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VITAMIN E AS AN INDEX OF TISSUE PEROXIDATION: THE EFFECT OF VITAMIN C DEFICIENCY AND ISCHEMIA/REPERFUSION

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

University of Ottawa
Ottawa, Ontario, Canada

January,1993

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ABSTRACT

Changes in tissue levels of vitamin E, the major lipid-soluble anti-oxidant present in mammals, have been studied under two conditions that are believed to alter the autoxidation/antioxidant balance in mammals: in guinea pigs, the induction of a severe vitamin C deficiency, and in rats, ischemia/reperfusion of the brain.

Levels and turnover of vitamin E (α-T) were studied in guinea pigs placed for three weeks on a diet containing a scorbutic level of vitamin C (10 mg/kg diet), and either a low level (5 mg/kg diet; LE group) or a high level (36 mg/kg diet; HE group) of hexadeuterium-labelled vitamin E (d_6-*RRR*-α-T acetate). Previously, the guinea pigs had been maintained for two weeks on an identical diet containing 250 mg/kg of vitamin C and either 5 or 36 mg/kg of unlabelled vitamin E (d_0-*RRR*-α-T acetate). Tissues and blood were recovered from animals sacrificed at various times over a 21 day period and were analyzed for vitamin C by high performance liquid chromatography (HPLC) using electrochemical detection and for d_0- and d_6-α-T using gas chromato-graphy/mass spectrometry (GC/MS).

The levels of vitamin C in the ten tissues analyzed declined very rapidly at rates that were the same in both the LE and HE groups, indicating that the level of dietary vitamin E had no effect upon tissue vitamin C levels.

There were some striking differences in the behaviour of total α-T, d_0-α-T and d_6-α-T in "scorbutic" guinea pigs compared to animals maintained on normal levels of dietary vitamin C in an earlier study. On the vitamin C deficient diet, the total α-T (d_0- + d_6-α-T) declined significantly over 21 days in the HE group in two tissues with high P-450 enzyme activity (adrenal

gland and testis) and in one tissue with a high partial pressure of oxygen (lung), whereas on a vitamin C-sufficient diet with the same concentration of vitamin E the levels of total α-T remained steady in the same tissues. The levels of total α-T were stable in all other tissues analyzed, in blood and in feces of the HE "scorbutic" animals. In the LE "scorbutic" group, the total α-T declined only in heart and kidney, whereas in the vitamin C-sufficient LE group there was a decline of total α-T in all tissues analyzed except brain.

Surprisingly, although the levels of d_0-α-T declined more rapidly in the first seven days in many of the tissues in the "scorbutic" animals, thereafter the levels tended to decline more slowly than in the "normal" animals. Even more surprising was the significant increase with time of d_0-α-T in sciatic nerve. It thus appears that a low level of vitamin C in the diet has the overall effect of causing a redistribution and conservation of vitamin E in the animals. This phenomenon may have been triggered, at least partly, by the dramatically lower amount of dietary d_6-α-T that was taken up from the vitamin C-deficient diet into the blood and tissues during the 21 day period. The strongest effect was seen in red blood cells and the smallest in biceps femoris muscle.

These findings help answer the long standing question of whether or not there is an interaction between vitamin C and vitamin E *in vivo*. The results show that in guinea pigs, at least, vitamin C is indispensable for proper uptake of vitamin E from the gut and absorption into tissues. The stability of d_0-α-T levels suggests that once inside the cells, vitamin E appears not to depend strongly on vitamin C protection and that a

mechanism for the conservation and redistribution of vitamin E exists within the animals.

Changes of vitamin E levels also were studied in six anatomical regions of the brain of rats subjected to controlled ischemia/reperfusion by occlusion of both carotid arteries, coincidental with hypotension. Analysis of vitamin E in the cerebellum, diencephalon-mesencephalon, hippocampus, pons-medulla, cortex and striatum, and comparison of the results with sham-operated rats showed that ischemia/reperfusion caused statistically significant losses of vitamin E in all regions, except the pons-medulla, and the extent of loss correlated well with the previously determined deterioration of the blood-brain barrier in the corresponding regions. These findings are consistent with the existence of a substantial free radical-mediated component of tissue injury during and/or after ischemia/reperfusion.

To my husband Lukasz

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I would like to thank Dr. Graham Burton for giving me the help and guidance that made this thesis possible.

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1. INTRODUCTION

The appearance of photosynthetic organisms on Earth led to the generation of molecular oxygen (O_2) as a by-product of photosynthesis. The major adaptation of biological species to this change has been the use of oxygen as the terminal electron acceptor in mitochondrial respiration. The multienzyme complexes of the electron transport chain, located in the inner mitochondrial membrane, accept and transfer electrons, with the net result that water is formed from the four electron reduction of molecular oxygen by the cytochrome oxidase complex (1).

It is believed that some components of the electron transport chain can "leak" electrons onto oxygen instead of passing them on to the next component of the chain. This leakage can result in a one electron reduction of oxygen, yielding superoxide radical, O_2^-. In this regard, the mitochondrial electron transport chain is considered the most important source of superoxide radical *in vivo* in most aerobic cells.

A similar mechanism of O_2^- formation is believed to be responsible for radical generation at sites where controlled oxidation of organic substrates is catalyzed by the P-450 enzyme system. For example, in the adrenal cortex electrons can "leak" from adrenodoxin, the non-heme protein which donates electrons required for adrenal steroid hormones synthesis from cholesterol (2).

Although it has been shown that superoxide radical is responsible for extensive biological damage, it is considered that O_2^- and its dismutation product H_2O_2 exert many of their harmful effects indirectly through the generation of more reactive species, e.g., hydroxyl radical, $OH^\bullet$ (2).

Some types of biomolecules have the potential to undergo undesirable and uncontrolled oxidation. For example, polyunsaturated fatty acids, constituents of cell membrane phospholipids, are especially vulnerable to attack by free radicals. Relatively small amounts of free radicals can cause extensive autoxidative damage to fat due to the chain reaction nature of autoxidation (3). Unchecked, phospholipid autoxidation (or lipid peroxidation) can change the properties of membrane bilayers in various ways (e.g., membrane fluidity and permeability) (4), causing serious physiological consequences. Lipid peroxidation also can damage membrane proteins (5). Obviously, oxidative destruction of membrane proteins which perform highly complex and diverse functions (e.g., in ion homeostasis or in signal transduction) can have profound physiological consequences. Also, several aldehydes, formed as products of lipid peroxidation, have been shown to react with nucleic acids (6) producing many different types of lesions that can be grouped into strand breaks and base modifications of DNA (7).

For protection against destructive oxidative processes, aerobic organisms have developed an elaborate system of endogenous antioxidants, i.e., substances (enzymic and non-enzymic) that are able to inhibit peroxidation of bio-organic substrates (2). Hence, cellular antioxidants counteract the continuous threat of autoxidation arising from the generation of free radicals during respiration and oxidative processes. It is believed that under normal conditions there is a dynamic balance between the destructive tendencies of autoxidation and protective effects of antioxidant systems (8).

Vitamin E (predominantly α-tocopherol, α-T) and vitamin C (ascorbic acid) are two natural antioxidants that have attracted a great deal of

attention. Vitamin E, a superb antioxidant *in vitro* (9), has been found to be the major and probably the only lipid soluble antioxidant present in mammalian blood (10, 11) and tissues (12, 13). Vitamin E therefore forms an important line of defense against peroxidative damage of polyunsaturated fatty acids. Hence, the levels of vitamin E in blood and tissues may be a sensitive marker of peroxidative stress. (The direct estimation of the extent of free radical formation *in vivo* is experimentally very difficult.)

Vitamin C, which is water-soluble, is believed to contribute to the mammalian antioxidant system since in vitro it directly "scavenges" free radicals present in the aqueous phase (14). Vitamin C also displays a cooperative effect with vitamin E (which intercepts oxy-radicals in the lipid phase) (15, 16). Thus, the ability of vitamin E and vitamin C to inhibit oxidation is enhanced when both vitamins are simultaneously present in homogeneous solutions (15, 17), aqueous micellar dispersions (18-20) and model membranes (21-23).

For example, in autoxidation experiments in which peroxidation was initiated either in the aqueous phase or lipid phase of a phosphatidylcholine liposomal suspension, the effects of vitamin E and vitamin C were either additive or synergistic (22, 23). The effect of vitamin C was additive when it trapped radicals presumably present in the aqueous phase (vitamin E also traps these radicals when they are present at the membrane surface). Vitamin C is unable to trap radicals that are generated in and confined to the liposomal membrane. It is proposed, therefore, that vitamin C regenerates vitamin E during the reaction, since under these circumstances the presence of vitamin C extends considerably the inhibitory effect of vitamin E.

The ability of vitamin C to regenerate vitamin E radical has been confirmed in homogeneous solutions using pulse radiolysis (24) and electron spin resonance (ESR) spectroscopy (25) and in model membranes using ESR (26, 27). Vitamin C appears to be a much better synergist of vitamin E action then most other natural, endogenous, water-soluble antioxidants. These other water-soluble antioxidants (e.g., uric acid, plasma proteins, cysteine, glutathione, bilirubin and biliverdin) also inhibit oxidation initiated in the aqueous phase, both in plasma (28-30) and in liposomal model systems (31-35). However, in peroxidations initiated within the lipid bilayer, a synergistic effect analogous to the vitamin C/vitamin E interaction has been observed only for conjugated bilirubin and biliverdin (37)

The possibility that vitamin C can "spare" vitamin E *in vivo* was suggested more than 20 years ago (38) and subsequently has been investigated in numerous studies. In a recent study using guinea pigs (which cannot synthesize ascorbic acid), no effect of vitamin C on levels or turnover of vitamin E was found (39). Although the behavior of vitamin E was studied at three different levels of dietary ascorbic acid, even the lowest level (50 mg/kg diet) had no effect on the level and turnover of vitamin E. Presumably, the reduction of tissue levels of vitamin C was not sufficient to adversely affect the overall protection against peroxidation. It is conceivable, however, that a more drastic reduction of dietary ascorbic acid to a scorbutic level might tip the autoxidation/antioxidant balance, if vitamin C indeed contributes to the antioxidant protection system of living organisms. It is possible, then, that the animals may show increased turnover and/or decreased levels of tissue α-tocopherol under these more stressful conditions.

Stroke-associated damage to the brain is caused by injury related to interruption of blood flow, i.e., a dramatic drop in oxygenation (ischemia) followed by a sudden restoration of oxygenation (reperfusion) of the affected brain region (5). There is compelling evidence that the physiological stress caused by ischemia/reperfusion triggers free radical-mediated oxidative events (40, 41) that may alter the autoxidation/antioxidant balance and therefore lead to damage of the affected tissue.

It has been observed in rats that in the application of the two vessel occlusion model of stroke that the symptoms of after-stroke injury (selective neuronal damage assessed histologically and by NMR-imaging (42, 43), and edema (44)) are associated with an increased permeability of the blood-brain barrier (44). If, indeed, ischemia/reperfusion does tip the autoxidation/antioxidant balance by promoting pro-oxidant events, does it lead to an increased consumption of vitamin E in the affected areas? If it does, is the extent of vitamin E depletion correlated with the extent of brain injury, e.g., the increased blood-brain barrier permeability? In other words, do vitamin E levels reflect the extent of damage of tissues subjected to ischemia/reperfusion?

Problem

The questions posed above suggest the following hypothesis: a physiological stress which alters the autoxidation/antioxidant balance in a tissue will affect the vitamin E dynamics of this tissue.

The work described in this thesis reports the results of two independent experiments designed to investigate this hypothesis.

Approach

Two conditions that are believed to strongly affect the autoxidation/antioxidant balance of an aerobic organism were employed:

1. Induction of vitamin C deficiency in experimental animals;
2. Controlled ischemia/reperfusion in experimental animals.

Objectives and Rationale

The objective was to see whether the physiological stress expressed by vitamin C deficiency and ischemia/reoxygenation affected the autoxidation/ antioxidant balance in experimental animals. More specifically, the vitamin E levels were used as a yard stick of the extent of the presumed autoxidation. Since guinea pigs can not synthesize vitamin C, their vitamin C status depends on their dietary intake. Therefore, these animals were used to study the effect on vitamin E under the influence of dietary vitamin C deficiency.

Two groups of animals were placed for three weeks on diets containing either a low or a high level of vitamin E and a scorbutic level of vitamin C. In the previous study using guinea pigs, it was found that the low dietary level of vitamin E caused a decline (except in nerve tissue) of tissue vitamin E, whereas the high level of dietary vitamin E maintained steady tissue levels of vitamin E (39). Since, the response of cellular α-tocopherol to dietary

manipulation appears to be tissue specific (39, 45) and also because the potential for unchecked autoxidative events may vary in different anatomical regions, as many as 13 tissues (adrenal gland, adipose tissue, brain, heart, kidney, liver, lung, muscle (biceps femoris), sciatic nerve, small intestine, spleen, spinal cord and testis), plasma, red blood cells (RBC) and fecal material were sampled on 10 selected days during the three week period. The concentration of vitamin E was measured in all samples and the concentration of vitamin C was measured in 10 tissues. Vitamin E was determined using a very accurate gas chromatography/mass spectrometry (GC/MS) method. Vitamin C content was measured using high pressure liquid chromatography (HPLC) coupled to a very sensitive electrochemical detector.
The results of this study are expected not only to provide information on the behavior of vitamin E under conditions of altered autoxidation/antioxidant balance but may also provide an answer to the question of the existence of a vitamin C/vitamin E interaction *in vivo*.

