ml of DECALIN (Eastman No. 1905 decahydronaphthalene) and shake for 10 minutes.

11. Discard aqueous phase and wash organic phase by shaking it for 2 minutes with 50 ml 0.1 N HCl.

12. Transfer organic phase to fluorometer tubes and centrifuge for approximately 2 minutes at moderate speed.

13. Zero fluorometer against DECALIN and read all tubes at 525 nm within 5 minutes.

Figure 2. Procedure for the analysis of selenium (Pond, 1977).

Table 1. Percent initial whole body ^{75}Se activity for male voles for 21 days following oral administration (N = 5).

Days Post-Administration	% whole body ^{75}Se activity ($\bar{x}$ ± SE)
1	81.8 ± 4.4
3	24.2 ± 1.5
5	17.3 ± 1.5
7	13.7 ± 1.3
12	7.8 ± 0.7
17	5.2 ± 0.5
21	3.5 ± 0.4

Table 2. Percent initial whole body ^{75}Se Activity for whole body and selected tissues of male voles from 21 days following oral administration: serial sacrifice.

Days post-administration	1	4	7	14	21
Testes					
% retention ± SE	0.25 ± .01	0.28 ± .03	0.26 ± .02	0.15 ± .02	0.07 ± .01
T retention per gram ± SE	1.27 ± .12	1.06 ± .07	1.41 ± .13	0.65 ± .07	0.36 ± .04
Epididymes					
% retention ± SE	0.02 ± .002	0.02 ± .002	0.03 ± .001	0.06 ± .01	0.03 ± .007
% retention per gram ± SE	0.74 ± .09	0.52 ± .06	0.70 ± .06	1.34 ± .22	0.87 ± .18
Kidneys					
% retention ± SE	2.49 ± .11	1.28 ± .06	0.71 ± .03	0.24 ± .02	0.09 ± .01
% retention per gram ± SE	7.57 ± .50	3.39 ± .15	1.96 ± .07	0.59 ± .06	0.28 ± .02
Liver					
% retention ± SE	8.06 ± .33	4.69 ± .01	3.16 ± .17	0.90 ± .01	0.27 ± .01
% retention per gram ± SE	5.26 ± .23	2.61 ± .22	2.02 ± .21	0.54 ± .04	0.19 ± .02
Whole Body					
% retention ± SE	38.69 ± .74	15.63 ± .35	11.62 ± .26	4.73 ± .13	2.88 ± .37

