SOME METABOLIC CHANGES ASSOCIATED WITH AVITAMINOSIS-E IN THE RAT DURING PREGNANCY

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

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1966

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ABBREVIATIONS AND SYMBOLS

cpm	= counts per minute
^{o}C	= degree centigrade
^{o}F	= degree fahrenheit
DNA	= deoxyribonucleic acid
gm	= grams
kg	= kilograms
μc	= microcuries
μg	= micrograms
mc	= millicuries
mg	= milligrams
ml	= milliliters
mm	= millimeters
mM	= concentration in millimoles/liter
$m\mu$	= millimicron = 10^{-7} cm
M	= concentration in moles/liter
N	= concentration in equivalent weights/liter
P	= probability
rpm	= revolutions per minute
RNA	= ribonucleic acid
TBA	= thiobarbituric acid
TCA	= trichloroacetic acid

INTRODUCTION

The requirement for vitamin E is commonly considered to be largely represented by a need for a biologically active lipid antioxidant (1, 2). Lipid peroxidation of membranes of cells and of subcellular particles is known to occur in several tissues of vitamin E-deficient animals (3). A free radical chain reaction catalyzed by hematin compounds is the mechanism known to be involved in the in vivo peroxidation of unsaturated lipids (4). The membranes of the microsomal, mitochondrial, and lysosomal subcellular fractions are especially labile to lipid peroxidation since they contain large quantities of unsaturated lipid. As might be expected, these same subcellular fractions also contain the highest relative concentrations of vitamin E (5). The structural damage to the membranes of cells and of subcellular particles would be expected to lead to widespread derangements of metabolism. In fact, it has been postulated that all of the gross syndromes of vitamin E-deficient animals are secondary effects resulting from lipid peroxidation damage (1, 6).

Naturally occurring vitamin E is actually a mixture of several chemically similar compounds collectively called the tocopherols. The most abundant and also the most biologically active form is alpha-tocopherol (2,5,7,8-tetramethyl-2-(4',8',12'-trimethyl-tridecyl)-6-chromanol). In higher animals, vitamin E is widely distributed among the tissues.

Its storage and distribution pattern is similar to those of the other fat soluble vitamins (7). Although several metabolites of vitamin E have been identified, its mechanism of action remains to be determined.

The most thoroughly studied symptom of avitaminosis E is muscular dystrophy. Relatively short depletion periods are required to produce a dystrophic condition in herbivorous animals such as the rabbit and the guinea pig. Longer periods on vitamin E-deficient diets are needed to produce this symptom in the carnivorous rat. The time required to produce this symptom in any species is largely dependent upon the unsaturated fatty acid composition of the diet (8). Pathological changes associated with a long depletion period such as is required to produce dystrophy in the rat are usually not reversible by vitamin E therapy.

Another symptom of avitaminosis E in the rat is fetal resorption. In fact, the observation of these strange resorptions led to the discovery of vitamin E by Evans in 1922 (9). The degree of deficiency required to produce fetal resorption is significantly mild in comparison to the exhaustive depletion required for the production of dystrophy in the rat. The administration of small quantities of vitamin E during the first days of gestation is followed by the birth of normal appearing young. If, however, vitamin E therapy is delayed until the middle of the gestation period,

a considerable variety of congenital malformations were found among the young at term (10).

Pregnancy is an anabolic process which requires not only maintenance of the maternal organism but synthesis of large amounts of new tissue. This process should stress the vitamin E reserves of the maternal organism already in a state of E-deficiency. Increased production of lipid peroxides with concurrent damage to the tissues of both the dam and fetus should result in measurable metabolic changes. This, at least, served as a beginning hypothesis for this investigation. Other evidence to support this approach was found in the published literature. Goldstein and McKay (11) have clearly demonstrated the ability of lipid peroxides to produce eclampsia of pregnancy in the rat. Dinning (12) showed an increase in DNA synthesis in the skeletal muscle of the dystrophic rabbit. Deterioration of the microsome fraction of rat livers by lipid peroxidation was reported by Tappel (13). Several other derangements of metabolism have been associated with increased lipid peroxides. For the most part, all these metabolic changes have been studied using dystrophic animals.

The research to be presented in this dissertation was undertaken as an attempt to relate the proposed lipid peroxidation function of vitamin E and metabolic changes associated

with a well established symptom of avitaminosis E, fetal resorption.

REVIEW OF LITERATURE

Vitamin E

The existence of an antisterility vitamin was first reported in 1922 by Evans and Bishop (9). Rats fed semi-synthetic diets deficient in this factor were not able to perform the normal reproductive functions. Both sexes were affected. The addition of a small quantity of lettuce or wheat germ oil to the diet restored fertility to the test animals.

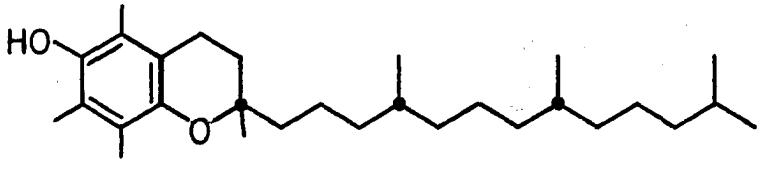
Attempts to isolate, purify, and chemically characterize the new factor followed verification of its existence. A mixture of allophanates was obtained when a wheat germ oil concentrate was reacted with cyanic acid (14). That this mixture did indeed contain a number of forms of vitamin E was firmly established by Emerson et al. (15). To date, eight forms have been isolated from one or more natural oils such as wheat germ oil, soybean oil, and rice oil. The generic name tocopherol was applied to this group of compounds and the individual members are distinguished by Greek alphabetical prefixes. Alpha-tocopherol is the predominant form of vitamin E and, in most cases, is actually the compound used in studies of vitamin E.

Although the initial interest in vitamin E arose from its requirement for reproduction, this deficiency characteristic is only one of many found to be associated with avitaminosis E in the rat. Cheng (16) reviewed the gross and pathological changes accompanying various degrees of vitamin E-deficiency in several animal species. Those symptoms which are pertinent to the present investigation will be described in detail in a later section of this review.

Chemistry of vitamin E

The most abundant and biologically active form of vitamin E is alpha-tocopherol (17). Using degradative techniques with comparison of the products obtained to known compounds, Fernholz (18) established the structure of alpha-tocopherol as:

● Optically active center



Alpha-tocopherol: $C_{29}H_{50}O_2$: Molecular weight 430:69

In the same year (1938) Karrer et al. (19) synthesized alpha-tocopherol by reacting phytyl bromide with trimethyl hydro-

quinone. Treatment of this synthetic dl-alpha-tocopherol with 3-bromo-(+)-camphor-sulfonyl chloride confirmed that it was a mixture of two optically active forms; the dextrorotatory diastereoisomer being identical to the natural compound.

Pure alpha-tocopherol was obtained from wheat germ oil by preparing a mixture of allophanates, selectively crystallizing the allophanate of alpha-tocopherol, and hydrolysis of the latter in methanolic potassium hydroxide (15). The pure form is a viscous oil which is soluble in organic solvents. Alpha-tocopherol has three asymmetric centers. Two are in the isoprenoid side chain and one is in the chromanol moiety. The configuration in the isoprenoid side chain is considered to correspond to that of natural phytol (17, 20). Nothing is known about the absolute configuration of the asymmetric center in the chromanol moiety. While it is known that the natural d-alpha-tocopherol has 36 percent higher vitamin E activity than synthetic dl-alpha-tocopherol (21), Isler et al. (17) have shown that the configurations of the two asymmetric centers in the side chain have no effect on the activity of either compound. Apparently the observed difference in activity is due entirely to differences in the chromanol asymmetric center.

As mentioned previously, eight tocopherols have been isolated from natural oils. The structures of beta-, gamma-, delta-, eta-, and $zeta_1$, tocopherol differ from that of alpha-

tocopherol by the number and position of the methyl groups attached to the aromatic portion of the chromanol moiety (22, 23, 24, 25, 26). Epsilon-, and zeta$_2$, tocopherol have an unsaturated isoprenoid side chain (25).

The eight tocopherols vary significantly in biological activity. Several investigations have shown that the natural form of alpha-tocopherol, d-alpha-tocopherol, has the greatest biological potency. Since this compound is sensitive to light and is oxidized by air at room temperature (27), the more stable acetate ester (28, 29) has been commonly used in experimental work. One mg of dl-alpha-tocopherol acetate is the international unit (I.U.) of vitamin E (30). Wiss et al. (7) have compared the absorption and distribution of alpha-tocopherol acetate in the chick with the pattern obtained for free alpha-tocopherol. They have shown a more rapid absorption of the free tocopherol. But with continuous feeding of the acetate form an equilibrium occurs so that the liver and plasma tocopherol levels agree with those from animals fed free tocopherols. Harris and Ludwig (31) reported that d-alpha-tocopherol was 1.36 times more potent than the synthetic dl-alpha-tocopherol. Pudelkiewicz et al. (29) have found d-alpha-tocopherol acetate to be 1.34 times more potent than dl-alpha-tocopherol acetate as measured by chicken liver tocopherol content. The use of several forms of vitamin E at widely variable levels of supplementation has made it ex-

tremely difficult to make valid comparisons among reports in the research literature.

The metabolism of vitamin E

Although it is widely distributed among the tissues of animals and much is known about the symptoms of its deficiency, very little is known about the metabolic route of vitamin E. The relative ease with which the tocopherols and their metabolites can be oxidized *in vitro* has complicated efforts to elucidate their mechanism of action (32).

Simon *et al.* (33) isolated and characterized two metabolites of *alpha*-tocopherol from both rabbit and human urine. These two oxidation products were given the common names topheronic acid and tocopheronolactone. Using d-*alpha*-tocopherol-5-methyl-C^{14} succinate, the same authors found that the urine contains mainly metabolized tocopherol; while intact tocopherol accounted for the majority of the radioactivity in the feces. Csallany *et al.* (34) have identified two new metabolites of *alpha*-tocopherol isolated from the liver of the rat after injection of *alpha*-tocopherol-5-methyl-C^{14}. About 25 percent of the recovered activity was unchanged *alpha*-tocopherol, about 19 percent was *alpha*-tocopheryl quinone, and about 50 percent was dl-*alpha*-tocopherone, a dimer of oxidized *alpha*-tocopherol.

The oxidation of alpha-tocopherol in vitro by peroxiding fatty acid radicals yields primarily the dimer, but also some alpha-tocopheryl quinone (35). This was cited as evidence that the mechanism of action of vitamin E may be through the prevention of lipid peroxidation in vivo.

Identification of these four metabolites of alpha-tocopherol has been very important in the understanding of vitamin E metabolism. However, no definite evidence relative to the vitamin's mechanism of action has been obtained since the breakdown products of a compound often have nothing to do with its biological function (36).

An excellent review of the alpha-tocopherol metabolites has been published (37).

Function of vitamin E

The literature contains reports of attempts to assign a definite metabolic role to vitamin E. These studies have lead to the development of two divergent interpretations of the role of vitamin E in mammalian metabolism. The "anti-oxidant" school, which designates the vitamin as being a nonspecific biological antioxidant, has gained strong support in recent years (3, 38, 39). The "specific metabolic function of vitamin E" school has attempted to assign a specific metabolic function to vitamin E (40, 41, 42). However, the majority of the known specific effects of the vitamin

can be interpreted as being secondary to the role of vitamin E in the inhibition of lipid peroxidation (43, 44).

Olcott and Mattill (45) discovered the antioxidant activity of vitamin E in 1941. The evidence supporting the lipid antioxidant role of vitamin E was first reviewed by Dam (46) in 1957. More recently, Tappel (1) has summarized additional information supporting this theory.

The mechanism of lipid peroxidation is fairly well understood (4). In biological material, hematin compounds such as hemoglobin and cytochromes catalyze the peroxidation of the unsaturated lipid present (47). The free radical chain reaction thought to be involved in these <u>in vitro</u> oxidations can be summarized as follows:

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$RH + ROO^{\bullet} \longrightarrow ROOH + R^{\bullet}$$

RH: Unsaturated fatty acid

A small amount of an antioxidant such as vitamin E could inhibit the peroxidation by breaking the reaction chain (48):

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$$

AH: Antioxidant

Ingold (4) discusses these reactions in more detail.

The secondary products of unsaturated fatty acid oxidation have been studied (49). The products resulting from the hematin catalyzed breakdown of linoleate hydroperoxide in the

absence of oxygen were mainly oxirane, hydroxyl, and carbonyl compounds with the original carbon chain intact. However, some cleavage of the carbon chain and some polymerization took place. An important secondary product is malonaldehyde (50) since this is the compound which reacts with thiobarbituric acid to give the red chromogen produced in the assay commonly used in studies of lipid peroxide content (51).

Lipid peroxides are highly toxic to cellular subfractions and to various enzyme systems in the cell. Roubal and Tappel (38) have found damaged proteins, enzymes, and amino acids when transient free radicals were generated in peroxidizing lipid-protein mixtures. Other workers have demonstrated the ability of lipoperoxides to inactivate the electron transport system in the liver mitochondria from vitamin E-deficient chicks (52). The microsomal subfraction of the rat liver contains 30 to 40 percent lipid and twice the amount of unsaturated lipid as does the mitochondria (13). Zalkin et al. (53) showed large increases in several of the lysosomal, hydrolytic enzymes in leg muscles of vitamin E-deficient rabbits. Peroxidation of the lipid containing membrane of isolated lysosomes has been demonstrated (54). Desai et al. (55) have prevented muscular dystrophy, lipid peroxidation, and increased lysosomal enzyme activity in the muscle tissues of vitamin E-deficient chicks by supplementation with d-alpha-tocopherol.

Tappel et al. (56) have suggested the following sequence of events in the development of the various symptoms of avitaminosis E: a) lipid peroxidation damage to the subcellular constituents; b) rupture of the lysosomes and release of lysosomal enzymes; and, c) hydrolysis of cellular components which leads to manifestation of the disease symptoms.

Synthetic chemical antioxidants can prevent and cure most of the symptoms of vitamin E deficiency. Crider et al. (57) have carried rats through a resorption-gestation and then obtained a successful reproductive cycle when the rats were fed 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinone (ethoxyquin) or N,N'-diphenyl-p-phenylenediamine (DPPD). Csallany and Draper (58) have shown that DPPD in daily doses of 20 mg prevented all characteristics of muscular dystrophy in rabbits fed a vitamin E-deficient diet. The production of congenital abnormalities in vitamin E-deficient rats was prevented by dietary ethoxyquin or DPPD (59). Although several structurally unrelated, synthetic antioxidants have been found to substitute for tocopherols in the prevention of specific deficiency diseases, DPPD and ethoxyquin appear to be the most effective (6).

No definite theory regarding the relationship between vitamin E and the structurally dissimilar antioxidants has been established. Early evidence indicated that these latter

compounds protected very small amounts of tocopherol present in the diet (60). However, this theory has recently been challenged. Draper et al. (6) prepared a highly purified diet which was analytically shown to be free of vitamin E. Feeding this diet along with daily supplementation of DPPD restored fertility to female rats of proven sterility. These results caused the authors to suggest a direct metabolic substitution of DPPD for tocopherol. Csallany and Draper (58) also used a sensitive analytical procedure to verify the lack of vitamin E in the diet which they used to produce dystrophy in rabbits. The tissues from the rabbits in which muscular dystrophy had been prevented by daily doses of DPPD were also shown to be completely exhausted of any tocopherol.

Relevant symptoms of vitamin E-deficiency in the rat

Retardation of growth has been shown to be a characteristic of vitamin E-deficiency in the rat. After 2 to 4 months on a vitamin E-deficient diet, the growth of the rat reaches a plateau and remains stationary for many months before slowly declining (61, 62). The normal growth rate can be restored by vitamin E therapy.

Several investigators have associated creatinuria with a state of vitamin E-deficiency in the rat (2, 16). The degree of creatinuria is commonly obtained by measuring the ratio of creatine-to-creatinine found in a 24-hour urine

specimen. A ratio greater than 0.40 is considered to be an indication of positive creatinuria in the rat (2).

The end product of creatine metabolism in mammals is creatinine (63). The formation of creatinine from creatine is believed to be an irreversible, nonenzymatic reaction occurring mainly in the skeletal muscle (64). Apparently creatine leaks from the muscle in certain muscle wasting diseases. Fitch and Dinning (65) demonstrated the reduced ability of the skeletal muscle of vitamin E-deficient animals to retain creatine-1-C^{14}. A direct correlation between the development of positive creatinuria and the amount and nature of the dietary unsaturated fatty acid has been found in the rat (2). The fats which produced the earliest creatinuria were those in which a given amount of unsaturation was concentrated into a small percentage of the dietary fatty acid. As an example, rats fed a diet containing 7.5 percent polyenoic fatty acid showed positive creatinuria after 5 weeks; while similar animals fed diets containing 19 percent saturated fatty acid developed this symptom only after 49 weeks. The level of tocopherol supplementation required to prevent or cure creatinuria increased with the concentration of dietary unsaturated fatty acid.

Creatinuria usually precedes any observable gross or histological alterations in experimental animals being fed vitamin E-deficient diets. In herbivorous animals, increased

urinary excretion of creatine precedes muscular dystrophy by about 2.5 weeks (66). Positive muscular dystrophy is usually considered to correspond to the inability of the animal to right itself when placed on its side (67). Evans and Burr (68) showed that the young of vitamin E-deficient females rats developed paralysis during the suckling period. Other workers later showed that this paralysis was due to lesions of the skeletal muscle similar to those observed in herbivorous species (69). The muscle lesions in adult rats develop very slowly. Rats fed a vitamin E-deficient diet containing 15 percent stripped corn oil showed positive creatinuria in 4 weeks, but the atrophy characteristic of gross dystrophy was not evident until the 53rd week (70). Vitamin E therapy arrests, but does not repair, the muscle lesions in the dystrophic rat (71, 72).

Nucleic acid synthesis in the muscles of animals exhibiting acute stages of muscular dystrophy induced by a vitamin E-deficiency has been studied (73). The ability to synthesize DNA was increased by 50 to 60 percent in these muscles. A corresponding increase of 40 to 50 percent in the synthesis of both RNA and protein was also found. Although these increases in synthesis lead to higher concentrations of RNA and DNA in the muscles, protein concentration actually decreased. This decrease could be due to increased activity of proteolytic ensymes in the affected muscles (74). Dinning (40) has summarized his investigations of the role of vitamin E in regulating

nucleic acid metabolism in vitamin E-deficient rats, rabbits, and monkeys. Both RNA and DNA concentrations are increased in the skeletal muscle and bone marrow of the dystrophic animals. The incorporation of radioactive precursors into DNA was increased in these same tissues. Diehl and his co-workers (67, 75, 76) have found increased incorporation of glycine-1-C^{14} into the protein of all subcellular fractions of skeletal muscles from dystrophic rabbits.