In order to study changes of vitamin E in a tissue subjected to ischemia /reperfusion, the 2-vessel occlusion model of stroke in the rat (44) has been used. In this model, cerebral ischemia is induced by the occlusion of both common carotid arteries simultaneously with a reduction in blood pressure, which is accomplished by aspirating blood from the tail using a heparinized syringe. Vitamin E concentration was determined by the GC/MS method in six anatomical regions of the brain (cortex, striatum, hippocampus, diencephalon/mesencephalon, cerebellum and pons-medulla) in a group of four rats subjected to controlled ischemia and in a control group of four

sham-operated rats. Since the same experimental animal model has been used in a previous, independent study that investigated changes in blood-brain barrier (BBB) permeability, it was possible to make a comparison between the change in BBB permeability and the response of vitamin E levels to ischemia/reperfusion in each brain region.

Decreased levels of vitamin E in the tissues subjected to ischemia/ reperfusion, if detected, will contribute to the evidence that oxidative events are responsible for the damage to ischemic tissue. The possibility would also arise of administering antioxidants at the earliest possible opportunity to persons afflicted by a stroke.

Finally, the study of the changes of vitamin E in both of these conditions of presumably altered autoxidation/antioxidant balance will help reveal the advantages and limitations of using vitamin E as a marker (or detection tool) of increased autoxidation *in vivo*.

2. REVIEW OF PERTINENT LITERATURE

2.1. Mechanism of Autoxidation (Peroxidation)

The spontaneous chemical reaction of an organic substrate with molecular oxygen is known as autoxidation (or more commonly as lipid peroxidation in biological systems). The reaction proceeds by a free radical chain mechanism (Fig. 1) (3, 36, 46-49)

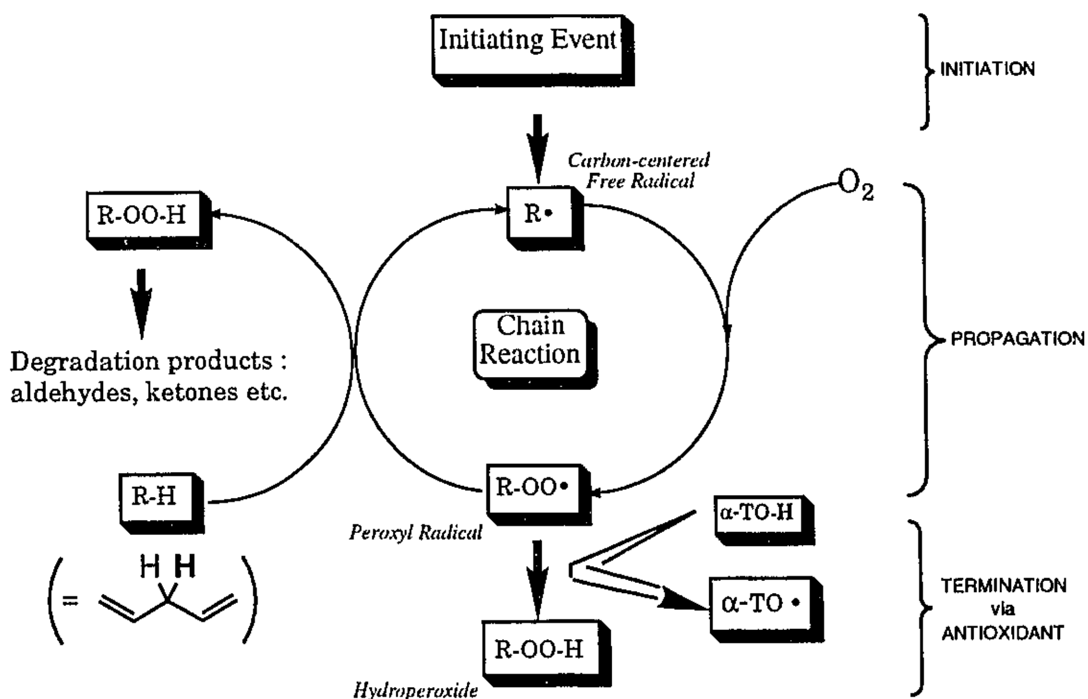


Figure 1. Scheme showing the three phases of the free radical, chain mechanism of lipid peroxidation. RH, R•, ROO•, ROOH, represent lipid, lipid carbon-centered free radical, peroxyl radical and lipid hydroperoxide, respectively. α-T and α–T• are α-tocopherol and α–tocopheroxyl radical, respectively.

The process begins with the formation of a low concentration of carbon-centered free radicals (R•) from a precursor molecule during the

initiation phase. The carbon-centered free radical reacts rapidly with molecular oxygen, being transformed from a relatively unreactive radical to a very reactive, chain-carrying peroxyl radical (ROO•). This is the first step in the *propagation* phase of autoxidation. The peroxyl radical then attacks any available peroxidizable material, producing a new carbon-centered radical, usually by abstraction of a hydrogen atom from a polyunsaturated fat molecule. The newly formed radical repeats the same propagation sequence of reactions. Thus, a single initiation event can oxidatively damage many substrate molecules. *Termination* is the last phase of autoxidation, which ends the *propagation* phase either by a reaction between two peroxyl radicals, yielding nonradical products, or by reaction with an antioxidant.

Membrane phospholipids contain large amounts of polyunsaturated fatty acid that are characterized by the presence of two or more carbon-carbon double bonds separated by a methylene, $-CH_2-$, unit. The methylene hydrogens in the bis-allylic arrangement, $-CH=CH-CH_2-CH=CH-$, are very susceptible to abstraction by free radicals, which leaves an unpaired electron on the methylene carbon.

The carbon-centered radical reacts very rapidly with oxygen, yielding a rearranged peroxyl radical.

2.1.1. Free radical repair concept

The concept of "free radical repair" through electron transfer or through H atom transfer to a radical arose in the 1950's as an explanation for the protective actions of some added substances (e.g., cysteine) in the radiation-induced decomposition of polymers (50-52).

Follow-up studies of chemical, viral and cellular systems provided further evidence in agreement with the radical repair concept (53-58). Attention has centered on the involvement of endogenous compounds, such as vitamins E and C and reduced nicotinamide adenine dinucleotide (NADH).

The advent of the pulse radiolysis technique in the early 1960's (59) provided quantitative information on the chemistry and kinetics of free radicals in solution and contributed to the development of some of the mechanistic interpretations of biological protective phenomena.

The use of absorption spectra of radicals formed during pulse radiolysis of reaction mixtures has shown that free radical repair by glutathione, vitamins C and E and NADH takes place in homogeneous solution and proceeds either through hydrogen atom or electron transfer reactions (60).

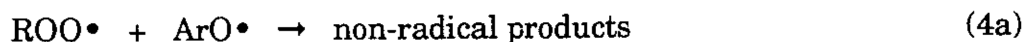
2.1.2. Mechanism of antioxidant action

Protection of living systems against autoxidation is provided by relatively small amounts (compared to lipid) of certain, specific compounds termed antioxidants. Antioxidants, depending on their mode of action, are divided into two classes. *Preventive antioxidants* function by reducing the rate of chain *initiation* (i.e., the number of free radicals produced), whereas *chain breaking antioxidants* act by interfering with one or more steps of the *propagation* phase through direct reaction with radicals (36, 61). Many

phenols (ArOH) are chain-breaking antioxidants because they are able to "trap" the chain-carrying peroxyl radicals. Further generation of carbon-centered radicals is inhibited, limiting the *propagative* phase of peroxidative lipid damage.

$$ROO\bullet + ArOH \rightarrow ROOH + ArO\bullet \quad (3)$$

The phenolic hydrogen atom is transferred to the peroxyl radical and the phenoxyl radical that is generated is too unreactive to continue the chain, i.e., to abstract hydrogen from another molecule of polyunsaturated fatty acid, because the unpaired electron is delocalized over the atoms of the aromatic structure. The phenoxyl radical is eventually removed, usually by reaction with a second peroxyl radical (reaction 4a)



$$ROO\bullet + ArO\bullet \rightarrow \text{non-radical products} \quad (4a)$$

or sometimes by reaction with another phenoxyl radical (reaction 4b)

$$ArO\bullet + ArO\bullet \rightarrow \text{non-radical products} \quad (4b)$$

It has been shown that each phenol molecule "traps" two peroxyl radicals (reactions 3 and 4a) (9). Most phenols react in this clean stoichiometric manner, thereby inactivating two chains.

Alternatively, the phenoxyl radical can undergo repair to give the starting phenol (in the presence of ascorbate (AH^-); e.g., reaction 5) (16, 33, 61).

$$ArO\bullet + AH^- \rightarrow ArOH + A^{\bullet -} \quad (5)$$

For a compound to be an effective chain-breaking antioxidant, it has to compete successfully for peroxyl radicals against a much greater amount of the peroxidizable substrate. Therefore, the rate constant for the reaction of a peroxyl radical with an antioxidant (reaction 3) has to be much larger than

the rate constant for reaction of a peroxyl radical with a polyunsaturated lipid molecule (Fig. 1).

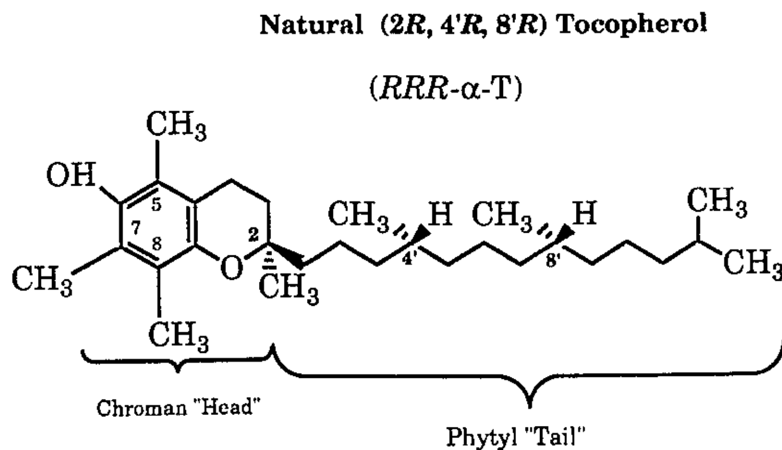
2.2. Vitamin E

2.2.1. Discovery, structure, pathology of deficiency and function

Evans and Bishop in 1922 first demonstrated the existence of a dietary, fat-soluble factor essential for normal reproduction in rats (62, 63). The absence of this factor in the diet of female rats resulted in fetal death and resorption. The factor was termed vitamin E. Isolation and purification of vitamin E from a number of plant oils revealed that it is represented by a series of structurally related phenolic compounds (64-66). The most biologically active constituent of vitamin E is α-tocopherol (**1**).

Natural (2*R*, 4'*R*, 8'*R*) Tocopherol

(*RRR*-α-T)



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In addition to the intrauterine death and resorption of the fetus observed in rats (62, 63, 67) and also described in mice (68, 69) and guinea pigs (70), the pathology of vitamin E deficiency also includes sterility in male rats (marked by testicular atrophy), acute central nervous system lesions in

growing chicks (nutritional encephalomalacia), chronic necrotizing myopathy in rabbits, guinea pigs and ducks (resulting in muscular degeneration), lesions of the cardiovascular system in chicks, hepatic necrosis in rats and the accumulation of excessive amounts of lipopigments in rats (71). In humans, apart from hemolytic anemia due to fragility of the erythrocyte membrane in premature babies, there are no specific deficiency symptoms resulting from a short term absence of vitamin E in the human diet (2, 71).

2.2.2. Antioxidative property of α-tocopherol and its vitamin E biological activity

The fact that the damaging effects of vitamin E deficiency in animals can be partially or completely alleviated by feeding them certain synthetic antioxidants has been taken to suggest that vitamin E functions to protect against lipid peroxidation *in vivo* (72). There is considerable evidence supporting this suggestion. For example, the incidence of encephalomalacia in vitamin E deficient chicks has been positively correlated with the amount of polyunsaturated fatty acid in their diet (73). Levels of pentane and ethane, which are degradation products of peroxidized fatty acids, are increased in the expired air of vitamin E deficient rats (74, 75), whereas they are reduced in the breath of humans supplemented with vitamin E (76, 77). Also, a lipid-soluble phenolic antioxidant analogous to tocopherol which has superior antioxidant ability *in vitro* has been shown to possess enhanced vitamin E activity in the rat plasma pyruvate kinase assay (78, 79).

Vitamin E, being hydrophobic, resides in cellular membranes (80) and is transported in blood lipoproteins (81-83). The protection by vitamin E of polyunsaturated fatty acids present in membranes stems from its high

reactivity toward oxygen-centered free radicals (9). Vitamin E reacts with peroxyl (ROO•) and alkoxyl (RO•) radicals more rapidly than can polyunsaturated fatty acids, and by doing so terminates the propagation reaction (3, 36, 48, 61).