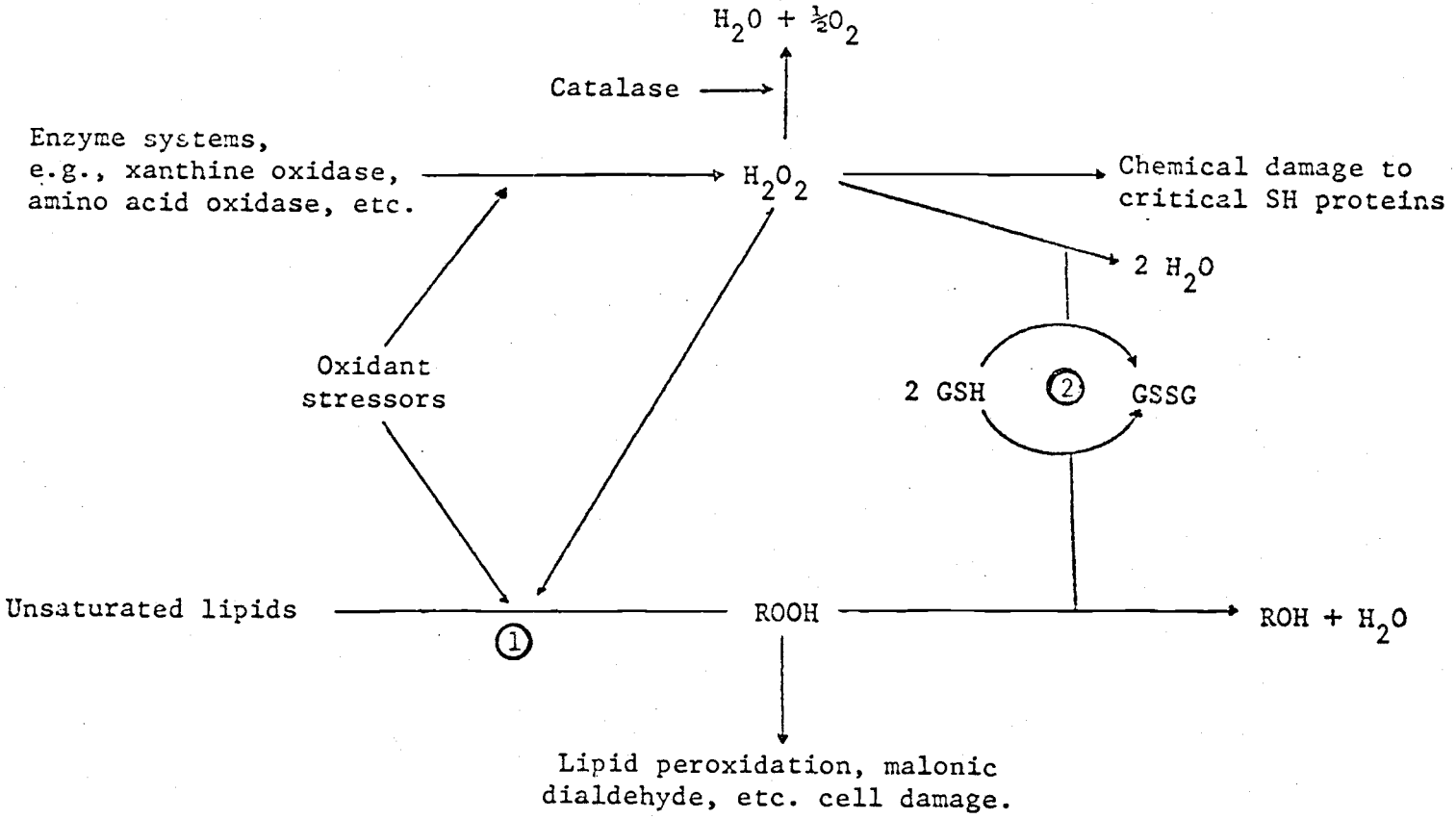
Table 3. Effect of sulfite and selenite challenge on 2-week retention of previously administered ^{75}Se (N = 5).

	No challenge		1 μmole Na_2SO_3		1 μmole Na_2SeO_3	
	% WB retention ± SE	% retention per gram ± SE	% WB retention ± SE	% retention per gram ± SE	% WB retention ± SE	% retention per gram ± SE
Whole body	53.87 ± 1.97	1.97 ± 0.14	60.07 ± 2.76	2.17 ± 0.12	25.18 ± 1.99	0.81 ± 0.04
Testes	2.2 ± 0.54	19.18 ± 4.84	3.01 ± 0.37	16.20 ± 0.94	0.97 ± 0.17	4.46 ± 0.29
Epididymes	0.21 ± 0.6	8.62 ± 1.79	0.37 ± 0.05	12.64 ± 0.84	0.31 ± 0.07	7.82 ± 1.65

Table 4. Intracellular distribution of orally administered ^{75}Se in spermatozoa of voles under two different dietary regimes of vitamin E.*

Cell Compartment	Torula yeast 0 ppm added vitamin E	Torula yeast 60 ppm added vitamin E
Nuclei + debris	69.0%	48.1%
Mitochondria	21.2%	44.8%
Microsome	2.3%	2.2%
Cytosol	7.1%	4.9%

*For both groups a composite sperm sample from 4 voles was collected.