Increased specific activity of respiratory carbon dioxide from vitamin E-deficient rabbits following injection of glycine-C^{14} has been reported (77). Diehl (78) found increased oxidation of radioactive glycine, leucine, and lysine in dystrophic rabbits. Elevated oxygen uptake by skeletal muscle preparations from dystrophic rabbits has also been reported (79). In summary, it is evident from all of these observations that the histopathological lesions of muscular dystrophy are associated with a wide spectrum of metabolic derangements.

Another symptom of avitaminosis-E in the rat, fetal resorption, was first reported in 1922 (9). Evans et al. (80) found that estrous, ovulation, fertilization, migration, and implantation took place in normal fashion, but the young were never born, resorption occurring instead. These authors described the stage of gestation at which the products of conception begin to stray from the normal. They also described

the pathological changes of the uterus, embryo, and placenta which accompany the prenatal death. Briefly, all reproductive events are normal up to the time of implantation at about the 6th day of gestation. Retardation of fetal development was observed on about the 10th day and death of the embryo occurred on the 13th day. Death was followed by rapid necrosis and resorption of the embryos, and a more gradual regression of the placentas. At term, only fragments of the maternal placentas remained in the uterus (81).

A single dose of vitamin E administered to the deficient female during the first six days of gestation lead to a normal termination of pregnancy. The dosage level depends upon the age of the female (82, 83). At 10 weeks of age, 0.3 mg of d-alpha-tocopherol was required for a completed gestation; while 6.0 mg was required at 45 weeks of age. Ames (83) has suggested this is an actual increase in physiological requirement.

Thomas and Cheng (10) reported a considerable variety of teratogenic changes in developing rat embryos induced by a deficiency of vitamin E. When tocopherol administration was delayed until gestational day 9, 10, or 11, gross congenital malformations were manifested in the surviving fetuses at term (84). However, when alpha-tocopherol acetate supplementation was administered at any time through the 8th day of gestation, no abnormally developed young were found.

Cheng et al. (85) have determined the vitamin E content of maternal liver, serum, and skeletal muscles and of fetal liver, serum, and carcasses from three groups of pregnant rats at term. Surprisingly, the maternal serum and liver levels were lowest in a vitamin E-sufficient group, highest in a vitamin E-deficient group, and intermediate in a group with the abnormal young. The carcasses of the abnormal young contained much less tocopherol than the normal carcass. However, since the values were all very small, the difference was not statistically significant. These investigators suggest that their results confirm the earlier observation that only a slight degree of maternal vitamin deficiency would produce congenital abnormalities in the young. The mechanism of the role of vitamin E in the production of congenital abnormalities remains to be determined.

Protein and Nucleic Acid Metabolism during Gestation in the Rat

Beaton (86) has described pregnancy as "a state of extreme nutritional stress". He emphasized the marked physiological changes which take place in the pregnant female during the gestation period. These gross physiological changes are associated with both increased nutritional requirements and altered metabolic reactions. New tissue in the form of

the fetuses, placentas, and fetal membranes are synthesized during pregnancy. Adequate dietary nutrients and vitamins are known to be of extreme importance during this period of anabolism.

Many studies with experimental animals have demonstrated the effects of malnutrition on pregnancy. For example, dietary inadequacy can prevent conception (87), cause fetal resorption (88), lead to congenital malformations (89), and induce abortion (90). More catastrophic disease processes during gestation can result in the death of the maternal as well as the fetal organism (91). Postnatal death of the young has also been associated with a maternal dietary deficiency (68). It seems unlikely that these various types of reproductive disorders are caused by a common series of metabolic events. Also, it would appear that complex interrelations among the various body systems are involved. Fisher and Leathem (92) found that a protein-free diet fed during pregnancy resulted in a 95 percent incidence of fetal resorption in the rat. However, viable litters could be obtained by the injection of hormones without addition of protein to the maternal diet. In the case of vitamin E, it is known that the structural state and the hormonal activity of the endocrine system of E-deficient rats are normal (93). Repeated resorptions in vitamin E-deficient rats do not af-

fect sexual function or the ability to complete pregnancy if sufficient vitamin E is supplied (94).

Only recently have the metabolic alterations characteristic of a normal gestation been intensively studied. Some of the pertinent observations are presented in the following sections of this review.

Protein metabolism during gestation

Beaton et al. (95) studied protein metabolism in the pregnant rat. Only slight increases in total body weight, fetal weight, and maternal nitrogen retention were observed during the first fifteen days of the gestation period. This was not unexpected since the structural organization of the developing embryo takes place between approximately the 7th and the 16th day of gestation. This period of organogenesis occurs with little increase in embryonic size. Primary fetal malformations are also determined during this 9-day period (96). During the final week of the gestation period, there was a marked increase in maternal body weight, fetal weights, and fetal moisture content (97). The ability of the female rat to retain dietary nitrogen was also greatly increased during the last week of gestation (98). This increased nitrogen retention was accompanied by decreases in blood amino nitrogen, liver alanine-glutamic transaminase, and the rate of urea formation in maternal liver slices. While the

15th day of gestation marks the beginning of the period of rapid fetal growth, the reverse was found to be true for the placenta (99). Its growth was very rapid during the first two weeks of gestation, with very little increase during the last week.

Poo et al. (98) studied protein anabolism in organs and tissues of pregnant rats at different levels of protein consumption. The liver was found to increase in size and correspondingly, in protein content during gestation. While livers from rats sacrificed on the first day of gestation had an average weight of 6.567 gm and contained an average of 1.256 gm of protein, livers from the rats on the 21st day of gestation had an average weight of 9.565 gm and contained 1.838 gm of protein. This increase in protein was associated with an increase in moisture content, phospholipid content, both RNA and DNA content, and a reduction in glycogen content (99). Fisher and Leathem (92) also measured the protein and nucleic acid content of livers from rats on the 21st day of gestation. Their data show an average liver weight of 11.596 gm and a protein content of 1.594 gm.

The importance of the placenta in the maintenance of pregnancy has been described by many investigators. It is a storage place for all vitamins, and supplies the fetus according to its needs. The rat placenta is divided into two parts: the yolk sac and the labyrinthine placenta. The

rodent yolk sac is an organ of exchange of metabolites between the maternal and the fetal organisms. However, the labyrinth part is the major location of exchange. When pregnant rats were fed a diet low in vitamin E, the animals showed pathological alterations of the placentas (100). Placental slices from these animals showed a depression of oxygen uptake and anaerobic glycolysis (101). Also, the in vivo transport of radioactive sodium across these placentas was depressed during the last one-third of gestation. An average placenta from a normal pregnancy in the rat weighed 541 mg and contained 54 mg of protein (92).

Uteri from rats sacrificed on the first day of gestation had an average weight of 0.290 gm and contained 40 mg of protein; while uteri from rats sacrificed on the 21st day of gestation had an average weight of 4.013 gm and contained 346 mg of protein (92, 98). The uterus increases in both cell size and number during pregnancy in the rat (102). Protein content increases during gestation have been correlated with an increase in collagen content (103). The total increase in size and protein content depends upon the distention of the two uterine horns.

In the course of their work on the effect of hormones on incorporation of L-valine-1-C^{14} into protein of rat liver and uterus, Little and Lincoln (104) used slices from tissues of rats sacrificed on either the 6th or the 16th day of gestation.

Both the liver and the uterine slices showed increased L-valine incorporation on the 16th-day in comparison to the 6th-day values.

Nucleic acid metabolism during gestation

A slight increase in DNA and a larger increase in RNA were found in the liver of the rat during gestation (97, 105). The increase in RNA appeared during the third week of gestation and was in excess of any increase associated with the rise in liver protein. The cause of this "excess RNA" in the liver was investigated by Campbell and Kosterlitz (106). Their findings suggest that a secretion of viable placentas is responsible for this increased synthetic activity. In fact, removal of the fetuses leaving the placentas intact did not prevent the increased RNA synthesis (107). The liver RNA levels returned to normal during the first week of lactation.

Fisher and Leathem (92) have determined the RNA and DNA contents of the rat placenta and uterus on the 21st day of gestation. RNA-to-DNA ratios calculated for these organs were 3.49 and 3.50 respectively. Wakid and Needham (108) have shown that the amount of RNA per uterine cell increases sevenfold during pregnancy and total nitrogen increases fourfold; while the DNA content remains relatively constant.

Toxicity Associated with Lipid Peroxidation

Lipid hydroperoxides are prepared by air oxidation of various unsaturated oils such as those from soybean and cod liver. These hydroperoxides have been shown to be acutely toxic to the rat (109, 110). Intravenous infusion of only milligram quantities of methyl linoleate hydroperoxide can cause two symptoms characteristic of vitamin E-deficiency, creatinuria and red blood hemolysis, within a few hours (6). However, Olcott and Dolev (110) have found both tocopherol and ethoxyquin to be ineffective in lowering the LD_{50} of methyl linoleate hydroperoxide in the rat. These investigators suggest that hydroperoxides kill by attack on some vital tissue with sulfhydryl enzymes or cytochromes being the vulnerable tissue component.

Stamler (111) has produced eclampsia of pregnancy by placing pregnant rats on an oxidized cod liver oil diet low in tocopherol content. This toxemia was associated with many pathological changes strikingly similar to those found in human patients with toxemia of pregnancy (91). More recently, Goldstein and McKay (11) have determined the lipid peroxide content of several tissues from female rats in which the eclampsia had occured. Lipid peroxides were found in all of the tissues studied (spleen, liver, kidney, uterus, and

placenta), but only the kidney, liver, and placenta showed peroxide values significantly higher than those of the control animals. These authors concluded that the vitamin E-deficiency lead to cellular damage which caused the eclamptic state by some unknown mechanism.

Release of lysosomal enzymes

The cellular damage and the visible symptoms of avitaminosis E are known to precede, rather than follow, the appearance of any significant lysosomal enzyme activity. The properties of lysosomes have been examined in detail in recent reviews (56, 112). Briefly, the lysosomes are a heterogenous group of particles that are not readily separated from mitochondria and microsomes. The single unit membrane which surrounds the enzymes is composed of lipoprotein. Free radicals produced during autoxidation of unsaturated lipids are known to damage the lipoprotein membrane of the lysosomal particle and allow the enzymes to become available in the free form (113). In rat liver, the lysosomal particles are known to contain at least ten different enzymes which are all soluble acid hydrolases with a wide spectrum of catabolic activity (56).

Woessner (114) found two cathepsin activities in the rat uterus during involution. One of the cathepsin, which had an optimum activity at pH 3.5, could completely digest

uterine collagen at 37°C *in vitro*. In the female rat, the involution process was virtually complete by 100 hours following partuition. It is not known whether catheptic activity increases in the uterus of vitamin E-deficient rats during the process of resorption.

Finally, Tappel *et al.* (56) have correlated the changes in the rate of turnover of tissue components reported by other workers with increases in lysosomal enzyme activities. For example, these investigators found that catheptic activity was increased 15 times over control values in the leg muscle from vitamin E-deficient rabbits; while Dinning *et al.* (115) showed a 600 percent increase in the rate of incorporation of formate-C^{14} into the protein of this muscle four hours after injection of the radioactive material.

EXPERIMENTAL

Rearing and Mating the Rats

Management of the rats

Albino rats of the Sprague-Dawley strain (Holtzman Company) were used exclusively in these studies. Females of weaning age (20 days) weighing between 50 and 60 gm were purchased in lots of various sizes as the need arose during the course of the investigation. Upon arrival, each shipment of females was randomly divided into three groups of approximately equal size. The animals were housed in standard metal animal cages (14 in. x 22 in. x 12 in.) with 6 to 8 rats in a cage. Fresh feed and distilled water were available ad libitum. The air conditioned animal room in which the rats were housed at all times was maintained at 78°F whenever possible.

Rations

The three groups of females were maintained, following their arrival, on different dietary regimens. A powdered semi-synthetic, tocopherol-deficient test diet (General Biochemicals) was fed exclusively to the negative control group of rats. The composition of this diet is shown in Table 1.

Table 1. Composition of the ration used to produce avitaminosis-E in female rats

Ingredients	Percent by weight
Casein, vitamin free	20.0
Cerelose	56.0
Lard, stripped	10.0
Salt mixture, HM&W	4.0
Yeast	10.0
Carotene in oil	68[a]
Vitamin D (400,000 Units/gm)	0.1[a]

[a]Gm/100 pounds of diet.

Shipments of this diet were received approximately biweekly, stored in a cold room at 4°C, and fed during the month following their arrival. The experimental group of females receiving this diet was designated as E (-).

Portions of the topcopherol-deficient diet were supplemented with dl-alpha-tocopherol acetate (General Biochemicals). The tocopherol acetate was first blended into a small amount of the deficient diet. This concentrate was then thoroughly mixed with additional deficient diet to give a final dl-alpha-tocopherol concentration of 100 mg/kg diet. The supplemented diet was always fed within 10 days following its preparation.

This diet was fed exclusively to a positive control group of females. This group was designated as E (+).

Wayne Lab Blox, a standard, pelleted rat chow consisting largely of natural ingredients, was fed exclusively to a second positive control group of females. This group, designated as the pellet-fed females, served as a check on the responses elicited by the E(+) group. A supply of vigorous, adult male rats was kept for mating during the course of the investigation. The pelleted diet was also fed exclusively to the males.

Growth of the rats

Individual weights were measured weekly for all randomized females in an early shipment of rats (20 rats per dietary regimen). The weighings were begun when the rats arrived at 24 days of age and were continued until they had reached 77 days of age. All rats in later shipments were weighed only at approximately 75 days of age.

Development of creatinuria

Early in this investigation, the ability of the tocopherol-deficient test diet to produce positive creatinuria in the female rats was tested. Twenty-four-hour urine samples were collected from each of eight females which were re-

ceiving the tocopherol-deficient diet. Collections were begun when the rats reached 50 days of age and continued weekly until they reached approximately 150 days of age. Concurrently, six females which were receiving the deficient diet which had been supplemented with dl-alpha-tocopherol acetate (E (+) females) were used as controls.

Additional creatine and creatinine determinations were undertaken for later shipments of rats. Females were randomly selected from each dietary group and 24-hour urine samples collected.

Rat metabolism units (Hoeltge, Inc.) were utilized in the collection of the urine. Only four of these units were available making it necessary to stagger the collection periods. Each test rat was weighed at the time it was placed in the metabolism unit. A urine collection bottle containing 5 ml xylene to prevent evaporation and bacterial growth was placed under each separation funnel. All diets were withheld during the collection period. However, water was available ad libitum.

Urinary creatine and creatinine excretion was quantitatively determined by the method of Bonsnes and Taussky (116). The volume of urine voided during each 24-hour-collection period was measured to the nearest 0.1 ml. Two ml of each urine sample were diluted with water to 250 ml in a

volumetric flask. Creatinine was then determined by the following procedure:

1. 3.0 ml of the diluted urine were placed in a 15 ml culture tube.
2. 1.0 ml of 0.04 M picric acid was added to each tube.
3. 1.0 ml of standard 0.75 N sodium hydroxide was added to each tube and the mixture shaken.
4. The golden color was allowed to develop for 15 minutes.
5. The absorbance of this mixture was then measured at 525 mμ against a blank in which water was used instead of the diluted urine. A Bausch and Lomb Model 340 colorimeter was used to measure the absorbance values.

Creatine was determined by the above procedure after heating 3.0 ml of the diluted urine with 1.0 ml of 0.04 M picric acid in a tightly capped culture tube for 45 minutes in a vigorously boiling water bath. After cooling, 1.0 ml of 0.75 N sodium hydroxide was added and the color allowed to develop. The absorbance was measured as described above.

By applying these procedures to commercial creatinine (Fisher certified reagent) a standard curve was prepared. Standard creatinine was run with each group of determinations to assure reproducibility of the standard curve.

The total creatinine in each urine sample was calculated directly from the concentration found on the standard curve. The total creatine was found by subtracting the absorbance value of the unheated mixture from that of an identical mixture after heating. This difference represents the absorbance of the creatine which was converted to creatinine by the heating process. Since this conversion is only 80 percent complete (116), the values obtained were multiplied by a factor of 1.25 to obtain the reported creatine concentrations.

Mating the rats

Mating was begun when the females reached approximately 75 days of age at which time they were considered sexually mature (80). Therefore, each group of females had received its specific diet for not less than 55 days prior to its use for gestational studies.

Two techniques were used to obtain pregnant females for which the time of coitus was known within 10 hours. The inefficiency of the first lead to its replacement by the second technique. The first technique consisted of placing two or three male rats in each cage with the females and observing their reactions for at least 10 minutes. Females which appeared to be receptive were removed and placed for a longer time in another cage with vigorous males. After approximately one hour, the females were vaginal smeared for signs of

positive mating. The presence of either the vaginal plug or sperm in the vaginal smear was positive evidence of coitus. Initially, this procedure was employed during the morning, afternoon, and late evening. But the breeding activity during the evening hours far surpassed that observed during the day. These observations lead to the use of the second mating technique. Males were placed with the females at approximately 10 p.m. each evening and were removed the next morning. All of the "exposed" females were then vaginal smeared for positive mating. This breeding procedure was continued routinely until either all the females had been mated or they had reached 100 days of age at which time the slow breeders were discarded. The day on which sperm was found was considered as day zero of gestation. All mated females were weighed, marked for identification, and transfered to smaller cages. Each mated female continued to receive the same diet on which it had been maintained since its random allocation to a dietary regimen.

Analyses of Tissues for *In Vivo* Incorporation of Radioactive Valine, Lipid Peroxide Content, and Total Protein

Injection of L-Valine-1-C^{14}, and collection of tissues for analysis

The pregnant females receiving the different diets were sacrificed on either the 10th day or the 20th day of gestation

following injection of radioactive valine. Tissues were excised and appropriately stored for analysis. Approximately the first half of each group mated was terminated on the 20th day and the second half on the 10th day of gestation. Therefore, all rats were between 95 and 115 days of age when sacrificed.