2.2.3. Biological activity of vitamin E not obviously related to its antioxidative property

Although the evidence linking biological activity and antioxidant properties of vitamin E is compelling, there have been reports of other functions associated with the vitamin that do not appear directly related to its antioxidant activity.

For example, vitamin E has been shown to modulate the release of arachidonic acid in platelets by inhibition of phospholipase A_2 (84), a membrane-bound enzyme that mediates the liberation of arachidonate from plasma membrane phosphatidylcholine. Also, it has been demonstrated that dietary vitamin E enhances the synthesis of a member of the prostaglandin family, prostacyclin, PGI_2 (85), which dilates blood vessels and is a powerful inhibitor of platelet aggregation (86). In contrast, the release of thromboxane A_2 (TXA_2), the antagonist of prostacyclin that is formed upon platelet activation is reduced by increased dietary tocopherol (87). It thus has been suggested that the ratio of TXA_2 to PGI_2, which is important in controlling local blood flow and blood vessel wall-platelet interactions, can be reduced by dietary vitamin E (85), thereby diminishing the risk of thrombosis and atherosclerosis.

It also has been reported (88) that dietary α-tocopherol inhibits 5-lipoxygenase and, therefore, the formation of products of the lipoxygenase

pathway of arachidonate metabolism. 5-Lipoxygenase has attracted a great deal of interest because several products of this pathway, the leucotrienes have potent biological activities (86). Two fundamental events of the inflammatory process, i.e., adherence of neutrophils to the vascular endothelium and stimulation of the migration of neutrophils into extravascular tissue are leucotriene-mediated. Thus, the observed effect of dietary α-tocopherol on the attenuation of leucotriene synthesis coupled with increased release of prostacyclin PGI_2 from the ischemic myocardium of rat have been suggested to diminish the neutrophil-mediated inflammatory response in ischemic tissue (85).

There are reports (89, 90) that α-tocopherol (in physiologically relevant concentration) inhibits vascular smooth muscle cell proliferation induced by phorbol esters, which are potent tumor promoters. These compounds cause the persistent activation of protein kinase C, the enzyme controlling cell division and proliferation. It has been shown that vitamin E prevents translocation of inactive protein kinase C from cytosol to membrane, the site of the enzyme activation, thereby inhibiting the effect of phorbol esters (91). Thus, a regulatory function of vitamin E in protein kinase C activity has been proposed.

2.3. Vitamin C

2.3.1. Discovery, structure, pathology of deficiency and function

The prevention of scurvy in guinea pigs with the crystalline compound isolated from lemon juice, vitamin C (ascorbic acid), was reported for the first

time in 1932 by Waugh and King (92) in the USA and two weeks later by Svirbely and Szent-Györgyi (93) in Hungary.

Humans, primates, guinea pigs, and fruit-bats do not have L-gulonolactone dehydrogenase, the last enzyme in the vitamin C biosynthetic pathway (94). Therefore they must rely on dietary sources for the vitamin. Sources include the many plants and animals that synthesize ascorbic acid from glucose. Frank scurvy is the ultimate disease of vitamin C deficiency. It is characterized by bleeding gums and hemorrhages leading to death if untreated, but it is seldom seen nowadays. However, it is believed that subclinical ascorbic acid deficiency is still very common and predisposes those affected to atherosclerosis, amyloidosis, and to diabetes mellitus (95).

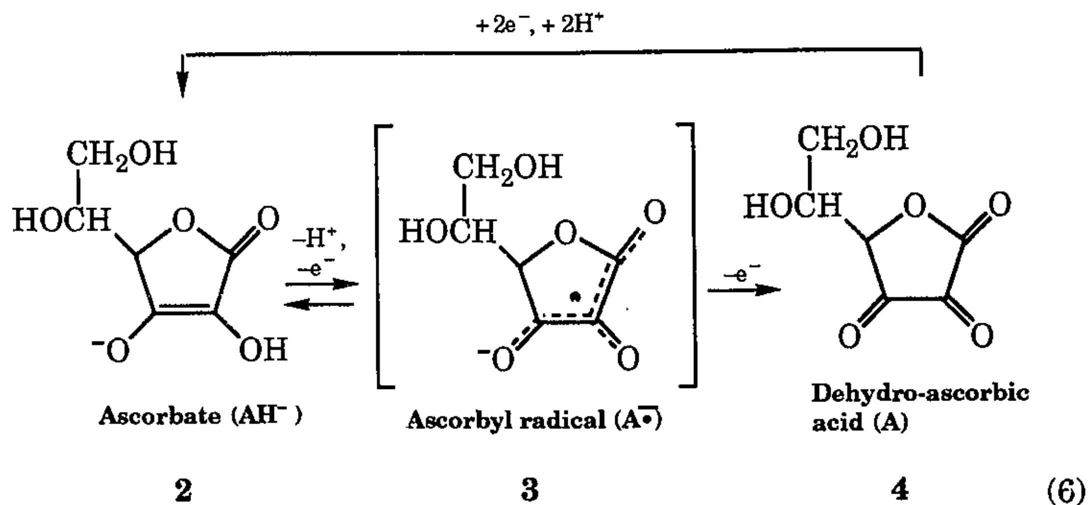
Ascorbic acid is required in the biosynthesis of collagen, the connective tissue protein containing hydroxylated proline and lysine residues. It functions as a cofactor of the enzymes, prolyl and lysyl hydroxylases. Both enzymes contain iron at their active site. Another enzyme containing iron, homogentisate oxidase, also requires ascorbic acid for maximal activity in catalysis of the oxidation of homogentisate to maleylacetoacetate during the degradation of phenylalanine (and tyrosine) to fumarate and acetoacetate. Similarly, two hydroxylases containing copper at their active site, the p-hydroxyphenylpyruvate hydroxylase and dopamine β-hydroxylase require ascorbic acid for their maximal activity. These enzymes catalyze, respectively, oxidation of p-hydroxyphenylpyruvate to homogentisate, another step in the phenylalanine catabolism, and dopamine conversion to norepinephrine in the synthesis of epinephrine in adrenal medulla (96). It is most likely that

ascorbate functions in enzymic reactions by keeping iron or copper at the enzyme's active site in its reduced form.

Ascorbic acid is also important for the absorption of dietary iron, which has to be reduced from Fe^{3+} to Fe^{2+} state for efficient absorption to occur (2, 97, 98). The inactivation of the carcinogenic nitroso-compounds (R–NO) by dietary ascorbic acid is also attributed to its ability to reduce them to an inactive form (99).

2.3.2. Antioxidant and pro-oxidant properties of ascorbic acid

Ascorbic acid, which under most physiological conditions exists as its conjugate base, ascorbate, AH^- (pK_a 4.25) (100) is a reactive reductant and can undergo a reversible two step oxidation-reduction involving a free radical intermediate (101):



(6)

The ascorbyl radical $A^{\bullet -}$, which is relatively unreactive toward lipid due to the delocalization of the unpaired electron over its structure, decays by disproportionation, yielding equal quantities of ascorbate and dehydro-ascorbate (14):

$$2\,A^{\bar{\bullet}} + H^+ \rightarrow AH^- + A \qquad (7)$$

It has been shown in vitro that ascorbate reacts with superoxide radical (O_2^-), hydroperoxyl radical ($HO_2^\bullet$), hydroxyl radical ($HO^\bullet$), singlet oxygen, and hydrogen peroxide (H_2O_2) (2, 14). All of these reactive species can be generated in biological system under aerobic conditions (2). The accumulation of lipid peroxides and of fluorescent pigments, as well as the formation of cross-linked, high molecular weight proteins in microsomal fractions, has been detected in many tissues of scorbutic guinea pigs. It has been concluded that this apparent increase in lipid peroxidation is a primary effect of vitamin C deficiency, and indeed, symptoms of scurvy have been discussed in terms of oxygen toxicity (102).

The efficiency of ascorbate in scavenging reactive oxy-radicals and the stability of the ascorbyl radical suggest a protective function for ascorbate against free radical damage in biological systems. However, in aqueous solution the ascorbate anion undergoes autoxidation generating superoxide radicals (16).

$$AH^- + O_2 \rightarrow A^{\bar{\bullet}} + O_2^- + H^+ \qquad (8)$$

This reaction is catalyzed by transition metal ions, especially iron or copper

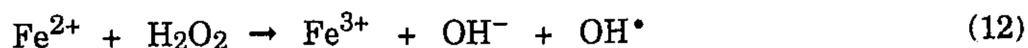
$$Fe^{3+} + AH^- \rightarrow Fe^{2+} + A^{\bar{\bullet}} + H^+ \qquad (9)$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^- \qquad (10)$$

The superoxide that is formed can dismutate to form hydrogen peroxide:

$$2\,O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (11)$$

which can then undergo Fenton chemistry, yielding the extremely reactive hydroxyl radical, $OH^\bullet$ (2):



$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet \qquad (12)$$

Vitamin C, in the presence of iron, has been used to initiate lipid peroxidation *in vitro* (13, 21, 103). It has been demonstrated that as the concentration of ascorbate is increased its efficiency as an antioxidant decreases (104), because it undergoes increased autoxidative destruction, making less of it available for peroxyl radical interception.

So far, there is little evidence that the pro-oxidant activity of vitamin C is of any significance *in vivo* (105). However, free iron ions, which facilitate single electron transfer from ascorbate to oxygen, can be released by superoxide radical from ferritin and by H_2O_2 from hemoglobin (2)

2.4. Vitamin C / vitamin E interaction

2.4.1. Studies *in vitro*

A synergistic effect of vitamin C upon vitamin E action was first reported in 1941 (106). Vitamin C was not effective in preventing autoxidation of lard *in vitro* but in the presence of vitamin E it greatly extended the induction period produced by vitamin E alone. It was later suggested that the cooperation of vitamins C and E may exist also *in vivo*, through regeneration of vitamin E from the tocopheroxyl radical (38).

A number of studies have found that the inhibition of autoxidation is enhanced in the presence of both vitamins as compared to the inhibition separately produced by vitamin E and C in homogeneous solutions (15, 17) and in aqueous micellar and liposomal dispersions (18-23).

It has been shown by pulse radiolysis (24) and by electron spin resonance spectroscopy (ESR) (25) that the tocopheroxyl radical is reduced by ascorbate in homogeneous solution. During the autoxidation of a methyl

linoleate monolayer on silica gel the addition of vitamin C quenched the ESR spectrum characteristic of the α-tocopheroxyl radical (the tocopheroxyl radical ESR spectrum was replaced by the ascorbyl radical spectrum) (26).

Another study (27), using ESR to follow the catalyzed autoxidation of aqueous phosphatidylcholine (PC) liposomes as model biomembranes, has shown that only ascorbyl radical is detected when both vitamins are present. The ESR spectrum of the tocopheroxyl radical began to emerge when nearly all of the ascorbate was used up and then gradually disappeared as the vitamin E was consumed. However, it was not possible to determine whether the sparing of vitamin E was caused by ascorbic acid directly trapping the peroxyl radical in the aqueous phase or by regeneration of oxidized vitamin E, or by both phenomena occurring simultaneously.

Two independent studies have shown that vitamin C can not only inactivate peroxyl radicals generated in the aqueous phase but also it can regenerate α-tocopherol from α-tocopheroxyl radical (22, 23). The studies used water- and lipid-soluble chemical radical generators (initiators) which at 37^0 C decompose to generate a steady flux of water- or lipid-soluble peroxyl radicals in a phosphatidylcholine liposomal system (107).

Model liposome systems have been used to study the abilities of different chain-breaking antioxidants, present in either the aqueous or lipid phase to trap radicals generated in either phase using either water- or lipid-soluble free radical generators to produce radicals at the specific site of interest. This approach has made it possible to study interactions between antioxidants *in vitro* which, due either to their hydrophilicity or hydrophobicity, are segregated in their respective aqueous or lipid phases.

Autoxidation experiments using micelles or multilamellar liposomes and water- or lipid-soluble thermal initiators have shown that lipid-soluble α-T, which resides within the phospholipid bilayer, effectively "scavenges" peroxyls generated both outside and inside the lipid phase. Water-soluble vitamin C, however, is only effective as an antioxidant when radicals are generated in the aqueous phase. When vitamins E and C are both present the antioxidant effect is enhanced. A synergistic effect is observed for peroxidation generated within the lipid bilayer, i.e., the period of inhibition of oxidation produced by the vitamin mixture is much longer than the sum of their individual effects. However, the combined effect of the two vitamins is simply additive when the radicals are generated in the aqueous region.

The synergy between vitamins E and C in autoxidation reactions initiated in the lipid phase is attributed to regeneration of tocopherol from tocopheroxyl radicals by the ascorbate, most likely at the lipid/water interphase since vitamin C appears to be unable to trap radicals resident within the membrane (61).

$$(\alpha\text{-T}\bullet)_{lipid} + (AH^-)_{aq} \rightarrow (\alpha\text{-T})_{lipid} + (A\bullet^-)_{aq} \quad (13)$$

The question arises: can an interaction between vitamins E and C also occur *in vivo* ?