1. Vitamin E blocks (1)
2. Selenium, as a component of GSH-Px, Catalyzes (2)

Figure 3. Proposed interrelation of selenium and vitamin E in the prevention of oxidative damage to tissues (from Hoekstra, 1975).

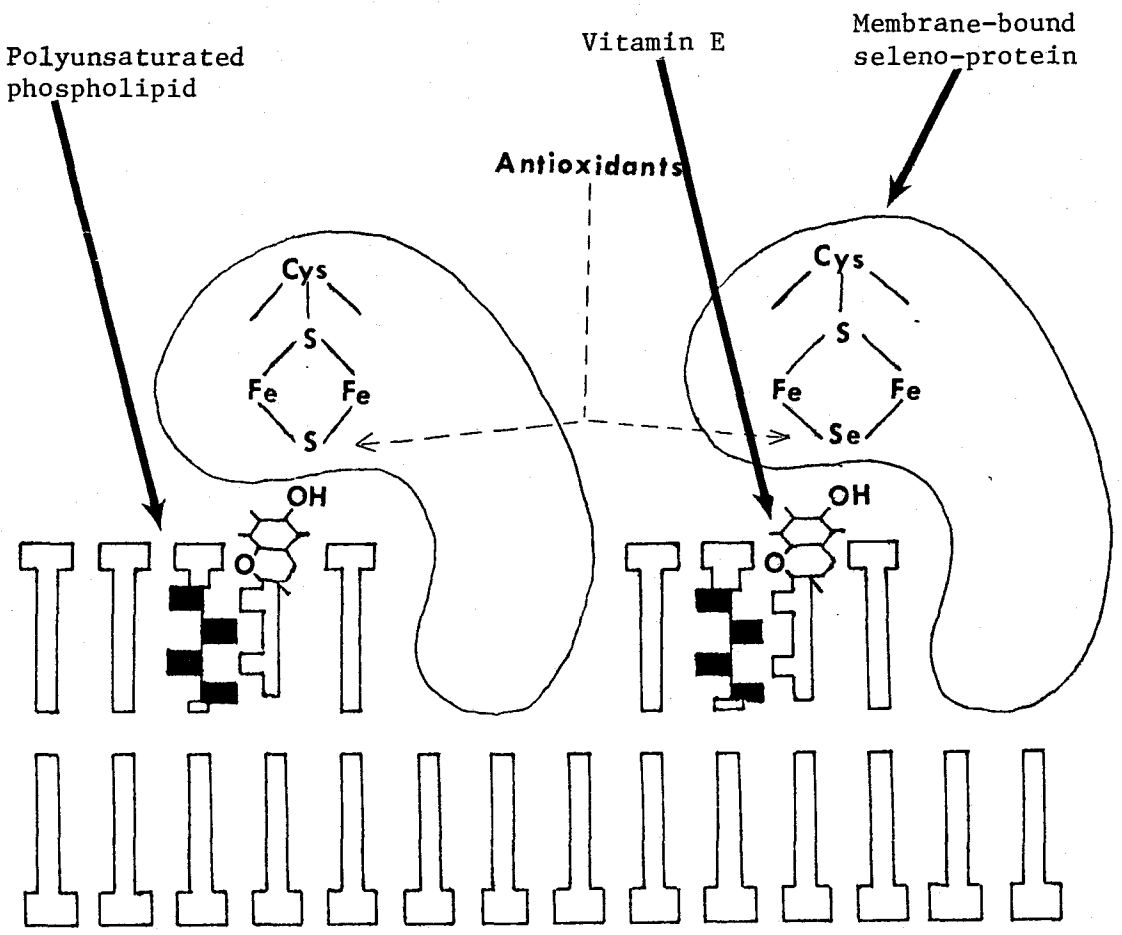


Figure 4. A diagrammatic representation of the proposed interactions between vitamin E, synthetic antioxidants, selenide- and sulphide-containing proteins, and polyunsaturated phospholipids in a biological membrane (from Diplock and Lucy, 1973).