The initial work was designed to examine the effect of a vitamin E-deficiency on the in vivo protein metabolism of of the liver, placenta, and uterus during pregnancy. L-valine-1-C^{14} (Calbiochem) with a specific activity of 9.8 mc per mM was used exclusively for these experiments. The radioactive valine was dissolved in physiological saline to a final concentration of 2.0 μc per ml. The pregnant rats were injected intraperitoneally with 0.5 ml of the valine solution per 100 gm body weight using a 2 ml hypodermic syringe. After either a 1, 2, or 4 hour interval during which the females were fasted, they were sacrificed by decapitation and the desired tissues excised. A median ventral longitudinal incision was made through the abdominal and the thoracic walls. The uterus and liver were excised quickly, the extraneous, adhering adipose and connective tissues discarded, and the tissues blotted dry on absorbent paper. The liver was weighed to the nearest mg on a Mettler analytical balance and frozen between two pieces of dry ice. The uterine horns were opened longitudinally and their contents removed.

The uterus was then blotted dry, weighed, and frozen.

In the case of the pregnant females terminated on the 10th day of gestation, the entire uterine contents were frozen after the number of developing embryos and their combined weights had been determined. This material was mainly placental with microscopically visible embryonic tissue just beginning to develop. Only the placentas from the uterine contents of the 20-day females were saved for analysis. They were detached from the uterine wall and the umbilicus of the fetus, enumerated, blotted dry, weighed, and frozen. All the tissues excised for study were wrapped in aluminum foil and stored at -35°F.

Preparation of homogenates

The preserved tissues required homogenization prior to analysis. Each tissue was homogenized *in toto* in cold 1.0 M phosphate buffer, pH 7.4, using a motor driven, glass-glass Duall tissue grinder (Kontes Glass Company). The liver homogenates were diluted to 50 ml using phosphate buffer and portions of each dilution taken for analysis. The final dilutions of the uterine, placental, and uterine content homogenates were less than those of the liver since this was determined by the weight of the material. These dilutions were made in a volumetric flask to approximately 0.2 gm tissue per ml.

Lipid peroxide determinations

Portions of all homogenates were further diluted to 0.05 gm tissue/ml with cold phosphate buffer. The volumes of the homogenate and buffer required were measured to the nearest 0.01 ml. The procedure of Bieri and Anderson (117) was used to measure *in vivo* and *in vitro* production of lipid peroxides in the homogenates:

1. 1.0 ml of cold homogenate was pipetted into 1.5 ml of 10 percent TCA.
2. 1.5 ml of homogenate were also pipetted into a 25 ml Erlenmeyer flask and shaken for 1 hour in a water bath maintained at 37°C.
3. The Erlenmeyer flask was cooled on ice and 1.0 ml of homogenate was pipetted into 1.5 ml of 10 percent TCA.
4. After centrifuging the TCA-homogenate mixtures from steps (1) and (3), duplicate 1.0 ml samples of the clear supernatant TCA solutions were transferred to graduated tubes.
5. 2.0 ml of 0.7 percent TBA were added and the tubes placed in a boiling water bath for 15 minutes during which time a red color of different intensities developed.
6. The mixtures were cooled and diluted to 5.0 ml with distilled water.

7. The absorbance values were read at 535 mμ with a Beckman DU spectrophotometer against a blank in which phosphate buffer was substituted for the tissue homogenate.

The results of the TBA assay are reported as the absorbance at 535 mμ since no satisfactory standard is known. The absorbance values from the solutions in which the homogenate was added directly to the TCA reagent are considered to represent *in vivo* lipid peroxide content; while the *in vitro* values were obtained after the one hour incubation.

Fractionation procedure

The precipitates which were obtained when the tissue homogenates were added to the TCA solution in the first step of the lipid peroxide procedure were retained for protein and radioactivity analyses. Nucleic acids and lipids were removed by the method of Siekevitz (118). The procedure included the following steps:

1. Wash the precipitate with 3.0 ml of 10 percent TCA, 2 times.
2. Extract with 4.0 ml of 7 percent TCA for 15 minutes, at 92°C, 2 times.
3. Wash the remaining precipitate with 2.5 ml of 95 percent ethyl alcohol saturated with potassium acetate, 1 time.

4. Incubate the precipitate with 2.5 ml of 95 percent alcohol: chloroform (3:1) for 10 minutes at 60°C.
5. Wash the precipitate with 2.5 ml of 95 percent alcohol:ether (3:1), 1 time.
6. Incubate the precipitate with 2.5 ml of ether for 10 minutes at 60°C.
7. Dry precipitate at 60°C for 8 hours.

A 5 minute centrifugation at 2,500 rpm followed each operation.

Determination of radioactivity, and protein analysis

The dried protein was dissolved in 80 percent formic acid and diluted to 5.0 ml with formic acid. Duplicate portions (0.5 ml) of this solution were dried onto stainless steel, ringed planchets (Planchets, Incorporated) for counting. The planchets were counted with a Nuclear-Chicago, Model D-47 gas flow counter for a time period sufficient to give an accuracy of 2 percent. Another 0.5 ml portion of the formic acid-protein solution was pipetted into 2.3 ml of 5 N sodium hydroxide and diluted to 12.5 ml with water. The protein concentration of this solution was then determined by the method of Lowry *et al.* (119) as follows:

1. 0.5 ml of the diluted protein solution was pipetted into 2.5 ml of a solution of 2 percent sodium carbonate in 0.1 N sodium hydroxide. The latter solution contained 0.05 gm of copper sulfate per liter.

2. 0.25 ml of 1 N phenol reagent (Fisher Scientific Company) was added and the mixture shaken immediately.
3. After 30 minutes the absorbance of the solution was read at 750 mμ on a Beckman DU spectrophotometer against a blank in which 0.5 N sodium hydroxide was substituted for the protein solution.

A standard protein curve was obtained using bovine serum albumin (Armour Laboratories). Determinations using this standard protein were run simultaneously with each group of protein assays to verify the reproducibility of the standard curve. Since the dilution of the protein in each step of the procedure was known, it was possible to calculate both the amount of protein placed on each planchet and the total protein in each tissue.

Verification of the protein values

Two additional means of analysis were undertaken to assure the accuracy of the Lowry protein determinations. The first involved weighing the dried protein before dissolving in formic acid. The second involved the application of the biuret method (120) of protein analysis to the protein solutions. The three methods gave results which agreed within 5 percent.

In Vitro Incorporation of L-Valine-1-C^{14}

Incubation medium

The composition of the Krebs-Ringer bicarbonate incubation medium (pH 7.4) used in the organ maintenance experiments is shown in Table 2. Riggs *et al.* (121) have shown a reciprocal relationship between amino acid uptake and potassium exchange during *in vitro* incubations. For this reason, Little and Lincoln (104) have suggested replacing the 4 parts of potassium chloride commonly found in the Krebs-Ringer bicarbonate medium with sodium chloride. This modification was used in the present research.

Fresh medium was prepared on the day it was needed. Three and one-half ml of the medium were pipetted into sterile 25 ml Erlenmeyer flasks and the flasks were gassed with a mixture of 95 percent oxygen and 5 percent carbon dioxide (Matheson Company) for at least one hour. After gassing, the flasks were tightly stoppered and stored in a refrigerator prior to use.

Preparation of the tissues

The pregnant rats were terminated and the tissues excised in the same manner as described for the *in vivo* experiments. Liver slices 0.5 mm thick were made with a Stadie-Riggs microtome (122); 2 or 3 slices (approximately 0.3 gm) were

Table 2. Composition of the Krebs-Ringer bicarbonate solution

Components[a]	Parts by volume
0.9 percent sodium chloride	104
0.11 M calcium chloride	3
2.11 percent potassium dihydrogen phosphate	1
3.82 percent magnesium sulfate heptahydrate	1
1.3 percent sodium bicarbonate	21
0.1 M sodium fumarate	7
0.3 M glucose	5
Penicillin-streptomycin mixture	1.5

[a]Components are listed in the order in which they must be combined.

used for each incubation. Placentas from the females sacrificed on the 20th day of gestation were also sliced with the microtome and approximately 0.3 gm of slices used per incubation. The small size and fragile nature of the uterine contents from the females sacrificed on the 10th day of gestation prevented the preparation of slices. Therefore, this tissue was lightly homogenized *in toto* and the entire homogenate (approximately 0.5 gm) used for each incubation. Each uterus was spread on a piece of "powder" paper and cut into several longitudinal, narrow strips. Approximately 0.3 gm of

the uterine strips from the 10th-day females and 0.6 gm of strips from the 20th-day females were used for incubation.

Incubation of the tissue preparations

The flasks containing the tissue preparations were pre-incubated at 37°C in a constant temperature, gyrotory shaker (New Brunswick Model G-76) for not less than 10 minutes. Then, 0.5 ml of a sterile solution of L-valine-1-C^{14} in physiological saline (2 μc/ml) was added to each flask followed by a brief gassing with the oxygen-carbon dioxide mixture. The flasks were then incubated for 30 minutes in the presence of the radioactive valine. After the incubation period, the medium was poured off, the tissues rinsed in 5.0 ml of 1 mM solution of "cold" L-valine in 0.1 percent acetic acid, frozen on dry ice, wrapped in aluminum foil, and stored at -35°F for future analysis.

Radioactivity, and protein analysis

Protein was isolated from these tissue preparations in the same manner as was described for the *in vivo* experiments. Also, the methods of plating, counting, and protein determination were identical. Results were calculated as cpm/mg protein.

Patterns of the in vitro incorporation of L-valine-1-C^{14}

The patterns of the *in vitro* incorporation systems were investigated by incubating tissue preparations for either 15,

30, or 60 minutes in the presence of L-valine-1-C^{14} after a 10 minute preincubation. The conditions of these incubations were identical to those used for the earlier in vitro incubations. Livers, uterine contents, and uteri from both E (-) and pellet-fed females sacrificed on the 10th day of gestation were compared. The lipid peroxide content of 1.0 ml of each liver slice incubation medium was measured by the TBA method. The conditions used were identical to those described for determination of the in vivo lipid peroxide content of tissue homogenates.

Nucleic Acid Determinations

A later shipment of females was used to investigate the reproducibility of the in vivo L-valine-1-C^{14} incorporation data obtained earlier and also to obtain tissues for RNA and DNA analyses. The nucleic acids were extracted from homogenates of the tissues according to a modification of the procedure of Schneider (123). The tissues were finely homogenized as described for the protein and lipid peroxide determinations. Water was substituted for the phosphate buffer as a homogenization medium. The homogenates were diluted with water to a final concentration of 0.2 gm tissue/

ml. One ml of each homogenate was mixed with 1 ml of cold 20 percent TCA and centrifuged. The precipitates were resuspended in 2.5 ml of cold 10 percent TCA and centrifuged. Nucleic acids were removed from the precipitates by two heatings at 92°C in TCA solution. Preliminary investigations showed that the length of the heating period and the concentration of the TCA solution required to give complete extraction of the nucleic acids varied with the tissue. This agrees with the observations of Hutchinson and Munro (124). These reviewers also concluded that short TCA extractions lead to incomplete removal of DNA; while longer extractions caused destruction of the DNA. Therefore, it was necessary to investigate several heating periods and TCA concentrations to find the optimal conditions for the extractions. The conditions finally used were:

1. Liver: two 15 minute extractions with 7 percent TCA.
2. Uterine contents from the 10th-day females: two 15 minute extractions with 7 percent TCA.
3. Placenta from the 20-day females: two 10 minute extractions with 5 percent TCA.
4. Uterus: two 15 minute extractions with 10 percent TCA.

All extractions were made with 4.0 ml of TCA solution. The two extracts for each homogenate were combined, the total volume adjusted to 8.0 ml if necessary, and the solution used

for nucleic acid analysis. RNA was determined by the orcinol method according to the procedure of Mejbaum (125):

1. 0.2 ml of the TCA extract was added to 1.0 ml of the reagent which contained 0.1 percent ferric chloride and 1.0 percent orcinol in 12 N hydrochloric acid.
2. Each solution was diluted to 2.0 ml with water, the tubes capped, and the mixture heated in a boiling water bath for 20 minutes.
3. The tubes were rapidly cooled in an ice bath and an additional 2.0 ml of water added.
4. The samples were read at both 600 and 660 mμ against a blank in which the appropriate TCA solution replaced the nucleic acid extract. Absorbance readings were made in a Beckman DU spectrophotometer.

Portions of a RNA solution (Torula, B grade, Calbiochem) were run with each group of determinations to prepare a standard curve.

DNA was determined by the diphenylamine method according to the procedure of Burton (126):

1. 1.0 ml of the TCA extract was added to 2.0 ml of a solution which contained 1.5 gm diphenylamine, 3.0 ml 70 percent perchloric acid, 1.5 ml concentrated sulfuric acid, and 100 ml of glacial acetic acid.
2. Each tube was tightly capped and incubated for 17 hours at 37°C.

3. The samples were read at both 600 and 650 mμ as described for the RNA determinations.

Portions of a DNA solution (Salmon sperm, A grade, Calbiochem) were run with each group of determinations to prepare a standard curve.

The concentrations of the RNA and DNA standard solutions, which had been prepared by weighing the nucleic acids on a Mettler analytical balance, were verified by phosphorus determination. Portions of the standard solutions were taken to dryness and digested with 70 percent perchloric over an open flame. Total phosphorous was then measured according to the method of King (127). The RNA and DNA concentrations calculated from the phosphorous values were used in preparing the standard curves.

Treatment of Data

Tabled data are reported as sample mean ± standard error of the mean. Significance levels of the differences between the means were calculated using the 2-sample, 2-tailed t test (128). These levels are reported as (P <significance level). For example, the term P<0.050 indicates that there is less than a 5 percent probability that the difference found between two means is not real. No P values greater than 0.050 are

usually considered significant in biological research, although values as high as 0.100 are reported as being indicative of a marginal degree of confidence. These criteria were used in interpreting the results of the present research. All levels of significance between corresponding means are tabulated in the Appendix. Only P values less than 0.100 are presented in the body of this dissertation.

RESULTS

Effect of Avitaminosis-E on Growth, Creatine and Creatinine Excretion, and Pregnancy in the Rat

The tocopherol-deficient test diet employed in the present study (Table 1) had not been used previously in this laboratory. Consequently, investigations to verify the ability of this diet to support reasonably normal growth while producing a state of avitaminosis-E in the rats were undertaken.

Growth

The mean body weights which were calculated from the individual, weekly weights of 20 females on each of the three dietary regimens are shown in Table 3. These values cover the growth period from weaning to sexual maturity. As noted, the 77 day values for the pellet-fed and E (-) females were calculated from the weights of less than 20 females since some of the rats had been mated between days 70 and 77.

In this experiment, the mean body weight of the pellet-fed females was significantly higher ($P<0.001$) than the mean body weight of either the E (+) or E (-) females beginning with the 35-day values. No significant differences were

Table 3. Mean body weights[a] of female rats from three dietary regimens measured weekly from weaning to the 77th day of age

Age Days	Rats No.	Dietary regimen[b] Pf	E (+)	E (-)
24	20	57±1[c]	56±1	53±1
29	20	83±1	75±1	74±1
35	20	113±2	101±2	99±3
41	20	140±2	125±3	121±3
47	20	161±2	143±2	138±3
53	20	177±2	162±2	156±3
59	20	194±2	178±3	172±3
65	20	206±2	194±2	186±2
71	20	218±3	198±2	194±2
77	--[d]	224±2	204±2	198±2

[a]Body weights in gm.

[b]Pf indicates rats fed standard pellets (Wayne Lab Blox); E (+) indicates rats fed the tocopherol-deficient diet supplemented with 100 mg dl-alpha-tocopherol acetate per kg; and, E(-) indicates rats fed the tocopherol-deficient diet (Table 1).

[c]Sample mean ± standard error.

[d]17, 20 and 18 rats respectively.

found between the weights of the E (+) and E (-) females during the experimental period.

Table 4. A comparison of the mean body weights[a] of female rats from the three dietary regimens at sexual maturity

Shipment Birth date of rats	Age at weighing Days	Dietary regimen[b] Pf	E (+)	E (-)
7/5/65	70	203±2[c] (27)[d]	194±2 (27)	192±2 (34)
7/28/65	75	209±2 (37)	209±2 (38)	208±2 (38)
1/11/66	75	196±1 (23)	191±2 (20)	191±1 (22)

[a]Body weights in gm.

[b]Described in footnote b, Table 3.

[c]Sample mean ± standard error.

[d]Number of rats indicated in parenthesis.

Each of the females in three later shipments of rats was weighed on the day that mating was begun. The mean body weights calculated from these values are shown in Table 4. The mean weights of the E (+) and E (-) females agreed within 2 gm or less in all three cases. In the first series of values, the mean weight of the pellet-fed females was higher than the mean weight of either the E (-) females ($P<0.001$) or the E (+) females ($P<0.010$). No differences were found among the three groups in the second series of mean weight values (7/28/65 females). The failure to find differences among the values in this second series does not appear to be due to a

reduced rate of growth in the pellet-fed group, but rather to a higher growth rate in the other two groups. The mean body weight of the pellet-fed females was slightly higher than the weights of either the E (-) females ($P<0.050$) or the E (+) females ($P<0.100$) in the third shipment (1/11/66). The 20 gm difference between the mean body weight of pellet-fed females and that of either the E (+) or E (-) females found at 71 and 77 days of age in the earlier shipment of rats (Table 3) was not observed in any of the cases reported in Table 4.

Creatine and creatinine excretion

The results of the experiments in which the levels of creatinuria in urine samples from eight E (-) females and six E (+) females were compared between 7 and 21 weeks of age are shown in Table 5. Since only four metabolism units were available, it was not possible to collect all of the urine samples during a single 24-hour period. The day on which each separate set of analyses was begun was considered the date of analysis.

High creatine excretion was observed in the E (-) rats by 71 days of age or therefore, after having received the tocopherol deficient diet for approximately 45 days. The E (-) females excreted 18 mg of creatine per kg of body weight at 71 days of age and this value increased erratically to a

Table 5. Twenty-four-hour urinary creatine and creatinine excretion by female rats fed a tocopherol-deficient diet and females fed the same diet supplemented with dl-alpha-tocopherol acetate[a]

Age Days	Dietary regimen	Creatinine mg/kg[b]	Creatine mg/kg	Ratio $\frac{\text{Creatine}}{\text{Creatinine}} \times 10^2$	
50	E (-)[c]	36±1[d]	11±1	31±3	
58	E (+)[e]	35±2	12±2		34±6
59	E (-)	34±2	14±4	41±7	
65	E (-)	35±1	12±2	34±5	
71	E (-)	32±2	18±3	56±10	
78	E (-)	35±2	22±5	63±15	
83	E (+)	35±2	13±2		37±3
90	E (-)	36±1	20±3	56±7	
94	E (+)	35±3	8±2		23±3
106	E (-)	39±2	31±7	79±14	
108	E (+)	43±3	12±1		28±4
117	E (-)	37±1	28±6	76±15	
122	E (+)	41±2	8±2		20±10
134	E (-)	37±1	29±4	78±9	
141	E (-)	34±2	22±4	65±12	
156	E (+)	28±2	6±2		21±7

[a]100 mg dl-alpha-tocopherol acetate per kg of diet.