2.4.2. Studies *in vivo*

The results of *in vivo* experiments are not in agreement. There are reports suggesting protective (108-117), antagonistic (74, 117-121), and no effect at all (39, 122, 123) of vitamin C upon vitamin E. For example, dietary vitamin C has been found to enhance levels of vitamin E in plasma of rats (109), guinea pigs (108) and humans (113) and in the adrenal gland, spleen,

lung and liver of guinea pigs (115). The effect was particularly marked in the adrenal gland (115). In vitamin E deficient rats, vitamin C feeding partially reversed the changes in some of the biochemical parameters characteristic of vitamin E deficiency (111). In contrast, the high supplementation of vitamin C of animals with low or marginally adequate vitamin E status has been also reported to increase spontaneous erythrocyte hemolysis and peroxidation of tissue lipids (118). The same dietary treatment also significantly lowered the level of vitamin E in plasma and the level of reduced glutathione in erythrocytes in guinea pigs (119).

Studies using expired pentane and ethane levels as a measure of lipid peroxidation have demonstrated that increased dietary vitamin C appeared to enhance lipid peroxidation induced by intraperitoneally injected methyl ethyl ketone peroxide only in vitamin E deficient animals, having no effect in animals with adequate vitamin E status (74).

Recently, two studies using animals that do not synthesize vitamin C, i.e., guinea pigs (39) and a mutant strain of Wistar rat with "Osteogenic Disorder Shionogi" (ODS) (117) have indicated no effect or a modest effect, respectively, of dietary intake of vitamin C upon vitamin E tissue levels. In both studies, the effect of vitamin C upon vitamin E was evaluated by measuring the influence of three different dietary levels of ascorbic acid on the levels of α-T in blood and tissues of animals in which the vitamin E status was normal or declining. In the study using guinea pigs, switching the animals from a diet containing unlabelled α-T fed for 2 weeks beforehand to a diet containing deuterium labelled α-T fed for 8 weeks permitted not only measurement of vitamin E levels but also the rate of vitamin E turnover in

blood and tissues, since unlabelled and deuterated α-T can be easily distinguished by GC/MS analysis. A decreased turnover of vitamin E in those guinea pigs that were supplemented with a high level of vitamin C, would be expected if there was a sparing or synergistic interaction between the vitamins. However, neither the level nor the turnover of vitamin E in plasma and in any of the tissues examined were affected by the vitamin C status of the animals. Thus, it was concluded that the regeneration of vitamin E by vitamin C, clearly demonstrated in vitro, is negligible under "normal" physiological conditions in guinea pig (39; see Appendix 3).

Two alternatives were suggested to explain this result. One of the proposed possibilities is that, under normal conditions, the production of peroxyl radicals is so small that even the lowest level of vitamin C used in the study was sufficient to regenerate any vitamin E consumed by peroxidation. The other explanation is that the tocopheroxyl radical is reduced *in vivo* not by ascorbate but by some other, possibly enzymic, process.

By contrast, in the study using the ODS rats maintained on a high level of dietary vitamin E statistically significant increases in vitamin E levels were obtained in the plasma, red cells, heart, liver, kidney, spleen and lung of animals fed 600 vs. 300 mg vitamin C/kg diet for 6 weeks. The largest effects were seen in plasma and red cells, which may reflect a protective effect of vitamin C upon vitamin E during digestion and absorption from the small intestine. Similar differences associated with the two dietary levels of vitamin C were seen in the same tissues, except kidney, when the animals were maintained on a very low level of vitamin E. However, in this latter experiment the levels of vitamin E in plasma and red cells were lower in the

animals fed the high level of vitamin C, suggesting a pro-oxidant effect of vitamin C.

An earlier study from the same laboratory reported only a tendency for interaction between the two vitamins when guinea pigs were used as the experimental animals (123). Larger individual variations in vitamin E levels in guinea pigs than in rats was suggested as the reason for the failure to detect any sparing effect of vitamin C upon vitamin E in the earlier study.

2.4.3. Enzymic regeneration of vitamin E

Other mechanisms of sparing or regenerating vitamin E have been proposed. For example, a heat-labile, glutathione-dependent, free radical reductase appears to be able to convert α-tocopheroxyl radical to α-tocopherol (103, 124-131). This enzymic activity was originally found only in liver. However, recently the regeneration of α-tocopheroxyl radical by glutathione in platelet homogenates has been suggested to occur via an enzymic route (131).

2.5. Free radicals and ischemia/reoxygenation injury

2.5.1. Tissue injury and death

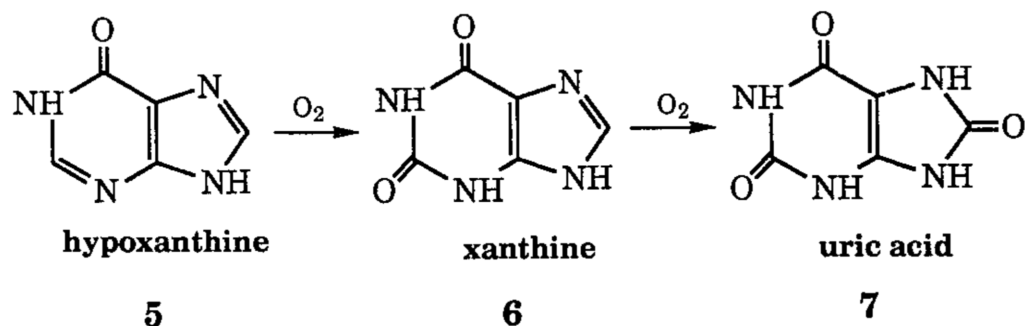
A blockage of the essential artery to an organ results in the restriction or complete cessation of the flow of blood leading, respectively, to partial (hypoxic) or complete (ischemic) O_2 deprivation in the affected tissue. Early responses of tissues to the lack of oxygen include an increase of glycogen degradation due to anaerobic glycolysis, resulting in production of lactate (leading to acidosis), a decrease in oxidative phosphorylation causing ATP levels to fall, an accumulation of hypoxanthine, a product of ATP degradation, and a rise in intracellular free Ca^{++} (causing profound

consequences which will be discussed later in this chapter) (2, 5, 42, 43). If oxygen deprivation continues for a sufficient period of time, the affected tissue is irreversibly injured and will die (for brain this can occur in only a few minutes). However, most of the affected tissue can be salvaged by reperfusion with blood, provided that the period of ischemia or hypoxia is not so long as to irreversibly injure the tissue. The reperfusion, however, can cause significant harm when oxygen is re-introduced into the ischemic tissue (132).

2.5.2. Xanthine/xanthine oxidase theory

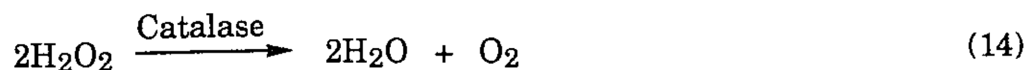
Reoxygenation injury is believed to be mediated by free radicals. During ischemia, the tissue is thought to become "primed" for increased rates of O_2^- and H_2O_2 generation upon reoxygenation (132).

One proposed mechanism suggests that, during ischemia, the influx of calcium into cells causes a protease, calpain, to cleave xanthine dehydrogenase to give xanthine oxidase, which, together with accumulated hypoxanthine from ATP degradation, provides an enzyme-substrate system that could generate superoxide radicals (132-136). Thus, re-introduction of oxygen during reperfusion could cause oxidation of hypoxanthine to uric acid with simultaneous reduction of O_2 to O_2^-

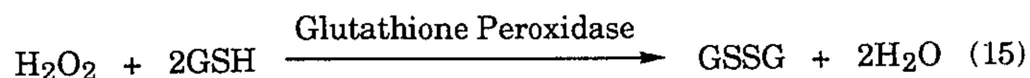


The production of O_2^- also can lead to the production of H_2O_2 through the dismutation reaction that is catalyzed by superoxide dismutase (reaction 11).

Brain damage resulting from ischemia/reperfusion (e.g., in stroke, where a cerebral blood vessel becomes blocked) is a major clinical problem and is more extensive than that produced by comparable insults to other tissues (2). The vulnerability of brain (and other nerves tissues) to radical damage may occur for a number of reasons. Firstly, the membrane lipids are very rich in polyunsaturated fatty acids that are highly susceptible to lipid peroxidation. Secondly, the brain and cerebrospinal fluid (CSF) are very poor in catalase activity and contain no significant glutathione peroxidase activity. Both of these enzymes catalyze the efficient removal of hydrogen peroxide:



$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad (14)$$



$$H_2O_2 + 2GSH \xrightarrow{\text{Glutathione Peroxidase}} GSSG + 2H_2O \quad (15)$$

Furthermore, superoxide dismutase is present only in moderate amounts (137). Thirdly, brain is rich in iron contained in ferritin complexes (138). The iron may be released as a result of injury to brain cells, e.g., by increased acidity due to lactate accumulation during ischemia (132).

In addition, brain and CSF have a high concentration of ascorbate. The combination of ascorbate and catalytic iron provides the ingredients essential for Fenton chemistry (reactions 9, 10, 11 and 12), yielding the extremely reactive hydroxyl radical (132). Indeed, the generation of hydroxyl radical

during stroke appears to have been directly demonstrated in the brain of the Mongolian gerbil (40, 41).

2.5.3. Glutamate theory

Although brain damage related to ischemia/reoxygenation injury may be mediated by the xanthine/xanthine oxidase system in the rat and gerbil (136, 139), in other mammals, including humans, the role of this mechanism has been questioned (140) on the grounds that very little xanthine dehydrogenase activity has been found in these species.

Therefore, another mechanism has been suggested involving the generation within the tissue of excitatory amino acids such as glutamate (5, 141-144). Inactivation by ischemia of glutamine synthetase, an enzyme that catalyzes the transformation of glutamate to glutamine (41), may contribute to an accumulation of glutamate in the affected neurons. The overproduction of glutamate and an accumulation of calcium can lead to neuronal death by a very complex process, the glutamate cascade, that has yet to be fully delineated.

The glutamate cascade consists of three basic stages (144). During the first stage (induction), oxygen-deprived neurons release an excess of glutamate that causes overstimulation of different types of glutamate receptors on other neighboring neurons. The affected receptors, which either control ion channels or the production of two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3), can cause opening of Ca^{++} and Na^{+} channels leading to an abnormal build-up of both ions and a triggering of the formation of DAG and IP_3. The second stage (amplification) further contributes to Ca^{++} overload by the Na^{+} activated transport that

exchanges Na^+ for Ca^{++}, by the internal positive charge activated voltage-gated Ca^{++} channels and by IP_3 releasing Ca^{++} from the intracellular storage site. The overload of calcium and the release of DAG activates enzymes that modify membrane proteins, increasing the sensitivity of the glutamate receptors to excitatory signals. Hence, further opening of voltage-gated Ca^{++} channels occurs, contributing to even more extensive calcium accumulation that, in turn, stimulates the release of more glutamate and spreading of the glutamate cascade to other cells. Ultimately, in the third stage (expression), during which irreversible damage occurs, Ca^{++} activates enzymes that degrade DNA, proteins and phospholipids, leading to the death of the affected cell. Phospholipid breakdown leads to the release and subsequent metabolism of arachidonic acid which can harm cells in two ways; by yielding eicosanoids that can promote blockage of previously healthy blood vessels, thus enhancing the spread of ischemia, and by the formation of free radicals.

2.5.4. Secondary radical production (immune response)

Neutrophils, platelets and monocytes are an alternative (or additional) source of free radicals (and other destructive substances, e.g., hypochlorous acid) which can contribute to tissue damage during ischemia/reoxygenation. Upon entering reoxygenated tissue, these blood components could adhere to endothelium, become activated and release O_2^-, H_2O_2, eciosanoids, and proteolytic enzymes (145, 146).

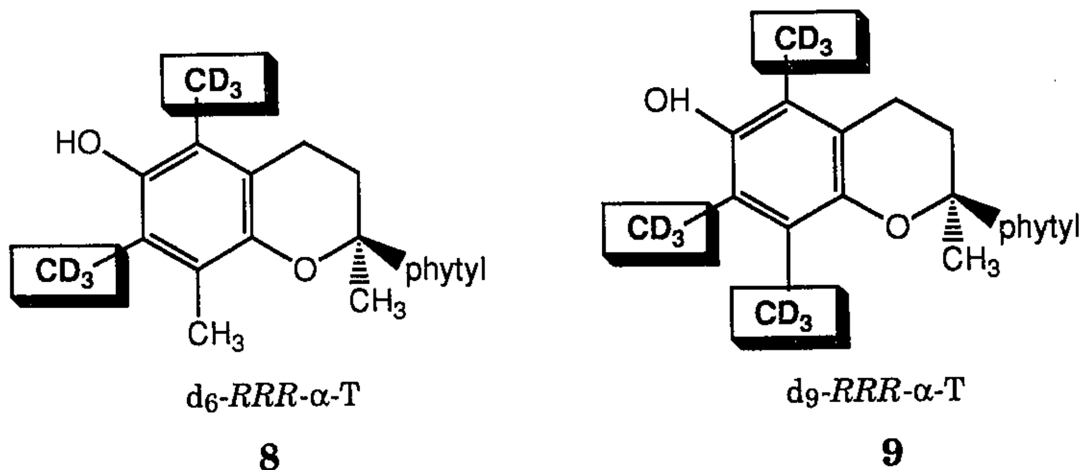
3. LEVELS AND TURNOVER OF VITAMIN E IN BLOOD, TISSUES AND FECES OF GUINEA PIGS DURING DEVELOPING VITAMIN C DEFICIENCY

3.1. Experimental procedures

3.1.1. Materials

Deuterated α-tocopherols.