[b]Data expressed per kg of rat weight.

[c]All means for the E (-) rats were calculated from eight females.

[d]Sample mean ± standard error.

[e]All means for the E (+) rats were calculated from six females.

maximum of 31 mg/kg at 106 days of age. Normal E (+) daily creatine excretion averaged between 12 and 13 mg/kg in the rapidly growing rats (up to 108 days of age), but declined to between 6 and 8 mg/kg when growth became slower. The amounts of creatinine excreted by both groups of females were relatively similar and constant during the experimental period.

The results of creatine and creatinine determinations on 24-hour urine samples from females randomly selected from later shipments of rats are shown in Table 6. Increased creatine excretion with correspondingly high creatine-to-creatinine ratios were again found for the E (-) females in comparison to either the E (+) or pellet-fed controls. The amounts of creatinine excreted by the three groups of females were very similar and correlated well with the values found for the E (+) and E (-) rats in the previous experiment (Table 5). The pellet-fed females were found to excrete more creatine than the E (+) females and less than the E (-) females. The similarities among the three series of data from the pellet-fed females are striking. Both the mean creatinine and the mean creatine values from the three series agree within 2 mg/kg. The ratios calculated from these creatine and creatinine excretion values also show good agreement.

One reason for collecting the creatine and creatinine data was to establish the ability of the tocopherol-deficient

Table 6. A comparison of 24-hour urinary creatine and creatinine excretions by female rats randomly selected from different shipments

Shipment Birth date of rats	Age at analysis Days	Dietary regimen[a]	Rats No.	Creatinine mg/kg[b]	Creatine mg/kg	Ratio $\frac{\text{Creatine}}{\text{Creatinine}} \times 10^2$
5/8/65	84	Pf	6	37±3[c]	19±1	51±3
5/8/65	84	E (+)	7	38±1	12±2	32±4
5/8/65	84	E (-)	7	35±3	25±3	71±11
7/5/65	94	Pf	6	39±1	18+4	46+10
7/5/65	94	E (+)	4	43±3	10±2	23±4
7/5/65	94	E (-)	5	37±2	33±3	89±16
4/3/65	86	Pf	8	37±2	20±4	54±8

[a]Dietary regimens described in footnote b, Table 3.

[b]Data expressed per kg of rat weight.

[c]Sample mean ± standard error.

diet to produce a state of E-deficiency in females receiving this ration. A second reason was to determine the approximate age at which positive creatinuria indicative of avitaminosis-E became evident in this group of rats. A third reason was to show that addition of dl-alpha-tocopherol acetate to the tocopherol-deficient diet prevented the production of increased urinary creatine-to-creatinine ratios. The low ratio consistently found for the E (+) group of females indicated a vitamin E-sufficiency in these rats.

The data presented in Tables 5 and 6 show that while the 24-hour creatinine excretions from the three groups of females were similar, creatine excretions were variable. The 24-hour creatine excretion from the E (-) females was greater than that from either of the two control groups after approximately 70 days of age. The creatine-to-creatinine ratios were highest for the E (-) group, intermediate for the pellet-fed group, and lowest for the E (+) group.

Significance calculations were performed on the differences between the means shown in Table 6. The levels of significance are given in Table 21. The relatively large standard errors associated with the creatine values and consequently, with the creatine-to-creatinine ratios made it impossible to establish confidence limits for the observed differences. Therefore, the conclusions drawn regarding the

occurrence of positive creatinuria in the females receiving the tocopherol-deficient diet are based on the consistency in which the elevated creatine excretions were found for the E (-) females in comparison to the two controls.

Weight gain during pregnancy

As described in the Review of Literature, the resorption process in the E (-) female begins on about the 10th day of gestation and is virtually complete by the 20th day of gestation. In contrast, the last one-third of a normal gestation in the rat is the period of rapid fetal growth. Therefore, the weight gain during the first ten days of gestation should be fairly similar for females on each of the dietary regimens followed by marked differences between the E (-) group and the two control groups during the last 10 days of gestation.

Data from 281 pregnant rats had been recorded throughout the course of these investigations. The majority of these animals had been sacrificed on either the 10th or the 20th day of gestation. A random sample of the weight gains of these rats during pregnancy was used to calculate the mean weight gains shown in Table 7.

A mean weight gain of 31 to 32 gm was found for each of the three groups of females during the first 10 days of gestation. While the pellet-fed and E (+) females continued

Table 7. Weight gained during pregnancy by rats from three dietary regimens

Dietary regimen[a]	Rats No.	Mean weight gained gm	
		First 10 days of gestation	First 20 days of gestation
Pf	20	32±1[b]	123±4
E (+)	20	31±1	106±3
E (-)	20	31±1	55±1

[a]Dietary regimens are described in footnote b, Table 3.

[b]Sample mean ± standard error.

to gain weight at a rapid rate during the second 10 days of gestation (91 and 75 gm respectively); the E (-) females gained only 24 gm during the same period ($P<0.001$).

The mean weight gain of the pellet-fed females was significantly higher ($P<0.001$) than that of the E (+) females during the first 20 days of gestation. The difference apparently occurs during the second half of the 20 day period. This increase can probably be explained by an observed tendency towards larger numbers of fetuses in the pellet-fed group.

A visual inspection of the fetuses which were removed from the pregnant rats on the 20th day of gestation was made

routinely. No differences in the number of resorptions, congenital abnormalities, or the number of dead fetuses were found between the E (+) and pellet-fed groups. Furthermore, the number of any of these complications was small in either case.

Six E (+) females were allowed to litter and to rear their pups to weaning. From all appearances, the young were healthy and they grew into normal appearing adults.

Lipid Peroxidation in Tissue Homogenates

Most investigators agree that the TBA reaction gives an index of lipid peroxidation. Malonaldehyde is formed during the decomposition of various polyunsaturated fatty acids (129). It is the malonaldehyde which reacts with TBA to form the red chromogen having an absorption maximum at 535 mμ (130). Therefore, the TBA method does not measure the lipid peroxides themselves, but rather, the products of peroxidation. In the present discussion, the term "lipid peroxide" is used with these facts in mind. It should also be restated that since no satisfactory standard exists the results of the TBA reaction are expressed as the mean absorbance at 535 mμ.

Tenth day of gestation

The in vivo and in vitro lipid peroxide measurements for the tissues from the three groups of females sacrificed on the 10th day of gestation are reported in Table 8. TBA reactants existed in all tissues tested. The mean in vivo value for the livers of the E (-) females was significantly elevated above both the E (+)($P<0.001$) and pellet-fed ($P<0.010$) liver values; while no significant differences were found among the in vivo uterine content and uterine lipid peroxide values for the three groups. All mean in vitro TBA values for homogenates of tissues from the E (-) females were significantly increased ($P<0.001$) over the corresponding values for either of the two control groups on the 10th day of gestation. It is also of interest that both the in vivo and in vitro mean TBA values for the liver of the pellet-fed group were significantly higher ($P<0.050$ and 0.010 respectively) than the corresponding mean values for the liver of the E (+) female on the 10th day of gestation.

More drastic differences are seen when the mean TBA (lipid peroxide) values for the tissues from the E (-) females sacrificed on the 20th day of gestation are compared to the values for the corresponding tissues from the two controls (Table 8). All of the in vivo and in vitro lipid peroxide values found for tissues from the E (-) females are markedly higher ($P<0.001$) than the corresponding values for either

Table 8. *In vivo* and *in vitro* measurement of TBA reactants in homogenates[a] of tissues from rats sacrificed on the 10th and 20th days of gestation

Tissue	Dietary regimen[b]	Gestational day	Rats No.	Lipid peroxide values Absorbance at 535 mμ[c] *In vivo*	*In vitro*
Liver	Pf	10	11	61±6[d]	168±25
	E (+)	10	11	45±3	69±6
	E (-)	10	13	101±11	401±32
	Pf	20	8	82±10	252±24
	E (+)	20	10	36±2	58±4
	E (-)	20	8	168±23	637±31
Uterine contents	Pf	10	14	33±2	67±6
	E (+)	10	11	30±3	57±3
	E (-)	10	16	40±3	98±5
Placenta	Pf	20	8	27±2	46±6
	E (+)	20	11	26±2	44±5
	E (-)	20	8	96±10	198±9
Uterus	Pf	10	11	29±2	41±2
	E (+)	10	8	36±3	43±5
	E (-)	10	11	42±4	72±4
	Pf	20	9	34±3	59±7
	E (+)	20	9	31±3	42±6
	E (-)	20	9	58±6	135±13

[a]Five percent in 0.1 M phosphate buffer, pH 7.4.

[b]Dietary regimens described in footnote b, Table 3.

[c]Mean values multiplied by 10^3.

[d]Sample mean ± standard error.

control. This rise in the number of significant differences can be accounted for by large increases in the amounts of TBA reactive material present in tissues from the E (-) females

between days 10 and 20 of gestation. The increases for the liver and uterus of the E (-) group between days 10 and 20 of gestation were: liver, *in vivo* 66 percent and *in vitro* 58 percent; uterus, *in vivo* 38 percent and *in vitro* 87 percent.

Twentieth day of gestation

Differences were found between the lipid peroxide values for tissues from the two control groups on the 20th day of gestation. Liver lipid peroxides were higher both *in vivo* and *in vitro* for the pellet-fed females ($P<0.001$) than for the E (+) females. While the *in vivo* liver value for pellet-fed females had increased by 34 percent and the *in vitro* value by 50 percent between days 10 and 20 of gestation, the corresponding values for the liver of the E (+) females had decreased by 20 and 16 percent. A higher *in vitro* lipid peroxide content ($P<0.001$) was also found in homogenates of uteri from pellet-fed females in comparison to corresponding values for the E (+) females on the 20th day of gestation.

The process of fetal resorption is therefore, associated with increased concentrations of TBA reactive material in the liver, placenta, and uterus of the vitamin E-deficient rat. Also, the smaller increases found for the liver and uterus of the pellet-fed females could possibly be interpreted to indicate that a normal pregnancy places an additional demand

on the vitamin E reserves of this group of rats.

In Vivo Incorporation of L-Valine-1-C^{14}

Tenth day of gestation

The mean specific activities of protein isolated from the livers, uterine contents, and uteri of rats which had received intraperitoneal injections of L-valine-1-C^{14} on the 10th day of gestation are shown in Table 9. The livers of the pellet-fed females incorporated more radioactive valine into their protein than did the livers of the E (-) females after the first hour of incubation, $P<0.005$. After the second hour of incubation, the protein from the livers of the E (-) females had a higher mean specific activity than did the protein from the livers of the pellet-fed females, $P<0.010$. The values for the liver protein from the E (+) females did not differ significantly from those of the other two groups after either the 1 or 2 hour incubation periods. The three mean liver protein specific activity values were very similar after 4 hours of incubation. These data indicate that the livers of the three groups of females did not differ in their ability to incorporate and retain radioactive valine during the three incubation periods investigated.

The specific activities of the proteins from the uterine contents of the three groups of females were nearly identical

Table 9. *In vivo* incorporation of L-valine-1-C^{14}[a] into tissue protein of rats on the 10th day of gestation

Time of incubation Hours	Tissue	Mean specific activity (cpm/mg protein) Dietary regimen[b] Pf	E (+)	E (-)
1	Liver	45.2±4.3[c](5)[d]	40.0±2.9 (5)	36.7±2.0 (6)
2		29.9±1.9 (7)	32.2±5.3 (5)	38.5±2.9 (6)
4		29.5±3.7 (6)	33.7±1.4 (4)	33.9±1.8 (6)
1	Uterine contents	58.8±3.7 (5)	61.0±2.1 (5)	61.7±3.7 (8)
2		54.2±0.6 (8)	53.4±6.2 (5)	68.8±5.6 (6)
4		63.3±2.1 (5)	68.6±0.9 (5)	71.7±3.5 (5)
1	Uterus	95.2±2.5 (4)	84.5±2.3 (5)	85.5±9.9 (6)
2		78.0±6.5 (7)	71.5±6.6 (5)	89.7±6.5 (5)
4		85.5±4.7 (5)	71.2+4.8 (5)	100.4±4.5 (7)

[a]Intraperitoneal injection of 1 μc per 100 gm body weight.

[b]Dietary regimens described in footnote b, Table 3.

[c]Sample mean ± standard error.

[d]Number of samples shown in parenthesis.

after the 1 hour *in vivo* incubation period. The uterine contents of the E (-) females had incorporated more radioactive valine than did the uterine contents of either control after the second hour, $P<0.100$ in both cases. After 4 hours, the mean specific activity value for the uterine content

protein of the E (-) females was slightly higher ($P<0.100$) than the value for the pellet-fed females, but did not differ significantly from the value for the E (+) females.

Some significant differences were also found among the mean protein specific activity values for the uteri of the three groups of females on the 10th day of gestation. After the first hour of incubation, the mean specific activity value for the protein from the uteri of the pellet-fed females was greater ($P<0.010$) than the corresponding value for the E (+) females. The mean uterine protein specific activity for the E (-) females was higher ($P<0.100$) than the corresponding value for the E (+) females after the second hour of incubation. The specific activity value for the uteri of the E (-) females was greater than the values for uteri from either the E (+) ($P<0.001$) or pellet-fed ($P<0.100$) controls after the fourth hour of incubation. In all three groups of females, the uterine proteins were found to have consistently higher specific activities than either the liver or uterine content proteins.

Twentieth day of gestation

Table 10 shows the *in vivo* radioactive valine incorporation data for rats sacrificed on the 20th day of gestation. After 1 hour of incubation, the mean incorporation values for the livers of both the pellet-fed and E (+) females were

Table 10. *In vivo* incorporation of L-valine-1-C^{14}[a] into tissue protein of rats on the 20th day of gestation

Time of incubation Hours	Tissue	Mean specific activity (cpm/mg protein) Dietary regimen[b] Pf	E (+)	E (-)
1	Liver	45.2±4.0[c](6)[d]	49.8±5.8 (5)	27.4±5.5 (4)
2		38.0±3.7 (6)	33.1±2.5 (6)	31.9±4.4 (4)
4		30.3±2.6 (6)	44.0±4.0 (6)	34.4±2.2 (5)
1	Placenta	30.3±1.3 (6)	43.5±1.9 (6)	4.8±3.6 (3)
2		36.1±3.0 (6)	35.9±1.9 (6)	4.6±2.2 (4)
4		38.7±1.1 (5)	40.6±2.2 (7)	7.7±3.3 (5)
1	Uterus	110.2±7.2 (5)	84.3±19.1(6)	51.9±3.6 (4)
2		130.6±9.3 (5)	110.1±3.7 (5)	51.6±10.4(4)
4		122.1±16.1(6)	134.6±15.9(6)	49.7±4.4 (5)

[a]Intraperitoneal injection of 1 μc per 100 gm body weight.
[b]Dietary regimens described in footnote b, Table 3.
[c]Sample mean ± standard error.
[d]Number of samples shown in parenthesis.

higher than the corresponding value for the E (-) females, $P < 0.025$ and 0.050 respectively. However, no significant differences were found between the liver values for the E(-) females and those for either control after 2 hours of incubation. The mean liver protein specific activity values for the pellet-fed

females and for E (-) females were lower ($P<0.025$ and 0.050 respectively) than the corresponding value for the E (+) females after 4 hours.

The mean specific activities of protein from the placental tissue residues remaining in the uteri of the E (-) females on the 20th day of gestation were low (5 to 8 cpm/mg protein); while the values for the placentas from the two controls were in the range of 30 to 44 cpm/mg protein after the three periods of incubation.

The effects of fetal resorption were also noted in the protein incorporation values for the uteri of the E (-) females in comparison to those of the two controls. While the mean uterine protein specific activity values for the pellet-fed and E (+) controls increased between days 10 and 20 of gestation, the mean protein specific activity value for the uteri of the E (-) females decreased during the same period. The resorption process was virtually complete in the vitamin E-deficient females by the 20th day of gestation. In contrast, the uteri of the control animals were greatly distended and contained rapidly developing litters of young. With these facts in mind, the large differences in activity found between the uteri of the controls and those of the E (-) females are not surprising.

In Vitro Incorporation of L-Valine-1-C^{14}

All of the tissues which had been studied *in vivo* were also studied *in vitro* to determine whether comparable results were obtainable by the two methods. The *in vitro* techniques were employed in hopes of obtaining conclusions more definite than those which had resulted from the *in vivo* experiments. Of special interest were the indications of increased uptake of L-valine-1-C^{14} by the uterine contents and uteri of the E (-) females on the 10th day of gestation (Table 9).

All of the *in vitro* incubations were 30 minutes in length following a 10 minute preincubation. On several occasions, the pH of the Krebs-Ringer bicarbonate medium was measured at the end of the incubation period. The initial pH of 7.4 was found to be maintained during the incubation. Other checks were made on the experimental procedure. Two sets of tissue preparations from rats sacrificed on the 10th days of gestation were run in the cold room ($4^{o}C$). Aside from the lower temperature, all other conditions were identical to those routinely used. None of the proteins extracted from these tissue preparations had a specific activity greater than 4 cpm/mg protein. In other experiments, dried proteins which had been prepared in the usual manner from tissue preparations after normal *in vitro* incubations were extracted with a solution containing 1 mM L-valine in 0.1 percent acetic

acid. The extracts were plated and counted. No radioactivity was found in any of the extracts. A final piece of evidence indicating complete removal of free L-valine-1-C^{14} will be presented in the following section of this Chapter. Linear patterns of protein specific activities, with the lines for each tissue apparently converging at zero specific activity, were obtained when in vitro incubations were carried out for three different time periods (Figure 1).