2*R*,4'*R*,8'*R*-α-(5,7-$(CD_3)_2$)tocopheryl acetate (d_6-*RRR*-α-TAc) used in the preparation of the experimental diet, was provided by the Natural Source Vitamin E Association (Washington). The internal standard 2*RS*,4'*R*,8'*R*-α-(5,7,8-$(CD_3)_3$)tocopherol (d_9-*ambo*-α-T), used as an internal standard for the GC/MS quantitation of d_0- and d_6-α-T in the samples analyzed, was previously synthesized and provided for use in this study by the organic synthesis group (led by Lise Hughes) in the Molecular Selectivity Group of the Steacie Institute for Molecular Sciences, NRC. These deuterated compounds were labelled with either 6 or 9 deuterium atoms, respectively, in metabolically inactive sites of the tocopherol molecule.



Details of the synthesis of the deuterated tocopherols are provided elsewhere (147).

The purity of d_6- and d_9-α-T was found to be >99%, as determined by thin layer chromatography (TLC) and by gas chromatography-mass spectrometry (GC-MS). The degree of incorporation of the nominal amount of deuterium into these compounds was 80-96% and the amount of remaining material containing one, two, three or four less deuterium atoms was determined by GC-MS. All measurements of deuterated α-T's were corrected to reflect the true amount of the d_6-α-T actually present in each sample.

Reagents.

All solvents were HPLC-grade (Fisher or Baker). Absolute ethanol, metaphosphoric acid (Fisher), sodium acetate trihydrate (Baker), glacial acetic acid (Anachemia), potassium hydrogen phosphate (Anachemia), n-octylamine (Aldrich), ascorbic acid (BDH), DL-homocysteine, (ICN Biochemicals) and sodium dodecyl sulfate (SDS) detergent (BDH) were of analytical grade.

Pyridine (silylation grade) and the silylating reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), containing 1% trimethylchlorosilane (TMCS), were obtained from Pierce (Rockford, Illinois).

3.1.2. Methods

3.1.2.1. Experimental design

Dietary regimes and handling of animals.

The care of the guinea pigs and the animal surgery was carried out by Mrs. Hedy Burton at the Animal Care Facility at the Institute for Biological Sciences, NRC, under the supervision of Dr. David Foster. The experimental

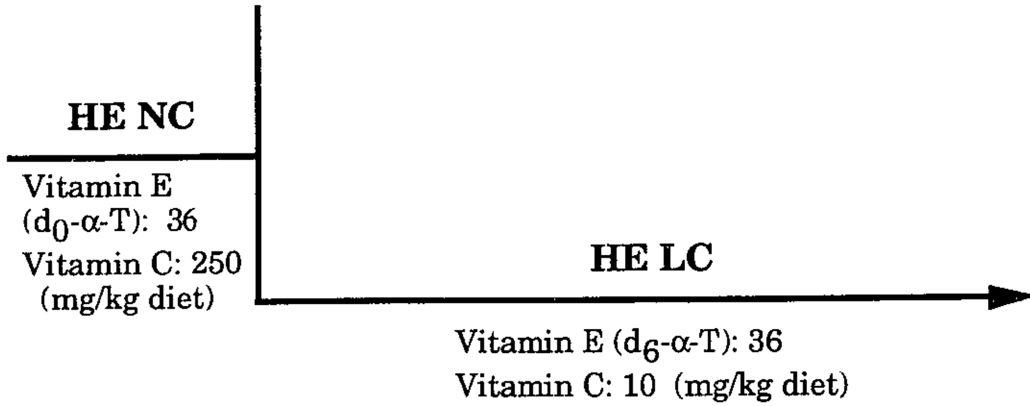
design, including dietary details and time points at which guinea pigs were sacrificed, is depicted schematically in Figure 2.

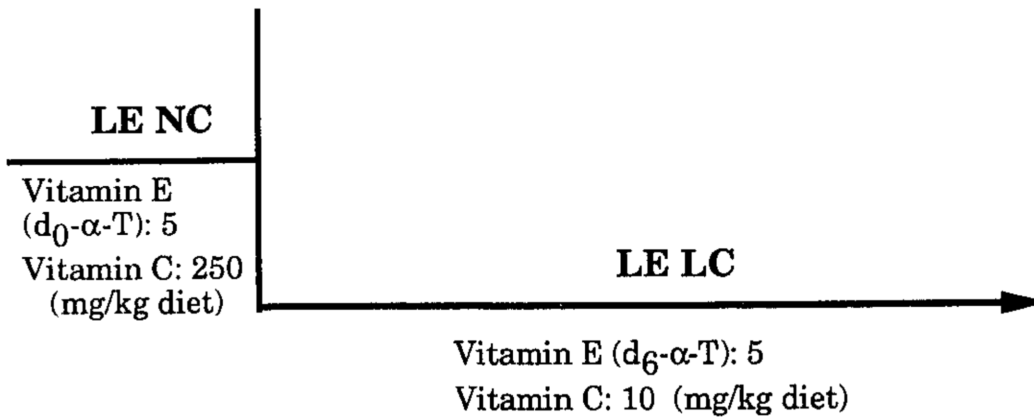
Forty-eight two week-old, male, specific pathogen free guinea pigs (170-222 g), obtained from Charles River Canada, Inc., P.Q., were divided into two groups, a high vitamin E (HE) group of 24 animals and a low vitamin E (LE) group of 24 animals. The animals were housed four per plastic cage (1720 cm^2 floor area) with ground cob bedding and were fed a Reid-Briggs Guinea Pig diet modified as follows: alfalfa meal (which contains α-T) was replaced by corn starch; corn oil (which also contains α-T) was omitted as were vitamins E and C from the vitamin mix and they were replaced by tocopherol stripped corn oil (7.3% by weight), containing d_0-*RRR*-α–TAc or d_6-*RRR*-α-TAc (5 or 36 mg/kg diet), which was added together with ascorbic acid (10 or 250 mg/kg diet). During the experiment the guinea pigs consumed about 15-20 g of diet per day.

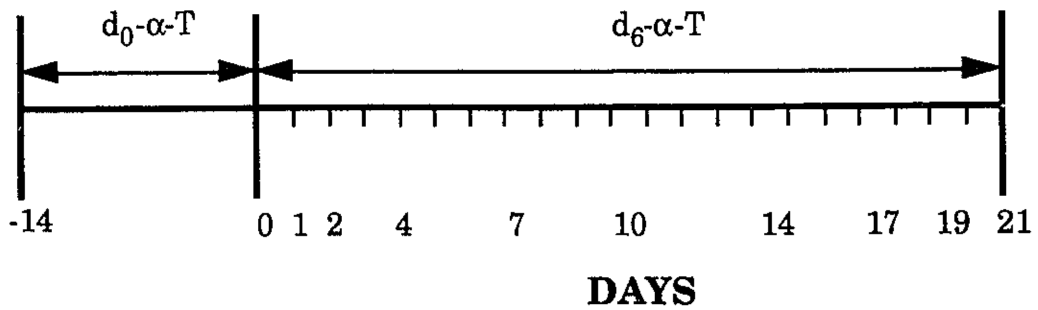
The animals in both the LE and the HE groups were fed for two weeks a diet containing 5 and 36 mg d_0-*RRR*-α-TAc/kg diet, respectively. Both groups received 250 mg ascorbic acid/kg diet. After this "lead-in" period, i.e., on day 0 of the actual experiment, both groups of animals continued to receive the same amount of vitamin E in their diet as before but solely in the hexadeuterated form, d_6-*RRR*-α-TAc (i.e., the d_0-*RRR*-α-TAc in each diet was replaced by an equivalent amount of d_6-*RRR*-α-TAc). However, the content of ascorbic acid was reduced to 10 mg per kg diet. Two guinea pigs from each group were sacrificed on days 0, 1, 2, 4, 7, 10, 14, 17, 19, and 21.

FIGURE 2. Scheme illustrating diets and sacrifice schedule used to determine the effect of dietary vitamin C upon tissue levels of vitamin E in male guinea pigs.

HE = high vitamin E; LE = low vitamin E;
NC = normal vitamin C; LC = low vitamin C.


HE NC
Vitamin E
(d_0-α-T): 36
Vitamin C: 250
(mg/kg diet)
HE LC
Vitamin E (d_6-α-T): 36
Vitamin C: 10 (mg/kg diet)


LE NC
Vitamin E
(d_0-α-T): 5
Vitamin C: 250
(mg/kg diet)
LE LC
Vitamin E (d_6-α-T): 5
Vitamin C: 10 (mg/kg diet)


d_0-α-T
d_6-α-T
-14
0 1 2 4 7 10 14 17 19 21
DAYS

Recovery of blood and tissue samples for analysis.

Animals were sacrificed in the morning without prior fasting. Blood samples were obtained by heart puncture of animals anesthetized with Innovar-Vet^{TM} (Pitman-Moore Ltd., Don Mills, Ontario). The animals were then sacrificed by arterial perfusion with isotonic NaCl. Blood was separated into plasma and red blood cell (RBC) fractions using a published procedure (45). Briefly, after collection, blood was placed in microcentrifuge tubes coated with disodium ethylenediaminetetraacetate (EDTA) and spun at 2000 rpm for 3/4 min in an Eppendorf microcentrifuge. The plasma was removed and stored at -80^0 C. The red blood cells were washed by resuspending them in ice-cold phosphate buffered saline (5 mM, pH 8.0), spinning for 30 s, removing the supernatant and repeating the procedure twice. The washed RBC, resuspended in the same buffer, were stored frozen at - 80^0 C after the hematocrit was measured.

Thirteen tissues (adrenal, brain, heart, kidney, liver, lung, muscle (biceps femoris), nerve tissue (sciatic nerve and spinal cord), small intestine (duodenum, including content), spleen, testis and renal white adipose tissue) and fecal pellets (recovered from the lower part of the large intestine) were removed from the animal, weighed and stored at -80^0 C prior to analysis.

3.1.2.2. Analysis of vitamin E

Lipid extraction.

Plasma (400 μl) was thawed and mixed with a known amount of d_9-*ambo*-α-T (approx. 7 nmol) in heptane. Lipids, including α-T, were extracted immediately by the ethanol-heptane procedure (11, 45). Typically, absolute ethanol (0.8 ml) was added to plasma (0.4 ml) and the mixture

vortex-stirred for about 30 s. Heptane (1 ml) was then added and the mixture was vortex-stirred again for a further 60 s. The aqueous and organic layers were separated by centrifugation (about 5 min) in a clinical, bench-top centrifuge and the top, organic layer (approx. 1 ml) was carefully drawn off with a Pasteur pipette and transferred to a glass vial that was sealed with a tightly fitting, foil-lined screw cap and stored at -20^0 C.

Frozen tissue samples were thawed (about 5 min) and placed on ice. One complete half of the brain, one adrenal gland, one testis and one kidney were used whereas weighed portions (0.006-1.0 g, depending on tissue size) were used for the other tissues and for fecal material. A known amount of d_9-*ambo*-α-T (50 µl of a 0.157 mM solution in heptane) was added to the sample immediately after it had been weighed. Tissues were homogenized in 2-8 ml of water, depending on the sample weight (ca 2 x 15 sec using a Brinkman/Kinematic Polytron PT 10/35 equipped with an anaerobic generator). The homogenate was placed in a glass centrifuge tube and the α-T was extracted using the SDS method (45, 148).

The SDS method takes advantage of the detergency of SDS to dissociate and solubilize membrane proteins, making the sample amenable to the aqueous alcohol/alkane procedure for general lipid extraction. SDS (0.5 M in a volume equal to about half of the homogenate volume), absolute ethanol (a volume equal to the combined aqueous volume), and *n*-heptane (a volume equal to, or in some cases, less than the volume of added ethanol) were each added to the homogenate in succession and vortex-mixed briefly (15 s each for SDS and ethanol and 60 s for *n*-heptane). The mixture was centrifuged in a clinical, bench-top centrifuge (about 5 min) and the heptane lipid extract was

removed with a Pasteur pipette and transferred to a glass vial that was sealed with a tightly fitting, foil-lined screw cap, and stored at -20^0 C prior to analysis.

The extraction of lipids from RBC also was performed using the SDS method (148). The RBC suspension in phosphate buffered saline (400 μl, hematocrit approx. 50%) was mixed with sodium ascorbate (approx. 100 mg; added to protect α-T from oxidation by hemoglobin-derived iron during extraction) and a known amount of d_9-*ambo*-α-T (50 μl of a 0.157 mM solution in heptane). SDS (0.1 M; 2 ml; 10 x volume of packed RBC), absolute ethanol 2.4 ml, equal to the total aqueous volume), and *n*-heptane (1 ml) were added to the mixture in succession and vortex-mixed briefly (about 30 s). The mixture was then centrifuged and the *n*-heptane lipid extract was recovered and stored in the same way as described in the procedure for plasma and tissue homogenates.