Tenth day of gestation

The results of the in vitro studies are shown in Table 11. No significant differences were found among the mean values for the in vitro incorporation of L-valine-1-C^{14} by liver slices from the females sacrificed on the 10th day of gestation. To the contrary, the three mean liver protein specific activities agreed within 10 cpm/mg protein (243 to 253 cpm/mg protein). In the case of the uterine contents, the homogenates from the E (-) females incorporated more L-valine-1-C^{14}/mg protein than did the uterine contents from the pellet-fed ($P<0.050$) or the E (+) females, $P<0.005$. The mean specific activities of protein from the uterine content homogenates were considerably higher than those of the liver slices. The former values ranged between 756 and 921 cpm/mg protein.

Table 11. *In vitro* incorporation of L-valine-1-C^{14}[a] into tissue protein of rats sacrificed on the 10th and 20th day of gestation

Tissue	Dietary regimen[b]	Mean specific activity (cpm/mg protein) 10th day of gestation	20th day of gestation
Liver	Pf	253±26[c](7)[d]	447±62 (6)
	E (+)	243±36 (5)	283±34 (6)
	E (-)	250±28 (6)	195±12 (6)
Uterine contents and Placenta[e]	Pf	801±25 (7)	478±51 (6)
	E (+)	756±16 (6)	458±32 (7)
	E (-)	921±50 (6)	53±12 (6)
Uterus	Pf	957±49 (6)	621±52 (5)
	E (+)	849±31 (5)	493±23 (8)
	E (-)	1070±90 (7)	282±20 (9)

[a]One μc per incubation flask.

[b]Dietary regimens described in footnote b, Table 3.

[c]Sample mean ± standard error.

[d]Number of samples shown in parenthesis.

[e]Uterine contents on the 10th day and placenta on the 20th day.

The protein from the uterine strips of the 10th-day E (-) females showed a higher mean specific activity than the uterine strip protein from either the pellet-fed or E (+) females. However, the former value had a high standard error

making calculations of significance difficult. Therefore, while the mean specific activity of the protein from the uteri of the E (-) females was 113 and 221 cpm/mg protein higher than the respective values for the pellet-fed and E (+) controls, the values were not statistically different.

Twentieth day of gestation

The in vitro incorporation data for the tissues excised from the females sacrificed on the 20th day of gestation are also shown in Table 11. The liver slices from the pellet-fed rats incorporated more radioactivity than either the slices from the E (+) ($P<0.050$) or the E (-) ($P<0.001$) females. In turn, the liver slices from the E (+) females incorporated significantly more L-valine-1-C^{14} than slices from the E (-) females, $P<0.050$. While liver slices from both the pellet-fed and E (+) females yielded protein with higher mean specific activities on the 20th day of gestation than on the 10th day (increases of 194 and 40 cpm/mg protein respectively), the corresponding value for the E (-) females decreased by 55 cpm/mg protein during the same period.

Slices of placental tissue residues from the E (-) females incorporated only 53 cpm/mg protein; while placental slices from the pellet-fed and E (+) females incorporated 478 and 458 cpm/mg protein respectively.

The uterine strips from the pellet-fed females incorporated more radioactive valine (621 cpm/mg protein) than the uterine strips from the E (+) females ($P<0.001$) or the strips from the E (-) females, $P<0.001$. The mean specific activities of the uterine protein from the latter two groups were 493 and 282 cpm/mg protein respectively. Both control values were significantly increased ($P<0.001$) over the value for the E (-) females.

Taken alone, the data presented in Table 11 would lead to the conclusion that the uteri of the three groups of females have a higher rate of L-valine incorporation on the 10th day of gestation than on the 20th day of gestation. The results of the *in vivo* incorporation studies presented earlier (Table 11) showed that while uterine uptake of L-valine-1-C^{14} was, indeed, decreased on the 20th day of gestation in the case of the E (-) females, marked increases were evident for the uteri of the two pregnant controls. The *in vitro* observations represent the actual situation (99, 104). It should be emphasized that the present *in vitro* investigations were not designed to compare responses on the two days of gestation, but rather, to compare the three dietary groups on each of the two days.

Patterns of the In Vitro Incorporation of L-Valine-1-C^{14} with Time

Females from the pellet-fed and E (-) groups were used on the 10th day of gestation to obtain patterns of the in vitro incorporation of L-valine-1-C^{14} into the protein of liver slices, uterine content homogenates, and uterine strips with time. These experiments were performed to answer three questions. What is the shape of the incorporation curve for each of the three types of tissue preparations? Are lipid peroxides formed during in vitro incubation of tissue preparations? If elevated amounts of lipid peroxides are formed by the preparations of tissues from the E (-) females, do these peroxides have any effect on the ability of these tissues to synthesize protein? The data for the incorporation of radioactive valine into the protein of the tissues being studied are shown in Table 12. Figure 1 shows the plots of the mean specific activities of the tissue proteins at each of the three incubation times.

Linear plots were obtained for each of the three types of tissue preparations from both pellet-fed and E (-) females. The similarities among the incorporation patterns for the corresponding tissues from the two groups of females can be seen. Slopes of 10.1 and 9.2 were calculated for the incorporation plots of the liver slices from the pellet-fed and

Table 12. *In vitro* incorporation of L-valine-1-C^{14}[a] into tissue protein of pregnant rats[b] after different periods of incubation

Time of incubation Minutes	Dietary regimen[c]	Mean specific activity (cpm/mg protein) Tissue: Liver	Uterine contents	Uterus
15	Pf	123±5[d](10)[e]	391±36 (6)	378±55 (6)
	E (-)	177±21(9)	397±52 (6)	284±57 (6)
30	Pf	298±23(9)	746±28 (5)	918±80 (5)
	E (-)	330±16(10)	784±42 (5)	1051±92 (5)
60	Pf	541±35(8)	1586±120(4)	1913±114(4)
	E (-)	570±27(10)	1777±88 (6)	1874±140(5)

[a]One μc per incubation flask.
[b]Rats sacrificed on the 10th day of gestation.
[c]Dietary regimens described in footnote b, Table 3.
[d]Sample mean ± standard error.
[e]Number of samples shown in parenthesis.

E (-) females respectively; while slopes of 28.1 and 25.9 were calculated for the plots of the uterine content activity values. Slopes similar to those found for the uterine contents were found for the patterns of the uterine strip incorporations. These slopes were 30.5 for the pellet-fed and 30.6 for the E (-) females. In only one case was a significant difference found between the specific activity values

Figure 1. Pattern of the *in vitro* incorporation of L-valine-1-C^{14} into tissue protein after different incubation times. Tissues obtained from pellet-fed and E (-) rats sacrificed on the 10th day of gestation

L_p (○—○) Liver slices from pellet-fed females

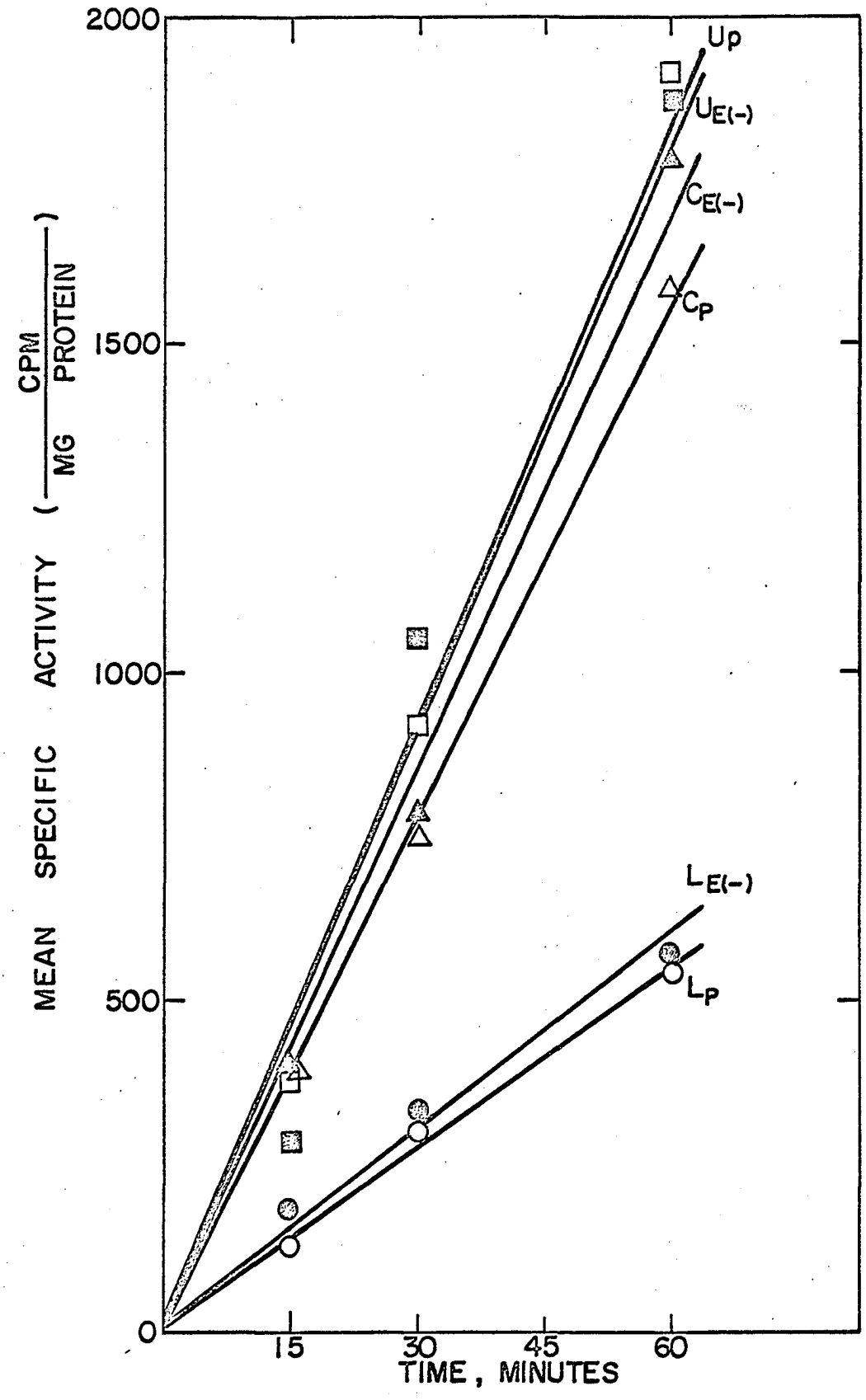
$L_{E(-)}$ (●—●) Liver slices from E (-) females

C_p (△—△) Uterine content homogenates from pellet-fed females

$C_{E(-)}$ (▲—▲) Uterine content homogenates from E (-) females

U_p (□—□) Uterus strips from pellet-fed females

$U_{E(-)}$ (■—■) Uterus strips from E (-) females


2000
1500
1000
500
0
MEAN SPECIFIC ACTIVITY (CPM / MG PROTEIN)
15
30
45
60
TIME, MINUTES
Up
UE(-)
CE(-)
CP
LE(-)
LP

for corresponding tissue proteins shown in Table 12. The liver slices from the E (-) females incorporated more radioactivity after 15 minutes of incubation ($P<0.025$) than did the liver slices from the pellet-fed females.

It was of interest to compare the data from the 30 minute incubations in the present experiments to the corresponding values obtained during the earlier in vitro experiments (Table 11). In both cases uterine strips incorporated the greatest amounts of radioactive valine followed by the uterine contents and liver tissue in decreasing order. The mean specific activity of the protein of the liver slices from the pellet-fed females was increased by 45 cpm/mg protein and the mean specific activity of the protein of the liver slices from the E (-) females was increased by 80 cpm/mg protein in the present experiments. However, decreases of 55 and 137 cpm/mg protein were found for the specific activities of the protein from the uterine contents of the pellet-fed and E (-) females respectively. Increases of 69 and 19 cpm/mg protein were found for the uterine protein specific activities for the pellet-fed and E (-) females respectively. In the present experiments, (Table 12) the significantly increased incorporation of L-valine-1-C^{14} by the uterine contents of the E (-) rats which was found earlier (Table 11) was not reproduced.

Table 13. Formation of lipid peroxides[a] by liver slices from rats sacrificed on the 10th day of gestation

Time of incubation Minutes	Dietary regimen[b]	Rats No.	Lipid peroxide content Absorbance at 535 mμ[c]
15	Pf	12	85±13[d]
	E (-)	12	178±13
30	Pf	8	103±13
	E (-)	15	265±17
60	Pf	8	210±26
	E (-)	13	403±19

[a]One ml of incubation medium analyzed by the TBA method.

[b]Pf (pellet-fed) and E (-) (tocopherol-deficient diet).

[c]Mean values multiplied by 10^3.

[d]Sample mean ± standard error.

Table 13 shows the results of the TBA assays performed on the media after the liver slice incubations. Ball and Engel (131) have demonstrated that lipid peroxidation takes place in the tissue being incubated rather than in the medium. However, a measure of the lipid peroxide content of the medium was found to be an index of the rate of peroxidation in the tissue preparations. The media used for incubations of liver slices from the E (-) females contained more TBA reactant

than did the media used for the slices from livers of the pellet-fed females, $P<0.001$ for each of the three incubation periods. Plots of the data shown in Table 13 (Figure 2) indicate that at zero time there is some TBA reactive material present in both livers. Since a portion of the unused Krebs-Ringer bicarbonate medium was used as a blank in the TBA assay of the media, the reactive material present at the start of the incubation period probably represents the *in vivo* lipid peroxides shown to be present in livers of pellet-fed and E (-) females (Table 8).

Effect of Treatment and Stage of Gestation on the Protein and Nucleic Acid Composition of the Tissues

Wet weights

The wet weights of the tissues being studied were needed for calculations of the protein and nucleic acid data since these latter values are expressed as the amount of component per unit of tissue weight. Also, comparisons of the tissue wet weights were of inherent interest since data of this type has been commonly used in biological research to measure the responses of animals to experimental treatments. The differences among the mean wet weights of the tissues studied in this investigation are presented in some detail in order to

Figure 2. Medium lipid peroxides measured after 15, 30, and 60 minute incubations of liver slices. Slices were prepared from livers excised from pellet-fed and E (-) rats sacrificed on the 10th day of gestation. One ml of incubation medium was used for analysis by the TBA method. Data are expressed as the mean absorbance at 535 mμ

(O — O) Pellet-fed females

(● — ●) E (-) females

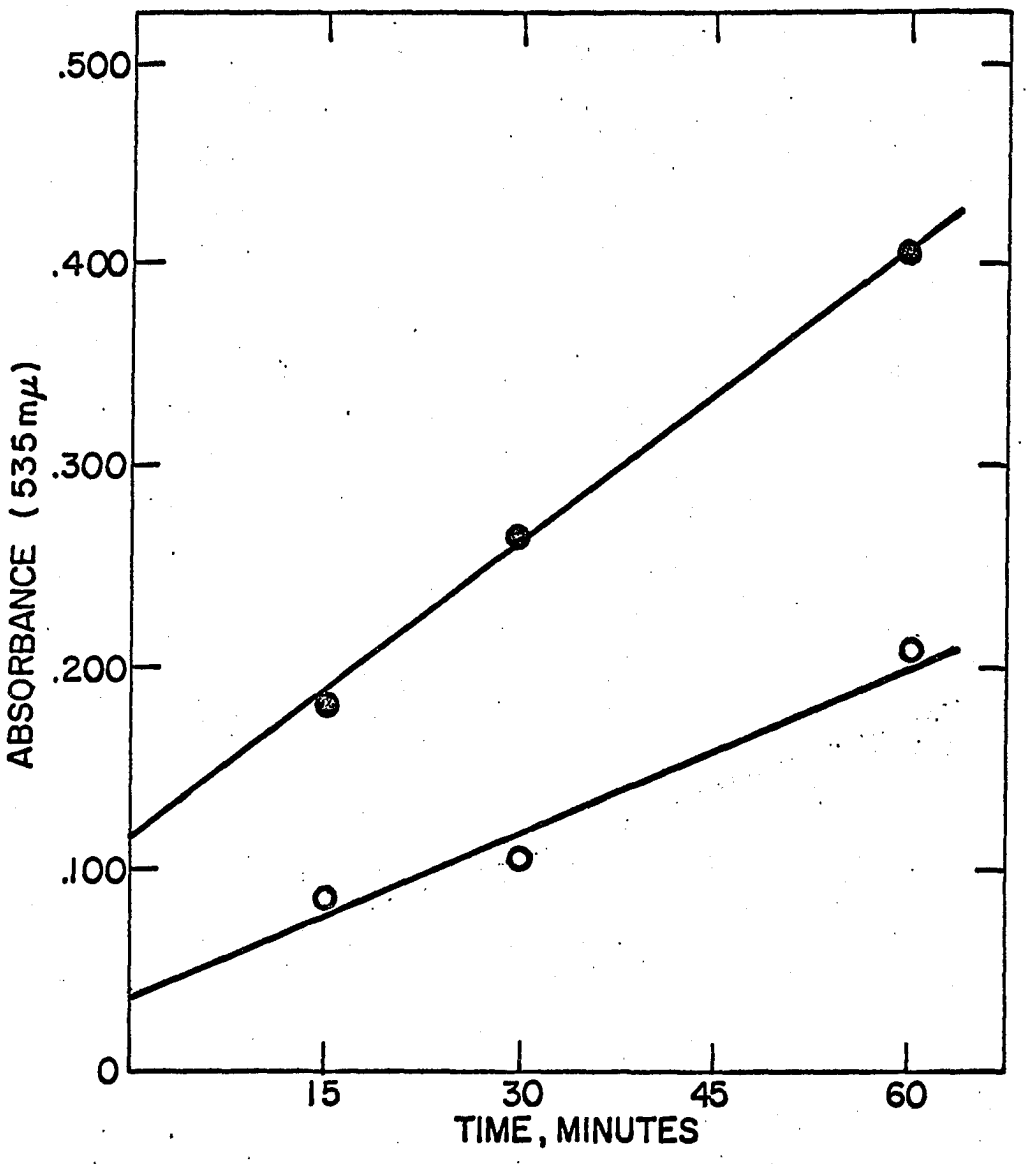

.500
.400
.300
.200
.100
0
ABSORBANCE (535 mμ)
15
30
45
60
TIME, MINUTES

Table 14. Influence of different dietary regimens on the wet weights and protein contents of tissues from rats on the 10th day of gestation

Tissue	Dietary regimen[a]	Rats No.	Mean tissue weight gm	Protein: Mean total mg	Protein: Mean conc.[b] mg/gm
Liver	Pf	9	9.226±0.303[c]	1390±71	151±5
	E (+)	10	8.349±0.284	1284±34	157±4
	E (-)	10	8.846±0.166	1380±40	156±4
Uterine contents	Pf	8	0.629±0.047	63±5	99±2
	E (+)	11	0.528±0.030	53±4	99±2
	E (-)	11	0.509±0.051	50±5	97±1
Uterus	Pf	9	0.741±0.060	69±5	94±3
	E (+)	9	0.658±0.041	59±4	89±3
	E (-)	8	0.773±0.072	70±4	90±3

[a]Dietary regimens described in footnote b, Table 3.