Earlier, it was found that the lipid extracts of white adipose tissue and of fecal material obtained by the SDS method contain a lot of substances that prevented adequate purification of the α-T by HPLC (45) . This problem was solved by subjecting samples of these materials (approx. 0.1 g of each) to alkaline hydrolysis prior to *n*-heptane extraction. This procedure was also applied to sciatic nerve (approx. 10 mg), which was not very susceptible to homogenization. The procedure was as follows. The sample was placed in a 15 ml glass tube equipped with a Teflon-lined screw cap. A known amount of d_9-*ambo*-α-T (0.157 mM, 50 μl for fat and fecal material; 25 μl for sciatic nerve), water (2 ml), absolute ethanol (2 ml) containing sodium ascorbate (1%), and saturated potassium hydroxide (1 ml) were each added in

succession to the sample and vortex-mixed briefly (about 30 s). The mixture was purged with nitrogen for 30 s, the tube capped and the mixture vortex-mixed briefly and heated at 75^0 C for 30 min. After cooling the tube on ice, water (1 ml) and *n*-heptane (4 ml) were added to the mixture which was then vortex-stirred for 60 s. After brief centrifugation, the heptane extract was recovered and stored at -20^0 C in a glass vial sealed with a tightly fitting, foil-lined screw cap. For sciatic nerve only 0.3 ml of saturated KOH solution was used and the amounts of other reagents used were reduced by half.

Purification of lipid extracts.

All extracts (including those from plasma and RBC) were purified by HPLC following a published procedure developed in this laboratory (45). The procedure was as follows. *n*-Heptane extracts were evaporated down to dryness under a stream of nitrogen and the residues were redissolved in 1 ml of *n*-heptane. The samples were then transferred to glass vials (12 x 32 mm) and sealed with screw caps equipped with Teflon septa. The vials were placed inside an HPLC autosampler (Varian, model 9090) and 500 μl samples were automatically injected into a high performance liquid chromatograph (Varian model 5000) equipped with a Lichrosorb Si 60 column (5μ; Merck, Darmstadt, Germany) and eluted with a mobile phase comprised of 90% *n*-heptane and 10% t-butylmethyl ether (vol/vol) at a flow rate of 2 ml/min. The eluent fraction containing α-T was automatically collected using a fraction collector (FOXY™, ISCO, Inc., series 2130-001). Peaks were detected with a Varian fluorescence detector, equipped with a deuterium lamp, using a 220 nm interference excitation filter and an emission filter which was a 2 mm thick Schott UG-1 glass band filter (λ_{max} approx. 358 nm).

The HPLC, autosampler, fraction collector and detector were interfaced with a Varian DS 654 control station and data-handling system.

Measurement of the tocopherols.

The relative amounts of deuterated (d_6-α-T and d_9-α-T) and nondeuterated (d_0-α-T) present in the purified lipid extracts were determined by the GC-MS method developed in this laboratory (45).

α-Tocopherol was analyzed as its trimethylsilyl ether derivative because this eliminated the peak "tailing" that occurs when tocopherol is analyzed in its free, underivatized form. This additional step gives more accurate peak area integration. The silylation was carried out as follows. The purified α-T extracts were evaporated down to dryness under a stream of nitrogen, then pyridine (100 μl) was added followed by the silylation reagent (50 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS)). Silylation was accomplished by heating the reaction mixture at 65^0 C for 15 min. After cooling, the samples were evaporated to dryness under a stream of nitrogen. The residues were redissolved in *n*-heptane (100 μl) and each vial was placed in an autosampler (HP 7673A) connected to a Hewlett Packard 5890 model gas chromatograph (GC). Samples (1 μl) were injected automatically into a Hewlett Packard Ultra 1 fused silica capillary column (12 m x 0.2 mm ID; cross-linked methyl silicone bonded phase) maintained at 280^0 C and eluted with helium using a 30:1 split ratio. The GC was connected to a Hewlett Packard 5970 A Series Mass Selective (MS) Detector programmed to monitor continuously the d_0, d_6 and d_9 molecular ions corresponding to the parent ion masses of the

respective α-tocopheryl silyl ethers (502, 508, 511 atomic mass units, respectively).

The GC, autosampler and the MS detector were interfaced with a Hewlett Packard 59970C MS ChemStation, the computer system controlling the analysis and the acquisition of the data. The GC-MS data, in the form of ASCII files, were transferred to an IBM PC clone (386 model) and were analyzed using the Lotus 123 spreadsheet program. The concentrations of d_0- and d_6-α-T in the original samples were determined by comparing the respective peak areas with the peak area of the internal standard, d_9-α-T, added in known amount to each sample just prior to lipid extraction. All measurements of deuterated α-tocopherols were corrected for the actual degree of incorporation of the nominal amount of deuterium in the molecule. The peak area data for the internal standard (peak 511) was also corrected for a 2.37% contribution from the M+3 ion of d_6-α-T. The M+3 contribution is due to the presence of natural abundance isotopes (^2H, ^{13}C, ^{29}Si).

The GC-MS technique permits detection of as little as 40 pg of α-T and it has been used, for example, to measure uptake of vitamin E into human heart from biopsy samples as small as 1 mg. In the present study, α-T in small samples of sciatic nerve (3.5-10 mg) was successfully measured and as little as 5 ng (ca 0.01 nmol) of α-T was detected.

The DOS computer files from the PC were transferred to a Macintosh computer for statistical analysis of results using the SYSTAT statistics program and for graphical presentation using KaleidaGraph, SYSGRAPH, MacDraw and ChemDraw programs.

3.1.2.3. Analysis of vitamin C

Vitamin C was analyzed by HPLC using a very sensitive amperometric detector. The analyses were performed in the laboratory of Dr. W. Behrens, Health and Welfare Canada, where the particular version of this method was first developed.

Extraction of vitamin C.

The tissues, after thawing (about 5 min), were immediately weighed (whole half of brain (approx. 1.6 g), one adrenal gland (approx. 0.05 g), one testis (0.1-0.2 g), one kidney (approx. 0.1 g), a sample of heart, liver, lung, (each approx. 0.5 g), small intestine (approx. 1.5 g), spleen and spinal cord (approx. 0.1 g) and placed in a tissue grinder (ground glass model, Duall^{R}, Kontes). An appropriate volume (0.1-1.0 ml) of cold metaphosphoric acid (17%) was added to give a final acid concentration in each homogenate of 0.85%. The samples were homogenized for about 30-60 s by the motor-driven pestle (Kontes). The homogenate was transferred to a graduated cylinder and made up to volume with water as follows: 0.1 and 1.0 g samples were made up to 3-5 ml, 0.05 g made up to approx. 2 ml and the "large" samples of brain and small intestine made up to approx. 20 ml of total homogenate volume. The samples were kept on ice throughout the procedure. The homogenates were then centrifuged at 20000 rpm for 15 min at 4^{0} C. The supernatant containing the ascorbate was separated by decantation and subsequently 400 µl of this was used for vitamin C analysis.

Reduction of oxidized ascorbic acid.

Any ascorbate which might have been oxidized to dehydroascorbic acid during sample manipulation was reduced back to ascorbic acid by reaction

with homocysteine. This was done as follows: to the aqueous acidic extract (400 µl) was added a freshly prepared, 1% solution of DL-homocysteine in phosphate buffer, pH 9.8 (115 µl). The mixture (final pH 7.1) was incubated in a shaking water bath for 30 min at 25^0 C after which time cold metaphosphoric acid (1.49 ml; 0.85%) was added and a 100-µl aliquot was withdrawn and diluted to 2-20 ml (depending on the sample weight originally taken for analysis and the anticipated content of ascorbate) with acetate buffer (85 mM, pH 4.8) containing 15% methanol.

HPLC analysis.

The diluted vitamin C extracts were transferred into glass vials (12x32 mm) which were closed with screw caps equipped with Teflon septa and then placed in an HPLC autosampler (Spectraphysics, model 8760) maintained at 4^0C. Aliquots (20 µl) were automatically injected into a 5µ C-18 reverse-phase column (Supelco: 250 mm x 4.6 mm ID). The column was eluted with a mobile phase (pH 4.6; flow rate of 0.9 ml/min) consisting of 85 mM sodium acetate buffer (initial pH 4.8) containing 1 mM n-octylamine, an ion-pairing reagent, 15% (vol/vol) methanol and metaphosphoric acid (0.015%, final concentration). The HPLC system was composed of a Spectraphysics XR extended range pump for liquid chromatography, a Spectraphysics model 4270 integrator and an electrochemical detector (LC-4B) equipped with a thin-layer glassy carbon working electrode (TL-5A) from Bioanalytical Systems (West Lafayette, IN). The working electrode potential was +0.7 V (50 nA) vs. the Ag/AgCl reference electrode. The HPLC system was interfaced to an IBM PC clone (model 386) using Labnet software (Spectraphysics) for instrument control and data collection.

Standardization and calculation of the results.

A series of five standard solutions of ascorbic acid containing 2.5-25 ng/ml of ascorbate were prepared by dissolving analytical grade ascorbic acid in sodium acetate buffer (85 mM; pH 4.8) containing 15% methanol. Aliquots (20 μl) of the five different standard solutions of ascorbate were automatically injected into the HPLC system and the area of each peak was recorded. The peak area data were transferred to a PC computer and, using the Lotus 123 spreadsheet program, the slope of the calibration curve was calculated. Excellent linear plots were always obtained for peak areas plotted against the standard ascorbic acid concentrations (data not shown). A calibration was performed every day prior to the analysis of a set containing about 20 samples of vitamin C tissue extracts. Vitamin C concentrations in tissues were calculated by dividing the ascorbic acid peak area by the slope of a corresponding calibration curve, making appropriate adjustments for sample dilutions. The DOS computer files were transferred to a Macintosh computer for graphical and statistical analysis of results using the software described already for analysis of the vitamin E results.

This method of vitamin C analysis permits detection of 50 pg of ascorbic acid in a 20 μl sample of a pure standard solution. In this study, 8 ng (ca 0.05 nmol) of ascorbic acid was detected in a 70 mg sample of heart tissue.

3.2. Results

The results of vitamin C concentrations versus time in 10 selected tissues and of vitamin E (α-T) concentrations versus time in plasma, RBC, feces, and 13 tissues are given in Appendices 1 and 2, respectively. The values are the means of the results obtained from two animals at each time

point, unless noted otherwise. Appendix 2 includes the concentrations of the labelled and unlabelled components of the total α-T, that is, the concentrations of the "old" d_0- and the "new" d_6-*RRR*-α-T.

Semi-logarithmic plots of vitamin C and total vitamin E (d_0+d_6-*RRR*-α-T) versus time are presented in Figures 3 and 4, respectively. Semi-logarithmic plots of d_0-*RRR*-α-T and normal plots of d_6-*RRR*-α-T versus time are presented in Figures 5 and 6, respectively. The lines in each of the graphs represent the least squares fit to each complete set of tissue data (i.e., not averaged data) of the LE and HE groups, respectively (i.e., linear regression plots).

The approximate linearity of the semi-logarithmic plots of concentrations of vitamin C, total α-T and d_0-α-T versus time makes it possible to perform simple statistical tests for the effects of dietary levels of vitamin C and E upon levels of vitamin C, total α-T and d_0-α-T in each tissue. The results of the regression analyses (slopes and y-intercepts) and the significance of each regression, that is, the probability that the slope of a regression line differs significantly from 0 (tested by analysis of variance, ANOVA) are given in Tables 1, 2, and 3 for vitamin C, total vitamin E and d_0-α-T, respectively.

Table 2 includes the results of analysis of covariance, ANCOVA of pooled LE and HE data. The results obtained by ANCOVA indicate whether or not the slopes of the two separate regression lines obtained for the LE and HE groups are statistically the same. Slopes that are the same for any particular tissue show that the rates of vitamin E change are very similar in the LE and HE groups (i.e., independent of the level of vitamin E in the diet). The ANCOVA

results also indicate whether or not the y-intercepts of two parallel regression lines are coincident. Coincident y-intercepts, together with parallel slopes, indicate levels of vitamin E that are very similar in the LE and HE groups. In this way, the effect of the level of dietary vitamin C and dietary vitamin E upon levels of tissue vitamin E can be assessed.

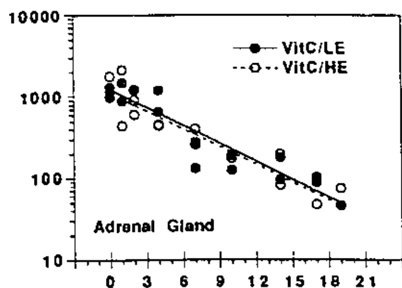
Levels of vitamin C.