[b]Protein concentration per gm of tissue wet weight.

[c]Sample mean ± standard error.

provide suitable background knowledge for the discussion of their protein and nucleic acid composition.

Table 14 includes the mean wet weights of the livers, uterine contents, and uteri from the three dietary groups of females sacrificed on the 10th day of gestation. The mean

liver weights ranged from 8.349 to 9.226 gm with the pellet-fed females yielding the heaviest livers and the E (+) females the lightest, $P<0.050$. The mean wet weight of livers from the E (-) females was intermediate and not significantly different from either of the other two liver weight values. The mean wet weight of the uterine contents from pellet-fed rats was higher than the corresponding value for either the E (+) ($P<0.100$) or the E (-) ($P<0.050$) females. In the case of the uterus, the three mean wet weights ranged between 0.658 and 0.773 gm, and there were no significant differences among the values.

Table 15 includes the mean wet weight data for tissues from the females sacrificed on the 20th day of gestation. Livers from the pellet-fed females were significantly heavier than livers from the E (+) and E (-) females, $P<0.025$ and 0.001 respectively. In turn, the mean weight of the livers from the E (+) females was higher than the mean weight of the livers from the E (-) females, $P<0.050$. Placental tissue from the E (+) and pellet-fed females weighed 4.971 and 5.301 gm respectively; while placental tissue residues from the E (-) females weighed only 0.775 gm. These values represent the total weight of the placental tissue found in the average uterus of each group of females. Uteri from the pellet-fed females were slightly heavier than uteri from the E (+) females (4.096 and 3.602 gm respectively). These values are

Table 15. Influence of different dietary regimens on the wet weights and protein contents of tissues from rats on the 20th day of gestation

Tissue	Dietary regimen[a]	Rats No.	Mean tissue weight gm	Protein: Mean total mg	Protein: Mean conc.[b] mg/gm
Liver	Pf	11	11.903±0.507[c]	1848±69	157±4
	E (+)	10	10.206±0.448	1609±75	156±9
	E (-)	10	8.947±0.219	1412±35	159±5
Placenta	Pf	10	5.301±0.022	494±27	92±10
	E (+)	9	4.971±0.216	445±27	89±3
	E (-)	8	0.775±0.117	121±17	155±3
Uterus	Pf	9	4.096±0.338	346±18	86±2
	E (+)	10	3.602±0.105	306±12	85±3
	E (-)	10	1.278±0.095	124±10	98±3

[a]Dietary regimens described in footnote b, Table 3.

[b]Protein concentration per gm of tissue wet weight.

[c]Sample mean ± standard error.

considerably higher than the mean wet weight value for the uteri from the E (-) females (1.278 gm). While the weight of uteri from both control groups increased between days 10 and 20 of gestation (3.555 for the pellet-fed females, and 2.944

gm for the E (+) females), uteri from the E (-) females showed an average increase of only 0.505 gm during the same period.

Since the RNA and DNA analyses were run on tissues obtained from later shipments of rats, it was possible to check the reproducibility of the mean wet weight values found for the tissues used for protein analysis (Tables 14 and 15). This second set of tissue weight data is included in Tables 16 and 17. For the most part, the mean wet weights for the tissues from the 10th-day females in the second set of data are in good agreement with the initial values. Livers from the pellet-fed females were again heavier than those from the other two dietary groups ($P<0.050$ for both comparisons); while no significant difference was found between the mean weights of livers from the E (+) and E (-) females. In general, the mean weights of uterine contents and uteri from the three dietary groups were more similar in the second series of weights than in the first. The higher weight initially found for the uterine contents from the pellet-fed females (Table 14) was not reproduced. While the mean weights of uteri from the three groups of females showed good agreement in the data shown in Table 14 (within 0.115 gm), this variation was only 0.078 gm in the data shown in Table 16.

Wet weight data for the rats sacrificed on the 20th day of gestation (Table 17) showed two discrepancies when compared

Table 16. Mean nucleic acid content of the liver, uterine contents, and uterus of rats from three dietary regimens on the 10th day of gestation

Tissue	Dietary regimen[a]	Rats No.	Mean tissue weight gm	DNA Mean total mg	DNA Mean conc.[b] mg/gm	RNA Mean total mg	RNA Mean conc. mg/gm
Liver	Pf	6	9.919[c] ±0.846	24.15 ± 3.26	2.58 ±0.12	115.29 ± 10.22	12.42 ± 0.29
	E (+)	6	8.080 ±0.246	21.98 ± 0.80	2.72 ±0.06	98.67 ± 8.75	12.15 ± 0.88
	E (-)	8	8.110 ±0.219	19.74 ± 1.30	2.44 ±0.14	109.06 ± 3.92	13.48 ± 0.36
Uterine content	Pf	6	0.434 ±0.065	1.05 ± 0.09	2.52 ±0.24	3.56 ± 0.61	8.35 ± 0.81
	E (+)	10	0.455 ±0.15	1.03 ± 0.05	2.26 ±0.29	3.80 ± 0.08	8.45 ± 0.47
	E (-)	11	0.495 ±0.061	1.09 ± 0.14	2.21 ±0.34	4.07 ± 0.50	8.29 ± 0.37
Uterus	Pf	6	0.717 ±0.081	1.60 ± 0.16	2.26 ±0.15	3.15 ± 0.27	4.51 ± 0.32
	E (+)	6	0.684 ±0.059	1.68 ± 0.12	2.50 ±0.15	2.83 ± 0.34	4.33 ± 0.25
	E (-)	8	0.762 ±0.049	1.64 ± 0.11	2.19 ±0.14	2.98 ± 0.26	3.90 ± 0.23

[a]Dietary regimens described in footnote b, Table 3.

[b]Nucleic acid concentrations per gm tissue wet weight.

[c]Sample mean ± standard error.

Table 17. Mean nucleic acid content of the liver, placenta, and uterus of rats from three dietary regimens on the 20th day of gestation

Tissue	Dietary regimen[a]	Rats No.	Mean tissue weight gm	DNA Mean total mg	DNA Mean conc.[b] mg/gm	RNA Mean total mg	RNA Mean conc. mg/gm
Liver	Pf	7	11.440[c] ± 0.393	21.87 ± 2.06	1.90 ±0.14	174.65 ± 12.47	15.37 ± 1.23
	E (+)	8	9.320 ± 0.165	18.06 ± 0.65	1.95 ±0.95	125.29 ± 3.74	13.46 ± 0.39
	E (-)	6	9.489 ± 0.631	18.52 ± 0.95	1.97 ±0.86	119.58 ± 7.89	12.66 ± 0.56
Placenta	Pf	7	6.325 ± 0.457	10.79 ± 1.52	1.68 ±0.15	37.85 ± 4.45	5.96 ± 0.05
	E (+)	8	5.242 ± 0.336	11.10 ± 1.46	2.10 ±0.17	31.92 ± 0.32	6.11 ± 0.04
	E (-)	6	0.776 ± 0.100	1.72 ± 0.22	2.22 ±0.11	1.84 ± 0.20	2.43 ± 0.22
Uterus	Pf	7	4.194 ± 0.196	4.97 ± 0.17	1.19 ±0.03	16.83 ± 0.59	4.04 ± 0.15
	E (+)	8	3.618 ± 0.086	4.32 ± 0.22	1.18 ±0.06	13.98 ± 0.06	3.87 ± 0.16
	E (-)	6	1.216 ± 0.155	2.62 ± 0.37	2.13 ±0.11	6.12 ± 0.57	5.13 ± 0.28

[a]Dietary regimens described in footnote b, Table 3.

[b]Nucleic acid concentrations per gm tissue wet weight.

[c]Sample mean ± standard error.

to the first series of data shown in Table 15. While the mean wet weight of livers from the E (+) females was 10.206 gm in the first series, the E (+) livers used for nucleic acid determinations had an average weight of only 9.320 gm. The latter value was not significantly different from the mean weight of the livers from the E (-) females (9.489 gm). The second discrepancy involved the weights of placental tissues from the two control groups. Wet weights of placentas from the pellet-fed controls were higher ($P<0.010$) than the placentas from the E (+) females in the second series; while the pellet-fed females had yielded only slightly heavier placental tissue (difference of 0.330 gm) in the first series. Agreement within 0.100 gm was found between corresponding mean uterine weights in the two series of determinations.

Tissue protein content

The total protein and protein per gm of tissue wet weight data for tissues from rats sacrificed on the 10th day of gestation are shown in Table 14. The total protein content of the tissues from the E (-) females did not differ significantly from the total protein content of corresponding tissues from either control female. It is of interest to note that the distribution of the total protein values for each tissue correlates directly with the wet weight data for the tissue. That is, the livers, uterine contents, and

uteri with the highest mean wet weights also contained the most protein. It is not surprising, therefore, that good agreement was found among the protein concentration per gm of tissue wet weight data. The three mean liver protein concentration values were found to be between 151 and 156 mg/gm; while the three mean uterine content protein concentration values averaged between 97 and 99 mg/gm. Uteri from the three dietary groups of females contained between 89 and 94 mg of protein per gm of wet weight.

Table 15 shows the total protein and protein per gm of wet weight data for tissues excised from rats sacrificed on the 20th day of gestation. Livers from the pellet-fed females contained more total protein than did livers from the E (+) or E (-) females, $P<0.050$ and 0.001 respectively. The total protein content of livers from the E (+) and E (-) females did not differ significantly. Total protein in the placentas from either the pellet-fed (494 mg) or the E (+) females (445 mg) was considerably higher than the 121 mg of protein found in placental tissue residues from the E (-) females. The total protein in uteri of both control groups of females were also significantly higher ($P<0.001$) than the protein content of uteri from the E (-) females. Calculations of the protein concentrations of tissues from the 20th-day females showed some interesting differences and similarities.

The concentration of protein per gm of liver were similar among the three dietary groups (156 to 159 mg/gm). These protein concentration values are in good agreement with the protein concentration data found for livers of the rats sacrificed on the 10th day of gestation (Table 14). The concentration of protein per gm of placental tissue was higher ($P<0.001$) for placental tissue from the E (-) females than for placental tissues from either group of control females. Also, the mean protein concentration value of 98 mg/gm found for uteri from the E (-) females was higher than the 86 and 85 mg/gm calculated for the uteri of the pellet-fed and E (+) females respectively, $P<0.010$ for both comparisons.

Tissue RNA and DNA

Table 16 shows the total RNA and DNA content of tissues from females sacrificed on the 10th day of gestation, as well as the number of mg of each nucleic acid per gm of tissue wet weight. No significant differences were found between any of the nucleic acid data for tissues from the E (-) females and the data for corresponding tissues from either of the two controls. Good agreement was also found in the nucleic acid values for the tissues from the pellet-fed and E (+) control females. While the mean DNA concentration per gm of liver, uterine content, and uterus tissue were all in the range of 2.19 to 2.74 mg/gm, the mean RNA concentrations of the three

tissues were markedly different. The livers from the three dietary groups of females contained 12.15 to 13.48 mg of RNA/gm; while the three groups of uteri averaged only 3.90 to 4.51 mg RNA/gm. The RNA values for the uterine contents (8.29 to 8.45 mg/gm) were intermediate between the liver and uterine content values.

Table 17 shows the nucleic acid data for the tissues excised from rats sacrificed on the 20th day of gestation. The mean total RNA content of livers from pellet-fed females (174.65 mg) was significantly higher ($P<0.001$) than the total RNA contents of 125.29 and 119.58 mg found for livers from E (+) and E (-) females respectively. However, since the mean wet weight of the livers from the pellet-fed females was also significantly higher than the weights of livers from either the E (+) or E (-) females ($P<0.005$ and 0.025 respectively), the concentration of RNA per gm of liver values were not significantly different among the three dietary groups of rats. Similar observations can be made in regard to the liver DNA values. That is, while the livers from the pellet-fed females contained more DNA than did livers from the E (+) and E (-) females, the DNA concentration values for livers from the three dietary groups were almost identical.

Drastically reduced RNA and DNA contents were found in the placental tissue residues from the E (-) females on the 20th day of gestation (Table 17). While the total RNA

content of placental tissues from the pellet-fed females was 37.85 mg and 31.92 mg for placental tissues from the E (+) females, the placental tissue residues from the E (-) females contained only 1.84 mg of RNA. The DNA content of the placental tissue residues from the E (-) females was only 1.72 mg in comparison to values of 10.79 and 11.10 mg for the placental tissues from the pellet-fed and E (+) females respectively. The nucleic acid composition of the placentas from the two controls were similar on the 20th day of gestation. The increased concentration of DNA (2.10 mg/gm) found for the placentas from the E (+) females in comparison to the value of 1.68 mg per gm of placenta from the pellet-fed females was not statistically significant.

The nucleic acid data for uteri from the three dietary groups of rats sacrificed on the 20th day of gestation are also shown in Table 17. The total RNA and DNA contents and the RNA and DNA concentrations per gm of uterus are in good agreement for uteri from pellet-fed and E (+) controls. Uteri from E (-) females contained less DNA than uteri from pellet-fed females ($P<0.005$) and uteri from E (+) females, $P<0.010$. Uteri from E (-) females also contained significantly less RNA than uteri from either control, $P<0.001$ for both comparisons. However, both the mean RNA and DNA concentrations per gm of uterus were higher for uteri from E (-) females than for uteri from either control; DNA ($P<0.001$ for both comparisons), and RNA ($P<0.005$ for both comparisons).

DISCUSSION

Effect of Avitaminosis-E on Growth, Creatine and Creatinine Excretion, and Pregnancy in the Rats

Growth

The importance of employing two different positive control groups of rats in this investigation can be seen from the mean body weight data shown in Tables 3 and 4. Although the pellet-fed females showed a higher rate of growth than either the E (+) or E (-) females, no significant differences were found between the latter two groups in any of the four replications. Therefore, it can be concluded that the deficiency of dietary vitamin E per se did not affect the growth of the E (-) rats during the 65 to 75 days they were fed prior to being sacrificed.

The palatibility of experimental diets has been found to be inferior to that of standard rat chows in some cases (16, 42). The slightly suboptimal growth found for the E (+) and E (-) females could be due to this factor. It was repeatedly observed that, given a choice, the rats preferred the pelleted chow to the semi-synthetic, powdered test diet. In any case, the differences between the growth responses of the pellet-fed females and those of the E (+) and E (-) females were small in three of the four shipments. Also the E (+) females

proved to be satisfactory controls as judged by all the criteria used in the present investegation.

Creatine and creatinine excretion

An infantile creatinuria which persists for approximately 3 weeks after weaning is known to occur in rats no matter what their diet (2). For this reason, the creatine and creatinine determinations reported in Table 5 were not begun until the rats had reached 50 days of age.

Witting and Horwitt (2) have shown a direct correlation between the rate of development of creatinuria and the degree of unsaturation of various fatty acids in diets fed to rats. The tocopherol-deficient test diet used in the present investigation (Table 1) contained 10 percent stripped lard, a relatively saturated animal fat containing monoenoic oleic acid as its main unsaturated fatty acid (132). Using the established criteria that a creatine-to-creatinine ratio greater than 0.40 is indicative of positive creatinuria (2), the data presented in Table 5 indicate that it required about 45 days for the tocopherol-deficient diet to produce this condition in the E (-) rats. Therefore, the E (-) females were creatinuric at the time of mating (Table 6).

The relatively low creatine-to-creatinine ratios which were calculated from the excretions of the E (+) group of females indicated a state of vitamin E-sufficiency. The low

lipid peroxide contents of the tissues examined from this group (Table 8) along with the demonstrated ability of these females to produce normal appearing young confirmed that the supplementation in their diet (100 mg dl-alpha-tocopherol acetate per kg) was adequate.

The indications of positive creatinuria found for the pellet-fed females (Table 6) was of some concern. However, other investigators have emphasized the marginal tocopherol content of some commercial rat chows (133, 134). This observation might provide a suitable explanation for the slightly elevated creatine excretion found in the urine of the pellet-fed females. Unfortunately, the vitamin E content of the pelleted diet (Wayne Lab Blox) used in the present research has not been determined to the knowledge of this investigator.

Fetal resorption, and weight gain during pregnancy

Evans et al. (80) followed the histopathology of gestation in rats deprived of vitamin E. Their studies showed that death of the fetuses occurred on about the 13th day of gestation and was followed by rapid resorption of the fetal tissues. Urner (135) has divided the weight curve for vitamin E-deficient rats during pregnancy into three sections; normal gain during the first 10 days of gestation, retarded increases

in body weight from day 10 to 15, and a decline from day 15 to 21.

The mechanism of the resorption process which occurred in the E (-) rats during the present investigation was considered to be identical to that described in the classical works of Evans et al. and of Urner. Complete fetal resorption occurred consistently and was accompanied by decreased weight gains (Table 7). Also, there was no reason to believe that the pregnancies of the E (+) and pellet-fed controls were not completely normal in all respects.

In Vivo and In Vitro Lipid Peroxide Content of Tissue Homogenates

Following the convention in this field, the term "lipid peroxidation" has been used in reference to the nonenzymatic oxidation of unsaturated lipid to a peroxide (1, 2, 3, 136, 137). The rate of lipid peroxidation is known to increase when cells are damaged (3). For this reason, homogenization of the tissues prior to their analysis for lipid peroxide content was accomplished as rapidly as possible, and all operations were performed in the cold (4°C). The friable livers, uterine contents, and placentas required only brief homogenization (3 to 4 minutes); while the tenacious uterus muscle required approximately 15 minutes. The possibility

remains, however, that data reported as representing the in vivo peroxide content of the tissues (Table 8) may partially represent peroxides formed during the homogenization process. Whatever their source, a valid comparison of the lipid peroxide content of the homogenates was obtained since all corresponding tissues were treated in an identical manner.

The existence of TBA reactants in homogenates of tissues from vitamin E-sufficient animals does not necessarily represent lipid peroxidation products. Zalkin and Tappel (130) have established the presence of reactants other than lipid peroxidation products which give rise to this background. These include: some sugars, aromatic aldehydes, and other compounds capable of yielding malonylaldehyde under the conditions of the assay procedure. However, because of its high sensitivity these investigators recommend the TBA method for measuring lipid peroxidation products.