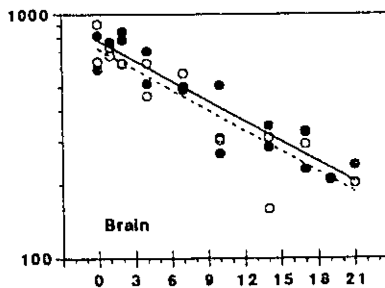
The results of the regression analysis of vitamin C vs. time (Table 1) indicate that the vitamin declines rapidly in all of the tissues measured and that the level of dietary vitamin E (LE vs. HE) has little or no influence (as is best seen in Figure 3). No ascorbate was detected for biceps femoris. It is possible that the difficulty experienced in measuring vitamin C in muscle and also in heart tissue was caused by the presence of high concentrations of iron in these tissues catalyzing the loss of vitamin C during the extraction procedure.

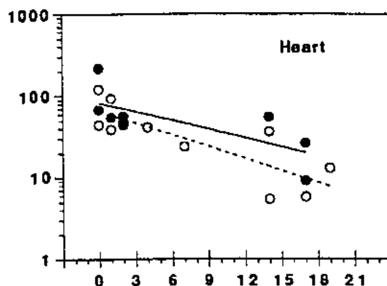
Levels of total vitamin E.

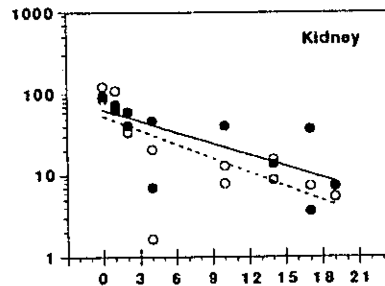
The results in Table 2 show that total α-T declines with time only in adrenal gland, lung and testis in the LE and HE groups of animals and in heart and kidneys in the LE group. That is, despite, the high dietary supplementation of vitamin E available in the HE diet, it was not possible to prevent a net loss of vitamin E in adrenal gland, lung and testis. ANCOVA showed the existence of common, significant negative slopes in all three tissues, indicating statistically identical rates of vitamin E decline on both LE and HE diets.

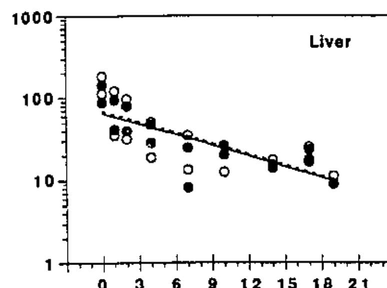
Figure 3. Semi-logarithmic plots of vitamin C concentration vs time for ten tissues of guinea pigs.

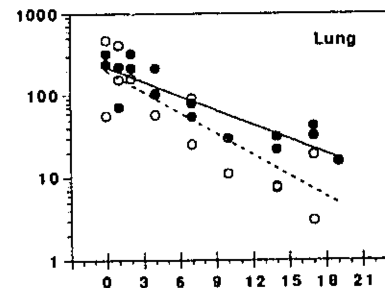


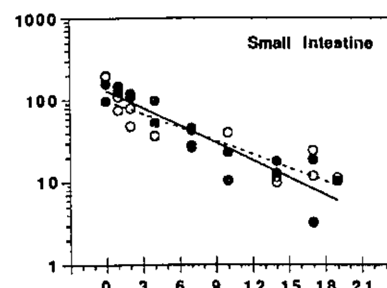


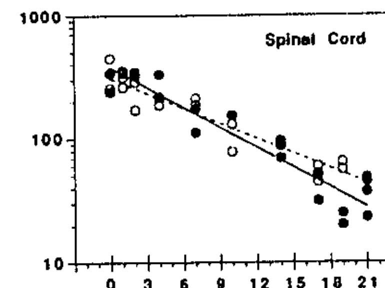


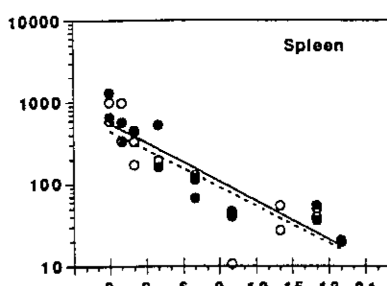


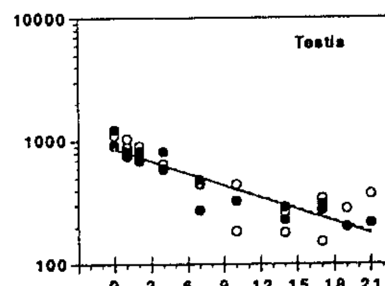












Vitamin C (nmol/g)

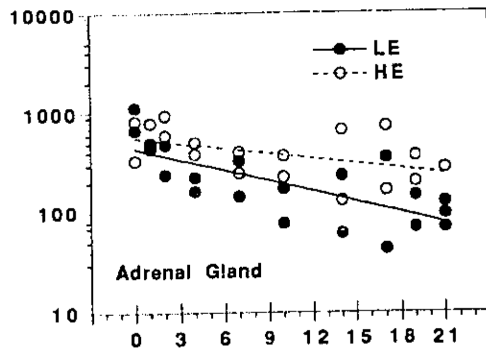
Day

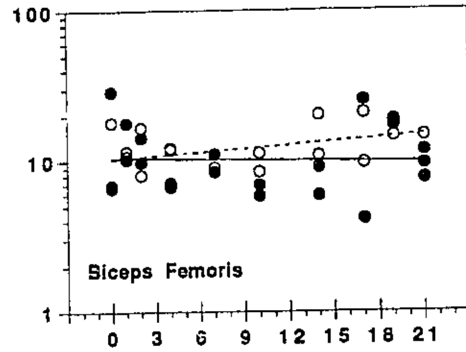
TABLE 1. Regression analysis of vitamin C concentration vs. time.

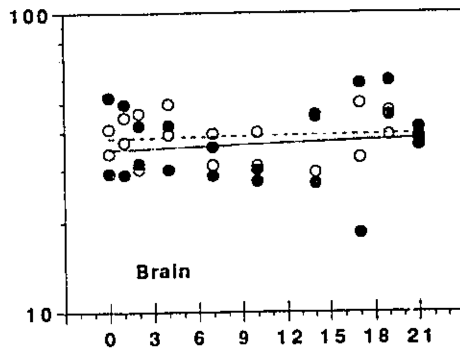
Tissue	Regression Coefficients [a] y-Intercept (nmol/g)		Slope (day^{-1})	
	LE	HE	LE	HE
Adrenal	1236	1108	-0.169	-0.168
Brain	781	720	-0.063	-0.064
Heart	83	66	-0.083	-0.113
Kidney	80	75	-0.116	-0.148
Liver	65	69	-0.099	-0.100
Lung	221	198	-0.134	-0.194
Small Intestine	132	101	-0.162	-1.126
Spinal Cord	148	307	-0.124	-0.093
Spleen	561	478	-0.182	-0.168
Testis	854	870	-0.075	-0.074

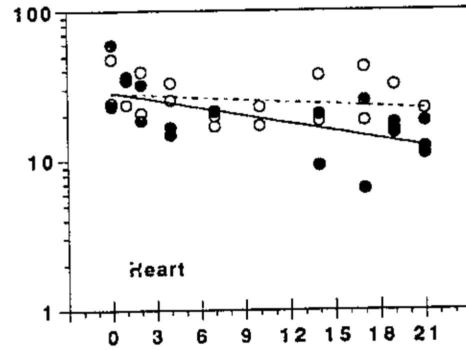
[a]The y-intercept and slope were obtained from the linear least squares fit of log vitamin C concentration versus time. All slopes were less than zero ($p < 0.001$, except for heart with $p < 0.05$ and $p < 0.01$ for LE and HE, respectively). Differences between slopes within tissues (LE vs. HE) were not statistically significant except for heart.

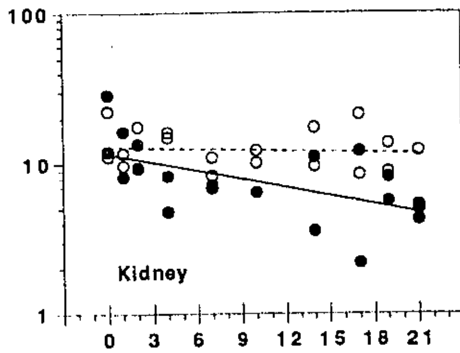
FIGURE 4. Semi-logarithmic plots of total (d_0- + d_6-) *RRR*-α-T concentrations vs. time for thirteen tissues, plasma, RBC and feces of guinea pigs.

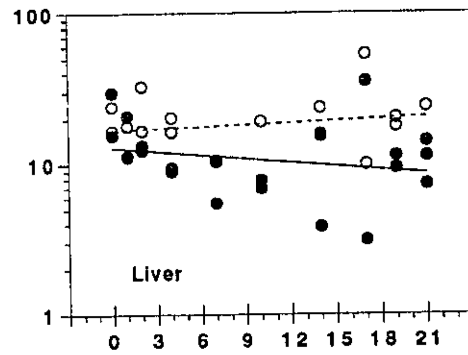


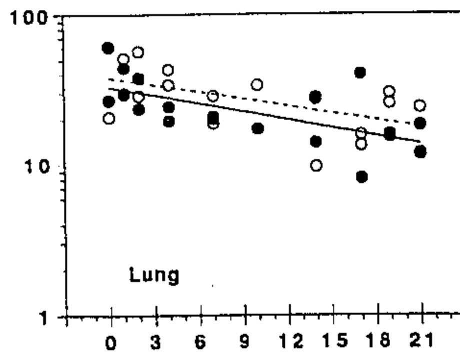


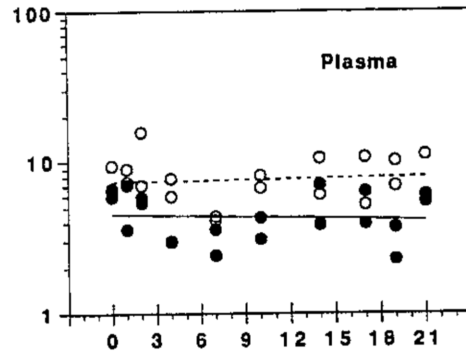






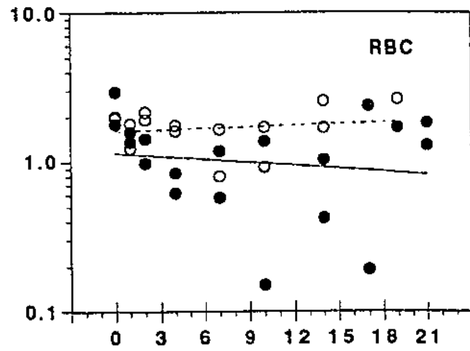


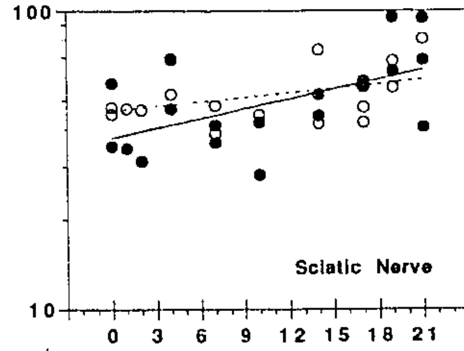


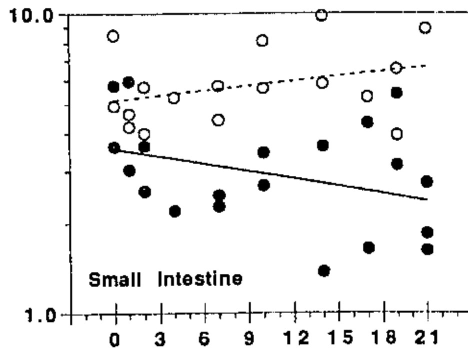


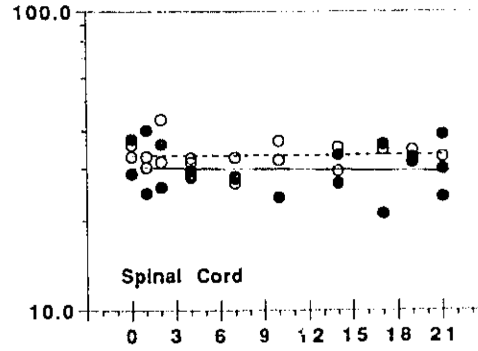
Total-α-T (nmol/g or ml)