Tenth day of gestation

The tissues removed from the E(-) remales on the 10th day of gestation (Table 8) were found to contain more lipid peroxide in vivo than did corresponding tissues removed similarily from control rats. The largest increases were found in the case of the liver. Other investigators have measured the in vivo lipid peroxide contents of tissues from normal and vitamin E-deficient rats (3, 11, 138). Their

results showed that the liver, kidney, and brain contain the largest concentrations of TBA reactants. No possible explanations were given for these observations.

The *in vitro* lipid peroxide values (Table 8) show that incubation at 37°C lead to increases in the peroxide content of all homogenates. It is known that large overdoses of *alpha*-tocopherol are required to suppress *in vitro* lipid peroxidation (117). Bird and Szabo (42) have concluded that differences in *in vitro* increases of TBA chromogen can be attributed to the inherent capabilities of various tissues. That is, the peroxidation of unsaturated fatty acids in a specific tissue is a function of its tocopherol-unsaturated fatty acid concentration ratio. These investigators found that while there were no significant differences in the *in vivo* lipid peroxide contents of tissues from vitamin E-deficient and E-sufficient guinea pigs, all tissues from the E-deficient guinea pigs contained increased amounts of TBA chromogen after 60 minute incubations. Presumably the vitamin E reserves of tissues are rapidly depleted when aerobic lipid peroxidation occurs *in vitro*.

The adequacy of the dl-*alpha*-tocopherol acetate supplementation in the diet of the E (+) females was initially suggested by the low creatine-to-creatinine ratios consistently found for this group (Tables 5 and 6). This assumption has been confirmed by the relatively small amounts of lipid

peroxides found in homogenates of their tissues. Perhaps the *in vivo* peroxide values for the tissues from the E (+) females could be considered to represent the backgrounds for the TBA reaction.

Twentieth day of gestation

While the *in vivo* and *in vitro* lipid peroxide contents of homogenates of livers and uteri from the E (+) females decreased slightly between days 10 and 20 of gestation, increases were found in homogenates of corresponding tissues from the pellet-fed rats (Table 8). The increases were most pronounced in the liver homogenates (34 percent for the *in vivo* lipid peroxide value and 50 percent for the *in vitro* value). The liver is a good storage depot for vitamin E (7). Cheng *et al.* (85) have clearly demonstrated that a normal gestation in the rat causes significant reductions in maternal liver and serum vitamin E contents. These workers suggested that liver tocopherol is labile and varies directly with the increased needs of the rat during gestation. Goldstein and McKay (11) have suggested that the lack of the antioxidant properties of vitamin E may be felt acutely under the metabolic "stresses" of pregnancy in the rat. The creatine-to-creatinine ratios determined for the pellet-fed females (Table 4) indicated that these rats were moderately creatinuric prior to mating. All of these observations taken together lead to the tentative conclusion that the pellet-fed

females were slightly deficient in vitamin E according to the criteria used in the present research. Further, the extent of this deficiency was decreased during gestation.

The process of resorption was accompanied by large increases in the amounts of malonaldehyde or precursors thereof present in homogenates of livers, placentas, and uteri of the E (-) rats (Table 8). The increases were most pronounced in the liver where the in vivo and in vitro lipid peroxide values are 366 and 988 percent higher than the corresponding values for the E (+) females, and 168 and 153 percent higher than those for the pellet-fed females on the 20th day of gestation. Whereas the increases for the placental remains and uteri of the E (-) females are not as large as those for the liver, they nevertheless are highly significant. It is difficult to completely interpret the meaning of the increased lipid peroxide content of the tissues from the E (-) females. As described in the preceding paragraph, there is little doubt that pregnancy with its predominately anabolic reactions would require more antioxidant protection than is normally required by the virgin female. Eclampsia of pregnancy has been shown to occur during the last 2 or 3 days of gestation in rats fed a diet containing oxidized cod liver oil (11). Elevated amounts of lipid peroxides were found in the livers and placentas of these females on the 21st day of gestation. Also,

the transport of radioactive sodium across the placentas of these rats was impaired during the last one-third of gestation (101). From the results of these experiments, Goldstein and co-workers concluded that a state of vitamin E deficiency might have been present in their rats and that cellular damage might have occurred due to insufficient antioxidant protection of vitamin E. Other references to published reports describing the toxic effects of lipid peroxides have been given in the Review of Literature (6, 109, 110). It is interesting to speculate that resorption might represent a mechanism of self preservation in the vitamin E-deficient rat. Pregnancy could be terminated to prevent the production of lethal or damaging amounts of toxic lipid hydroperoxides.

A second factor involved in the process of resorption is the catabolism of fetal and maternal tissues in the return to a virgin-like state. Others have found that the destruction of tissue is associated with release of lysosomal enzymes (55, 56) and invasion of the damaged tissue by phagocytic cells (139, 140). It is possible that the increased lipid peroxide values in the E (-) females on the 20th day of gestation might be partially explained by the presence of these products of catabolism. That is, the products of lipid degradation, if present, might be more easily oxidized than is the case for the intact lipid molecule. Tappel (137) has presented considerable evidence to show that hematin catalyzed, non-enzymatic peroxidation is the primary method of

pathological unsaturated fat oxidations in living organisms. It would be of interest to induce fetal resorption by some method other than vitamin E-deficiency and determine its effect on the lipid peroxide contents of the maternal tissues. Actual comparisons of the vitamin E content of tissues from E (-) females before and after resorption would also be of help in answering the questions raised here.

In Vivo Incorporation of L-Valine-1-C^{14}

The intraperitoneal injection of 1 μc of L-valine-1-C^{14} per 100 gm of body weight represents the introduction of only 35 μg of L-valine into a 300 gm rat since the specific activity of the radioactive valine was 9.8 mc/mM. After injection, the valine was absorbed into the blood, circulated through the body, concentrated to different degrees by the various body tissues, and incorporated into protein. One tissue may incorporate amino acids into protein in situ, whereas another tissue might incorporate amino acids into protein which may be lost from the tissue. An example of the latter alternative might be the synthesis of plasma protein by the liver.

Glycine-1-C^{14} was used as the radioactive tracer in preliminary in vivo incorporation experiments. Since extremely variable protein specific activities were obtained,

the glycine was replaced by the more "inert" L-valine. Valine has a small pool size and is not commonly converted to other amino acids (141). Other workers have reported variable incorporation results when radioactive glycine was employed as a carrier (104).

Tenth day of gestation

Indications were found that the uterine contents and uteri of the E (-) females incorporated more L-valine-1-C^{14} into their protein than did the corresponding tissues of either the pellet-fed or E (+) controls (Table 9). However, the differences were too small to be considered conclusive evidence of increased uptake. Urner (135) found pathological changes in uteri of vitamin E-deficient rats on the 10th day of gestation. The uteri developed a softening of the implantations sites which showed a blue discoloration due to blood in the amniotic cavity. Evans et al (80) observed enlarged blood sinuses in the decidua subplacentalis and trophoblast regions of 10th day placentas from E-deficient rats. Apparently these histopathological changes, assuming they also occurred in the E (-) females during the present investigation, did not affect the ability of the uterine contents of the E (-) females to concentrate L-valine-1-C^{14}.

Dinning et al. (142) have shown that the leg muscle of the dystrophic rabbit incorporates more C-14 into its protein

than does the leg muscle of the vitamin E-deficient rabbit. The muscles were excised 4 hours after intraperitoneal injection of the labeled precursor. Century and Horwitt (70) found increased uptake of formate-C^{14} into skeletal muscle nucleic acid of vitamin E-deficient rats at both 2 and 4 hours after intraperitoneal injection. This increased synthesis of rat muscle nucleic acid resembles that observed for DNA in skeletal muscles of dystrophic rabbits (12). Other examples of increased metabolic activity in tissues from E-deficient, experimental animals have been reported (73, 75, 76). In general, it is agreed that gross pathological changes caused by vitamin E-deficiency are associated with increased lipid peroxidation, increased lysosomal enzyme activity, and increased synthesis of protein and nucleic acid. It is conceivable, therefore, that the pathological alterations found in embryos, placentas, and uteri of E-deficient rats might also be associated with similar metabolic derangements. It would be of interest to study lipid peroxidation, lysosomal enzyme, and protein and nucleic acid synthesis patterns of uteri and embryos of E-deficient rats between days 8 and 14 of gestation.

Twentieth day of gestation

The placental tissue residues and uteri of the E (-) females showed decreased uptake of L-valine-1-C^{14} on the 20th

day of gestation (Table 10). While the specific activities of uterine proteins from the two control groups increased between days 10 and 20 of gestation, a decrease was found in the case of the E (-) females. Resorption was nearing completion in the E (-) females by the 20th day of gestation, and they were returning to a virgin-like state. In contrast, the development of the fetuses in the two groups of control females was nearing completion at this time. Therefore, the differences in protein synthesis found between the control and E (-) tissues can, most simply, be said to reflect the physiological states of these tissues.

It would be of interest to measure the catheptic activity present in the uteri of E (-) females between days 10 and 20 of gestation. The increased catheptic activity which occurs in the uterus of the rat following normal partuition (114) might also have occurred following prenatal fetal resorption.

In Vitro Incorporation of L-Valine-1-C^{14}

Short-term incubations of tissue preparation under carefully controlled environmental conditions is commonly referred to as organ maintenance. Experiments of this type are especially useful when the results can be compared to data obtained *in vivo*. One decided advantage of the *in vitro* technique is the ability to study the metabolism of a single

tissue *per se*. Also, the *in vitro* incubation of tissues allows the investigator to differentiate between differences due to actual metabolic variation and those due to transport and permeability phenomena in the intact animal. Diehl (78) emphasized the desirability of using *in vitro* methods for studying protein metabolism in tissues from dystropic rabbits. He argued that *in vivo* investigations could be influenced by possible effects of E-deficiency on blood supply and capillary permeability.

Tenth day of gestation

Data obtained from these short-term incubations *in vitro* (Table 11) were in good agreement with those found after longer-term incubations *in vivo* (Table 9). That is, indications that the uterine contents and uteri from E (-) females incorporated more radioactive valine into their protein than did corresponding tissues from the control rats were found. However, the increases were again too small and variable to warrant a concrete statement to the effect that elevated incorporation rates are a reality in these tissues.

In a recent publication, Diehl (143) presented evidence to indicate that the increased *in vivo* incorporation of radioactive glycine into the protein of skeletal muscles from dytrophic rabbits might be partly due to increased membrane permeability. A given dose of labeled glycine, or other

amino acid, might be incorporated faster because it reaches the amino acid pool of the cells more rapidly. Diehl incubated diaphragms isolated from E-deficient and control rabbits in media containing alpha-aminobutyric acid-1-C^{14}, a non-metabolizable amino acid (144). TCA extracts of diapragms from E-deficient rabbits contained more alpha-aminobutyric acid than extracts of diaphragms from E-sufficient rabbits after incubation. These data lead to the tentative conclusion that the increased incorporation of glycine into the protein of tissues deficient in vitamin E might be a secondary effect representing an attempt to compensate for glycine which has "leaked out" of the cells. This conclusion supports the hypothesis that lipid peroxidation damage to membranes might be the primary cause of the symptoms of vitamin E-deficiency. (1).

Twentieth day of gestation

The differences found between the in vitro L-valine-1-C^{14} incorporation data for the E (-) females and those for the E (+) and pellet-fed controls were more pronounced on the 20th than on the 10th day of gestation (Table 11). Slices of livers from the pellet-fed females showed a marked increase in radioactive valine incorporation between the 10th and the 20th days of gestation. Little and Lincoln (104) also found increased incorporation of L-valine-1-C^{14} into the protein

of rat liver slices as pregnancy progressed. These authors compared the incorporation at 6 days of gestation with the uptake at 16 days of gestation. While the incorporation of radioactive valine into the liver slice protein of the E (+) females was also greater on gestational day 20 than on day 10, the increase of only 40 cpm/mg protein was slight in comparison to the increase of 194 cpm/mg protein found for the pellet-fed rat. The reason for the failure to find a larger increase in the case of the E (+) females is unclear. Most interesting, however, was the observation that the incorporation of radioactive valine by liver slices from the E (-) females not only was significantly lower than either of the two controls on the 20th day of gestation, but was actually decreased in comparison to the incorporation found on the 10th day of gestation.

The lack of protein biosynthesis in the placental tissue residue removed from the uterus of the E (-) females on the 20th day of gestation (Table 11) was not unexpected since these tissues were in an advanced state of resorption. In contrast, the placental slices from the two control groups incorporated relatively large amounts of radioactive valine. Although these control placentas were not increasing in size during the last week of gestation (99), a considerable amount of transport and biosynthesis was occurring in this vital tissue (91).

The data presented in Table 11 also show that uteri of the E (-) females were less metabolically active than those of either of the two controls as judged by their ability to incorporate L-valine-1-C^{14} into protein during *in vitro* incubation. The reason for this reduced activity in the case of the uteri from E (-) females is possibly that they no longer contain developing litters of young and are returning to the less active, nonpregnant state.

Patterns of the *In Vitro* Incorporation of L-Valine-1-C^{14} with Time

Three types of tissue preparations were used in these *in vitro* studies; namely, slices, homogenates, and strips. All of these preparation methods have been used extensively by other investigators (104, 145, 146). It was necessary to determine whether the 30 minute incubation period selected for the *in vitro* incubations described previously (Table 11) represented an incubation time during which there was a linear pattern of uptake for each type of tissue preparation. Since Blackard *et al.* (147) have suggested that lipid peroxides formed during *in vitro* incubations might influence the metabolic activity of the tissues being incubated, it also was necessary to compare responses of tissues from control and E (-) females. Data in Table 8 show that homogenates of

tissues from the pellet-fed females produced greater amounts of lipid peroxides than did homogenates of tissues from the E (+) females. The same data demonstrate that, among the tissues studied, the liver produces the greatest amounts of lipid peroxides both in vivo and in vitro. Keeping these facts in mind, the initial experiments involving the L-valine-1-C^{14} incorporation patterns were designed. First, it was decided to compare the pellet-fed and E (-) females rather than the E (+) and E (-) females since any differences found between the former two groups due to lipid peroxidation damage would most likely be found between the latter two. Second, portions of the liver slice incubation media were taken for lipid peroxide comparison because it was decided that inherent differences would be more apparent here than in media used for either uterine content homogenates or uterine strips. Third, 15, 30 and 60 minute incubation periods were selected to show the pattern followed on either side of the 30 minute period used in the earlier experiments.

Almost identical linear incorporation patterns were obtained for corresponding tissues from the pellet-fed and E (-) rats (Figure 1). It can also be seen that the protein specific activity values for the 30 minute incubations (Table 12) are in good agreement with those found earlier (Table 11) for corresponding tissues. In the present experiments, however, no significant differences were found between the

specific activities of protein from uterine contents and uteri of E (-) females and those of pellet-fed controls. This observation sheds further doubt on the early indications (Tables 9 and 11) that the uterine contents and uteri of the E (-) females incorporate increased amounts of radioactive valine into their protein.

Elevated concentrations of malonaldehyde or precursors thereof were found in the incubation media used for liver slices from the E (-) females (Table 13). Apparently the increased lipid peroxide formation did not affect the ability of the liver slices to incorporate radioactive valine into protein. Blackard et al. (147) suggested that lipid peroxides formed during in vitro incubations might cause metabolic derangements in the tissue preparation being studied. Perhaps the effects of the increased peroxides would have been revealed had the incubations been continued for a longer duration.

It should be emphasized that additional experiments would have been undertaken had any of the types of tissue preparations failed to show linear incorporation patterns or if the patterns for the tissues from E (-) females differed from those for the pellet-fed controls.

Effect of Diet and Stage of Gestation on the Protein and Nucleic Acid Content of the Tissues

A study of the protein, RNA, and DNA content of the various tissues studied in this investigation was undertaken to provide a clearer picture of their composition.

Wet weights

Wet weights *per se* are not very satisfying criteria for comparison, especially in the case of friable tissues such as uterine contents on the 10th day of gestation. Evaporation of tissue moisture and the presence of adhering, extraneous material are probably the two largest sources of error in obtaining uniformly comparable weights. Reasonable precautions were taken during the present investigation to minimize these errors.

While the livers, uterine contents, and uteri from the three dietary groups had approximately equal weights on the 10th day of gestation, there were drastic reductions in the weights of placentas and uteri from the E (-) rats on the 20th day of gestation (Tables 14, 15, 16 and 17). Even the two positive control groups could be distinguished by differences in the mean wet weights of their livers on the 20th day of gestation. Wet weights of livers from the pellet-fed females consistently were greater than the weights of livers

from the E (+) females. It has been shown (Table 6) that each of the three groups of females gained an average of 31 to 32 gm during the first 10 days of gestation. Table 6 also shows that resorption in the E (-) females was associated with reduced weight gains during the second 10 days of gestation in comparison to the marked increases found for the two groups of controls. The pellet-fed females gained significantly more weight during the second 10 days of gestation than did the E (+) females. Therefore, there was a direct correlation between the mean body weights of the rats and the mean weights of the tissues studied from these rats on both the 10th and 20th days of gestation.

Protein content

Most investigators use either the micro-Kjeldahl or the bieret procedure to measure the protein content of animal tissues. The Lowry method was used in the present investigation because of its greater sensitivity. This increased sensitivity was needed to measure the protein content of the relatively dilute solutions employed. Fisher and Leathem (92) have determined the protein contents of livers, placentas, and uteri isolated from rats sacrificed on the 21st day of gestation. They employed the mico-Kjeldahl procedure. Results using the Lowry procedure (Table 15) are in good

agreement with those of Fisher and Leathem. The two sets of data are compared in Table 18.

No reports have been found in the literature in which the protein content of tissues excised from rats on the 10th day of gestation are presented. Perhaps the data shown in Table 14 are unique in this regard. The livers, uterine contents, and uteri of the E (-) females did not differ from those of either control in total protein content or in mg of protein per gm of tissue wet weight on the 10th day of gestation. These data were not unexpected since preceding data (Tables 9, 11, and 12) have shown that the tissues of the E (-) females are normal in their protein synthesizing ability on the 10th day of gestation.

The protein contents of livers from the three groups of females (Table 15) correlate with their wet weights. No differences in protein concentration/gm were found for livers from the E (-) females in comparison to values for livers from either control. However, significant differences were found when placental and uterine values were compared. Placental tissue residues in uteri of E (-) females were in an advanced state of resorption. While these residues contained much less total protein than did normal placentas, their protein concentration actually was significantly higher. This observation can be explained by a marked loss of moisture from the

Table 18. A comparison of the protein concentrations of rat tissues as determined by two different analytical procedures

Tissue	Group of rats[a]	Mean protein concentration mg/gm wet weight	Analytical procedure
Liver	Pf	157 ± 4[b]	Folin
	E (+)	156 ± 9	Folin
	FL	139 ± 6	micro-Kjeldahl
Placenta	Pf	92 ± 10	Folin
	E (+)	89 ± 3	Folin
	FL	99 ± 2	micro-Kjeldahl
Uterus	Pf	86 ± 2	Folin
	E (+)	85 ± 3	Folin
	FL	86 ± 3	micro-Kjeldahl

[a]Pf (pellet-fed) and E (+) females studied in the present investigation (Table 15); FL (Fisher and Leathem) studied on 21st day of gestation (92).

[b]Sample mean ± standard error.

resorbing placental material. The same trend was found for uteri from the E (-) females. That is, while the mean protein content of the uterus was decreased in comparison to control values, its mean protein concentration increased. Imbibition of water into the rat uterus is known to occur during preg-

nancy (148). Assuming this imbibition does not occur in the uterus of the resorbing female, the increased protein concentration of this tissue could be explained by differences in moisture content.

Nucleic acid content

The mean RNA and DNA contents and concentrations found in the liver, uterine contents, and uterus were comparable in all groups on the 10th day of gestation (Table 16). The magnitude of the DNA and RNA values found for the livers are in good agreement with previously published results (149). To the knowledge of the present investigator, the RNA and DNA values for the uterine contents and uterus are without precedent in the published literature.

Interesting results were obtained from the measurement of the RNA and DNA contents of livers, placentas, and uteri from the three groups of females on the 20th day of gestation (Table 17). In the case of the livers, the increases in wet weight between days 10 and 20 of gestation were associated with increases in RNA content, but total liver DNA content decreased in all three groups during the same period. The reason for this slight reduction in DNA content is not known. Other workers have found stationary or even slightly increased levels of liver DNA during gestation in the rat (97). An increase of 24 percent was found in the RNA concentration of

the liver of the pellet-fed females between days 10 and 20 of gestation; while a corresponding increase of 12 percent was found for the E (+) females. A more nominal increase (6 percent) was found in the liver of the E (-) group. Campbell and Kosterlitz (105) have reported the presence of "excess RNA" in livers of pregnant rats during the last week of gestation. These investigators suggest that secretions of viable placentas are responsible for this increased RNA concentration. The data in Tables 15 and 17 show that the pellet-fed females average more placental material than the E (+) females on the 20th day of gestation. In light of the suggestion of Campbell and Kosterlitz, the existence of more RNA per gm of placenta in the pellet-fed females could be postulated to correspond to more placental secretion. Of course, the placental tissue remaining in the uteri of E (-) females on the 20th day of gestation was not viable and could not support liver RNA synthesis.

Marked differences in nucleic acid content were found between the placental material from the E (-) females and that of either control group. Only small amounts of RNA and DNA remain in the placental tissue residues of the E (-) females. The placentas of the pellet-fed and E (+) females were similar in their nucleic acid composition. Fisher and Leathem (92) reported the RNA and DNA composition of placentas from rats sacrificed on the 21st day of gestation. They found

an RNA concentration of 6.65 mg/gm and a DNA concentration of 1.92 mg/gm. These data are in agreement with the values found in the present study; namely, RNA 6.11 mg/gm and DNA 2.10 mg/gm for the E (+) females and, RNA 5.96 mg/gm and DNA 1.68 mg/gm for the pellet-fed females.

The RNA and DNA content of uteri from both control groups increased between the 10th and 20th days of gestation, but the concentration of the nucleic acids per gm of uterus decreased. This decrease in concentration was probably due to an increased imbibition of water into the uterus as pregnancy progressed (148). Uteri of the E (-) females showed only slight increases in wet weight and nucleic acid content between days 10 and 20 of gestation. In contrast to the control uteri, the DNA concentration stayed constant and the RNA concentration increased in the case of the E (-) uteri. Therefore, these data clearly differentiate the uteri from the E (-) group from those of the two controls.

The protein and nucleic acid data presented in Tables 14 thru 17 represent the first reported attempt to compare tissues of normal pregnant rats with those of rats in which resorption has been induced by a deficiency of vitamin E during the gestation period.

SUMMARY

Studies were undertaken in an attempt to correlate the complex process of fetal death and resorption in vitamin E-deficient rats with some metabolic derangements commonly associated with avitaminosis-E.

Female, albino rats were maintained from weaning on one of three diets; namely, standard rat pellets, basal tocopherol-deficient ration and, basal tocopherol-deficient ration supplemented with dl-alpha-tocopherol acetate. The effects of the three dietary regimens on the growth of the rats were noted. While the pellet-fed females gained more weight than either the E (+) or E (-) females, no retardation of growth due to a lack of vitamin E was found in the E (-) group.

Increased urinary excretion of creatine has been associated with a state of avitaminosis E in several species of animals. Therefore, the ability of the tocopherol-deficient ration to produce positive creatinuria was tested. The E(-) females were found to be creatinuric by approximately 70 days of age; while the creatine-to-creatinine ratios for the E (+) females were consistently low. Indications of a moderate degree of creatinuria were found for the pellet-fed females at sexual maturity.

When the females reached 75 days of age, mating to normal males was begun. While the three groups gained approximately

the same amount of weight during the first 10 days of gestation, significant differences were found during the second 10 days of gestation. Fetal death and resorption occurred consistently in the E (-) females and were accompanied by reduced weight gains during the last week of gestation. Investigations involving the three groups of pregnant rats were confined to the liver, uterine contents, and uterus on the 10th day of gestation and to the liver, placenta, and uterus on the 20th day of gestation.

The main biological function of vitamin E is considered to be the inhibition of the oxidation of unsaturated lipids. Both the in vivo and in vitro lipid peroxide content of the tissues studied were determined using the TBA reaction. The mean in vivo lipid peroxide content of livers from the E (-) females was significantly higher than that of livers from either control group on the 10th day of gestation. By the 20th day of gestation, all of the tissues studied from the E (-) females contained higher in vivo levels of lipid peroxides than did corresponding tissues from either of the two control groups. More drastic differences were found among the in vitro lipid peroxide data. The mean lipid peroxide contents of all tissues studied from the E (-) females were significantly higher than the corresponding values for the tissues from either control on both the 10th and the 20th days of gestation. Further, the mean in vitro lipid peroxide

content of livers from the pellet-fed females was higher than the value for livers from the E (+) females on both the 10th and 20th days of gestation.

Since protein synthesis plays a vital role in the anabolic process of pregnancy, this parameter was selected as the initial metabolic system to be studied. The in vivo incorporation of L-valine-1-C^{14} into the protein of the tissues studied was measured at 1, 2, and 4 hours after intraperitoneal injection. The three groups of females did not show any consistent differences in their ability to incorporate and retain radioactive valine on the 10th day of gestation. Resorption was virtually complete in the E(-) females by the 20th day of gestation. The placental tissue remaining in the uteri of these rats was in an advanced state of resorption and did not incorporate radioactive valine. The uteri themselves incorporated significantly less valine than did uteri from either of the pregnant controls.

Organ maintenance experiments were begun to more clearly differentiate the tissues from the three groups of females. Liver and placental slices, uterine content homogenates, and uterine slices were incubated in Krebs-Ringer bicarbonate medium containing L-valine-1-C^{14}. A 10 minute preincubation was followed by a 30 minute incubation in the presence of radioactive valine. No reproducible differences were found among the three groups of rats on the 10th day of gestation,

but indications that uterine contents and uteri from E (-) females did incorporate more radioactivity than corresponding tissues from control groups were found. The incorporation of L-valine-1-C^{14} by preparations of tissues excised from the E (-) females on the 20th day of gestation were significantly lower than the corresponding values for the tissues from the two controls in which normal litters were nearing full development. Both liver and uterine preparations from the pellet-fed females incorporated significantly more radioactivity than did similar preparations from the E (+) females on the 20th day of gestation.

Patterns of the <u>in vitro</u> incorporation of L-valine-1-C^{14} by preparations of tissue from pellet-fed and E (-) females sacrificed on the 10th day of gestation were determined. Linear plots of protein specific activity against incubation time were obtained in each case. The lipid peroxide content of the medium was measured after each liver slice incubation. The mean peroxide content of the media used for the liver slices from the E (-) females was significantly higher than in those used for the liver slices from the pellet-fed females after each time period.

Total protein, RNA, and DNA were determined quantitatively in each of the tissues studied. Using the average wet weight of each tissue, the concentrations of the three components were calculated. The protein and nucleic acid composi-

CONCLUSIONS

The results obtained under the described experimental conditions lead to the following conclusions:

Specific Conclusions

Tenth day of gestation

1. Homogenates of the liver, uterine contents, and uterus of the vitamin E-deficient rat produce more lipid peroxide than those of control rats during aerobic incubations.

2. The total protein, RNA, and DNA contents of these tissues from the E-deficient rat are normal.

3. The incorporation of L-valine-1-C^{14} into the protein of these tissues from the E-deficient rat could not be distinguished from control values after *in vivo* and *in vitro* incubations.

Twentieth day of gestation

1. Lipid peroxidation is markedly increased in the liver, uterine contents, and uterus of the E-deficient rat between the 10th and 20th days of gestation.

2. The liver and uterus of the E-deficient rat contain less total protein, RNA, and DNA, and incorporate less L-

valine-1-C^{14} than those of control rats.

General Conclusions

1. The amount of vitamin E in the diet affects the amount of lipid peroxidation in the liver of the rat during a normal gestation.

2. Lipid peroxides formed in tissues of the E-deficient rat do not significantly alter their total L-valine-1-C^{14} incorporation during *in vitro* incubation.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Byron H. Thomas, my advisor and research director, for his assistance in all aspects of my Ph.D. program.

Valuable information concerning the techniques used in this investigation was obtained during enlightening discussions with Dr. Jack Horowitz and with Dr. Walter Hearn. The organ maintenance experiments were performed according to detailed directions given by Dr. John Arnold, Department of Zoology. Mr. Fred Sick cared for the rats and was of assistance in all aspects of the animal work. My fellow students were very cooperative in making laboratory equipment available when needed. I wish to express my appreciation to the persons mentioned above. Without their help this dissertation could never have become a reality.

My wife, Rosalee, deserves credit for typing the early drafts of this dissertation and for her daily encouragement and understanding during its preparation.

Financial support was provided by a Procter and Gamble Predoctoral Fellowship, and the Agricultural Experimental Station, Iowa State University.

APPENDIX

Table 19. Level of significance of differences between means shown in Table 3

Age Days	Means compared[a]		
	P and E (+)	P and E (-)	E (+) and E (-)
24	>0.500	>0.500	>0.500
29	<0.200	<0.400	>0.500
35	<0.001	<0.001	>0.500
41	<0.001	<0.001	>0.500
47	<0.001	<0.001	>0.500
53	>0.001	>0.001	<0.400
59	<0.001	<0.001	<0.400
65	<0.001	<0.001	<0.200
71	<0.001	<0.001	>0.500
77	<0.001	<0.001	<0.400

[a]P (pellet-fed females); E (+) (females fed tocopherol-deficient diet supplemented with dl-alpha-tocopherol acetate); and, E (-) (females fed tocopherol-deficient diet).

Table 20. Level of significance of differences between means shown in Table 4

Shipment Birth date of rats	Means compared		
	P and E (+)	P and E (-)	E (+) and E (-)
7/5/65	<0.010	<0.001	>0.500
7/28/65	>0.500	>0.500	>0.500
1/11/66	<0.100	<0.050	>0.500

Table 21. Level of significance of differences between means shown in Table 6

Shipment Birth date of rats	Nature of data	Means compared		
		P and E (+)	P and E (-)	E (+) and E (-)
5/8/65	Creatinine	>0.500	>0.500	>0.500
	Creatine	>0.500	<0.400	<0.200
	Ratio	<0.400	<0.500	<0.200
7/5/65	Creatinine	>0.500	>0.500	>0.500
	Creatine	<0.500	<0.400	<0.400
	Ratio	<0.200	<0.500	<0.025

Table 22. Level of significance of differences between means shown in Table 7

Nature of data	Means compared		
	P and E (+)	P and E (-)	E (+) and E (-)
First 10 days of gestation	>0.500	>0.500	>0.500
First 20 days	<0.001	<0.001	<0.001

Table 23. Level of significance of differences between means shown in Table 8

Tissue	Gestational day	Nature of data	Means compared		
			P and E (+)	P and E (-)	E(+)andE(-)
Liver	10	In vivo	<0.050	<0.010	<0.001
		In vitro	<0.010	<0.001	<0.001
	20	In vivo	<0.001	<0.001	<0.001
		In vitro	<0.001	<0.001	<0.001
Uterine contents	10	In vivo	>0.500	<0.400	<0.100
		In vitro	<0.200	<0.001	<0.001
Placenta	20	In vivo	>0.500	<0.001	<0.001
		In vitro	>0.500	<0.001	<0.001
Uterus	10	In vivo	<0.500	<0.005	<0.200
		In vitro	>0.500	<0.001	<0.001
	20	In vivo	>0.500	<0.001	<0.001
		In vitro	<0.010	<0.001	<0.001

Table 24. Level of significance of differences between means shown in Tables 9 and 10

Tissue	Gestational day	Time hours	Means compared: P and E (+)	P and E (-)	E (+) and E (-)
Liver	10	1	<0.500	<0.005	<0.400
		2	<0.500	<0.010	<0.400
		4	<0.200	<0.400	>0.500
	20	1	>0.500	<0.025	<0.050
		2	<0.400	<0.400	>0.500
		4	<0.025	<0.400	<0.050
Uterine contents	10	1	>0.500	>0.500	>0.500
		2	>0.500	<0.001	<0.100
		4	<0.100	<0.100	>0.500
Placenta	20	1	<0.010	<0.001	<0.001
		2	>0.500	<0.001	<0.001
		4	>0.500	<0.001	<0.001
Uterus	10	1	<0.010	<0.400	>0.500
		2	<0.400	<0.200	<0.100
		4	<0.050	<0.100	<0.001
	20	1	<0.500	<0.001	<0.400
		2	<0.100	<0.001	<0.001
		4	>0.500	<0.001	<0.001

Table 25. Level of significance of differences between means shown in Table 11

Tissue	Gestational day	Means compared: P and E (+)	P and E (-)	E (+) and E (-)
Liver	10	>0.500	>0.500	>0.500
	20	<0.050	<0.001	<0.050
Uterine contents	10	>0.500	<0.050	<0.005
Placenta	20	>0.500	<0.001	<0.001
Uterus	10	<0.200	<0.400	<0.100
	20	<0.010	<0.001	<0.001

Table 26. Level of significance of differences between means in Table 12

Time of incubation minutes	Tissue: Liver	Uterine contents	Uterus
15	<0.025	>0.500	<0.400
30	<0.400	<0.400	<0.500
60	<0.500	<0.400	<0.500

Table 27. Level of significance of differences between means shown in Table 13

Time of incubation minutes	Mean compared P and E (-)
15	<0.001
30	<0.001
60	<0.001

Table 28. Level of significance of differences between means in Table 14

Tissue	Nature of data	Means compared P and E (+)	P and E (-)	E (+) and E (-)
Liver	wet weight	<0.050	<0.400	<0.400
	total protein	<0.400	>0.500	<0.400
	protein conc.	<0.400	<0.400	>0.500
Uterine contents	wet weight	<0.100	<0.050	>0.500
	total protein	<0.200	<0.200	<0.500
	protein conc.	>0.500	>0.500	>0.500
Uterus	wet weight	<0.500	>0.500	<0.400
	total protein	<0.100	>0.500	<0.100
	protein conc.	<0.400	<0.400	>0.500

Table 29. Level of significance of differences between means shown in Table 15

Tissue	Nature of data	Means compared		
		P and E (+)	P and E (-)	E (+) and E (-)
Liver	wet weight	<0.025	<0.001	<0.050
	total protein	<0.050	<0.001	<0.200
	protein conc.	>0.500	>0.500	<0.500
Placenta	wet weight	>0.500	<0.001	<0.001
	total protein	<0.400	<0.001	<0.001
	protein conc.	<0.500	<0.001	<0.001
Uterus	wet weight	<0.200	<0.001	<0.001
	total protein	<0.200	<0.001	<0.001
	protein conc.	>0.500	<0.010	<0.010

Table 30. Level of significance of differences between means shown in Table 16

Tissue	Nature of data	Means compared		
		P and E (+)	P and E (-)	E (+) and E (-)
Liver	wet weight	<0.050	<0.050	>0.500
	total DNA	>0.500	>0.500	>0.500
	DNA conc.	<0.500	>0.500	>0.400
	total RNA	>0.500	>0.500	>0.500
	RNA conc.	>0.500	>0.500	>0.500
Uterine contents	wet weight	>0.500	<0.400	<0.500
	total DNA	>0.500	>0.500	>0.500
	DNA conc.	<0.200	<0.200	>0.500
	total RNA	>0.500	>0.500	>0.500
	RNA conc.	>0.500	>0.500	>0.500
Uterus	wet weight	>0.500	>0.500	>0.500
	total DNA	>0.500	>0.500	>0.500
	DNA conc.	<0.400	>0.500	<0.200
	total RNA	<0.500	<0.500	>0.500
	RNA conc.	<0.500	<0.200	<0.400

Table 31. Level of significance of differences between means shown in Table 17

Tissue	Nature of data	Means compared		
		P and E (+)	P and E (-)	E (+) and E (-)
Liver	wet weight	<0.005	<0.025	>0.500
	total DNA	<0.100	<0.200	>0.500
	DNA conc.	>0.500	>0.500	>0.500
	total RNA	<0.001	<0.001	<0.500
	RNA conc.	>0.500	>0.500	>0.500
Placenta	wet weight	<0.010	<0.001	<0.001
	total DNA	>0.500	<0.001	<0.001
	DNA conc.	<0.400	>0.500	<0.400
	total RNA	<0.400	<0.001	<0.001
	RNA conc.	>0.500	<0.001	<0.001
Uterus	wet weight	<0.010	<0.001	<0.001
	total DNA	<0.050	<0.005	<0.010
	DNA conc.	>0.500	<0.001	<0.001
	total RNA	<0.100	<0.001	<0.001
	RNA conc.	<0.500	<0.005	<0.005