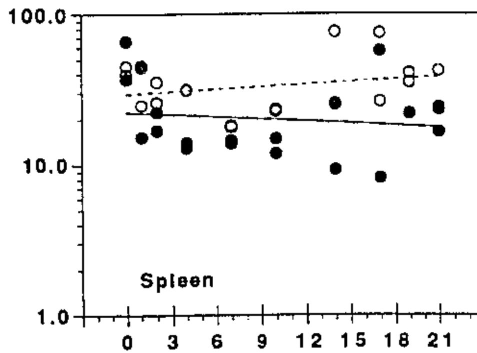
Day

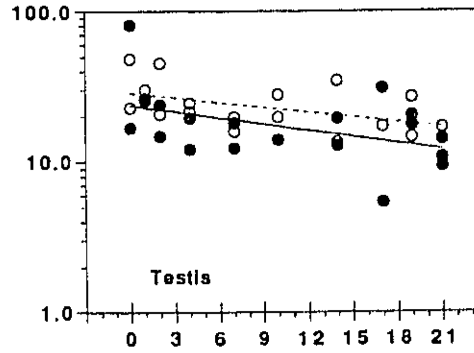


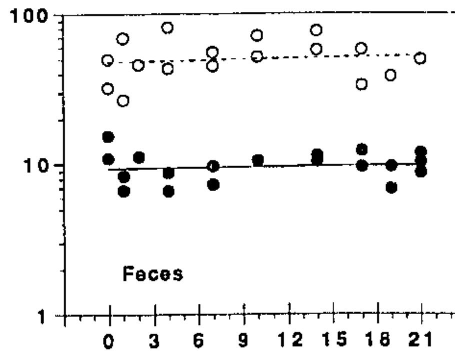


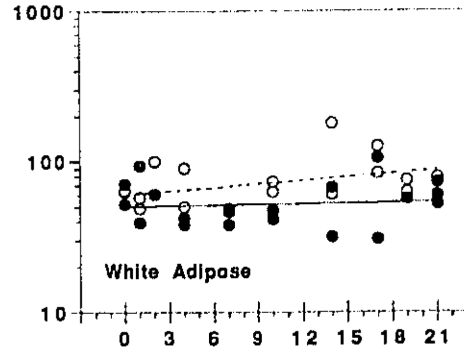












Total-α-T (nmol/g or ml)

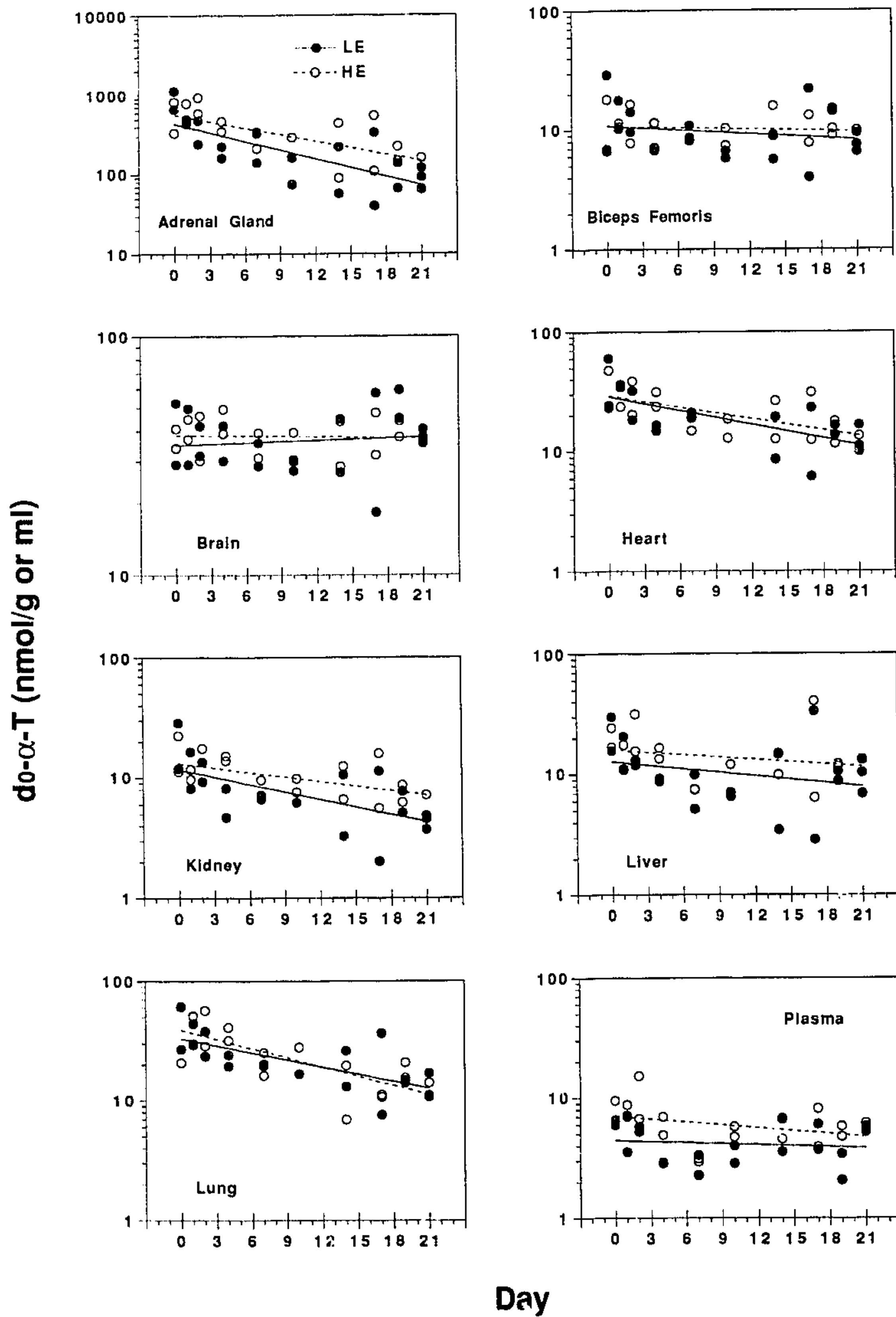
Day

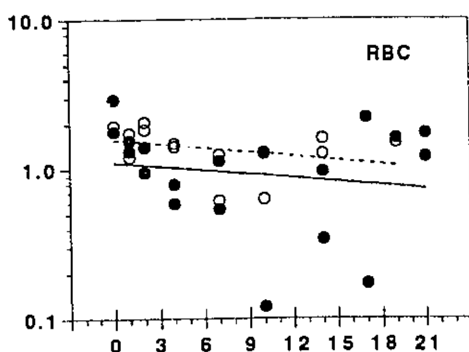
TABLE 2. Regression and covariance analyses of total *RRR*-α-T concentration vs. time.

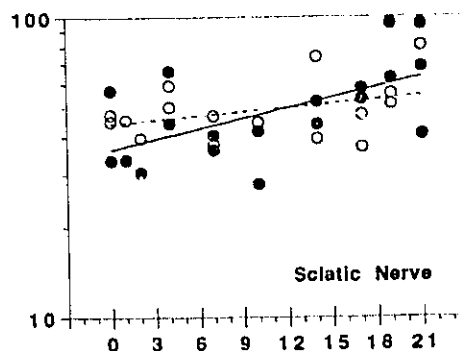
Tissue	Regression Coefficients [a]			
	y-Intercept (nmol/g or ml)		Slope (day^{-1})	
	LE	HE	LE	HE
Adrenal	437†††	560	-0.082***†††	-0.040*
Biceps Femoris	11	10	-0.003	0.018
Brain	35	38	0.004	0.001
Heart	29	28	-0.040**††	-0.012
Kidney	12‡	13	-0.043**	-0.005
Liver	13††	17	-0.019	0.010
Lung	33	38	-0.042***†††	-0.036*
Plasma	4.6†††	7.4	-0.004	0.004
RBC	1.1†	1.6	-0.016	0.008
Sciatic Nerve	38	46	0.025*†	0.011
Small Intestine	3.6‡‡‡	5.1	-0.019	0.012
Spinal Cord	30†	33	-0.001	0.0002
Spleen	22††	30	-0.011	0.012
Testis	24†	29	-0.032*†	-0.025*
Feces	9.4†††	48	0.002	0.004
Adipose	51††	61	0.003	0.018

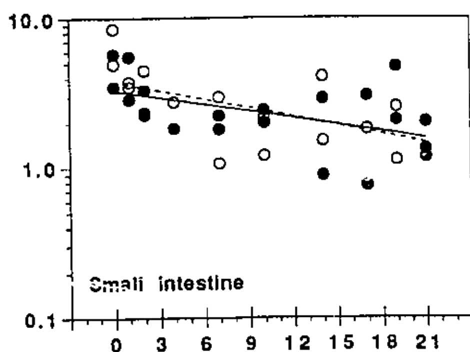
[a]The y-intercept and slope were obtained from the linear least squares fit of log vitamin E concentration versus time. The symbols*, ** and *** indicate non-zero slopes at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, calculated separately for LE and HE data points by ANOVA. The symbols †, †† and ††† indicate non-zero common slopes or lower total *RRR*-α-T levels for the LE vs. the HE group of animals at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, calculated for combined LE and HE data by analysis of covariance (ANCOVA). For kidney and small intestine the parallelism (homogeneity) of regression lines for the LE and HE groups was not established which is a prerequisite for ANCOVA, therefore the levels of α-T in LE group vs. HE group were compared by ANOVA; in these cases the symbols ‡, ‡‡‡ indicate a lower levels of total *RRR*-α-T for the LE group at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

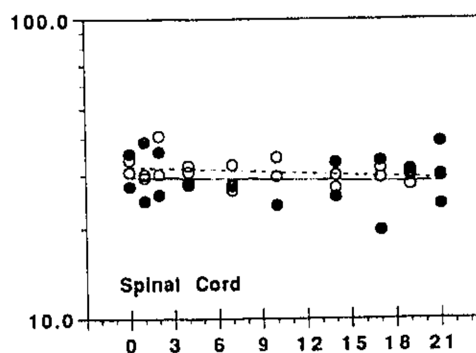
FIGURE 5. Semi-logarithmic plots of endogenous d_0-*RRR*-α-T concentrations vs. time for thirteen tissues, plasma, RBC and feces of guinea pigs.

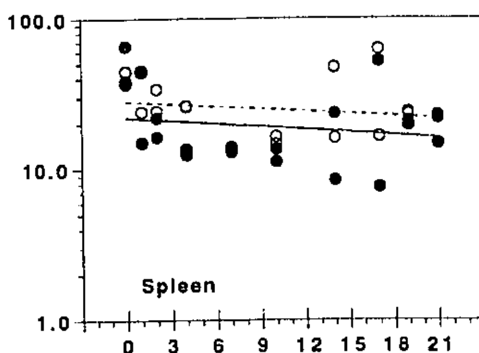

LE
HE
Adrenal Gland
Biceps Femoris
Brain
Heart
Kidney
Liver
Lung
Plasma
d0-α-T (nmol/g or ml)
Day

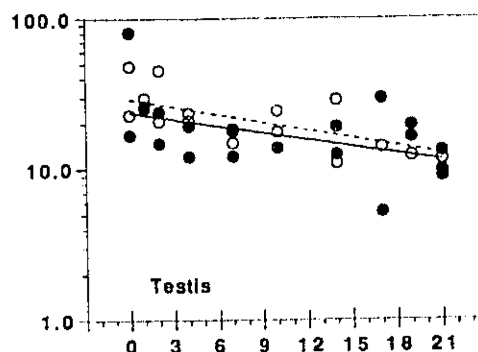


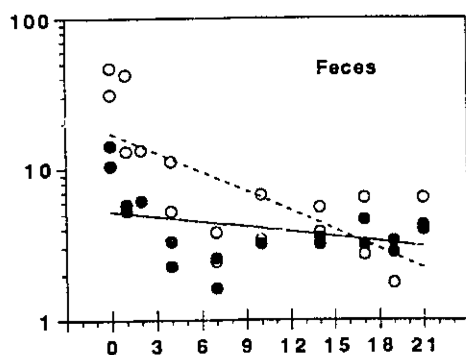


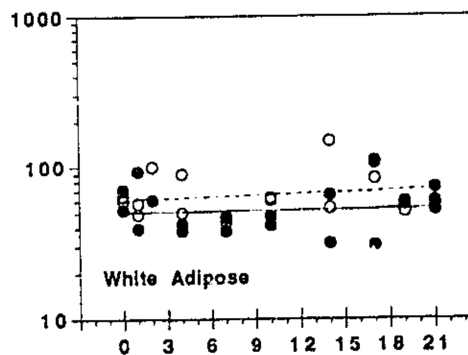












d0-α-T (nmol/g or ml)

Day

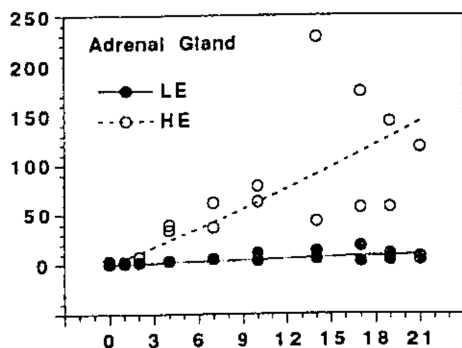
TABLE 3. Regression and covariance analyses of d_0-*RRR*-α-T concentration vs. time.

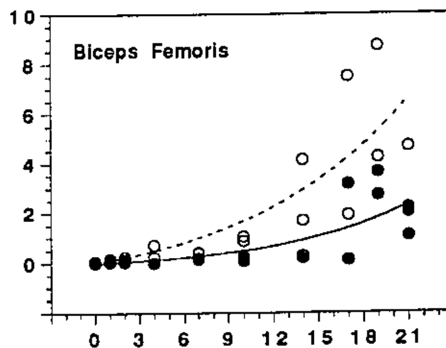
Tissue	Regression Coefficients [a]			
	y-Intercept (nmol/g or ml)		Slope (day^{-1})	
	LE	HE	LE	HE
Adrenal	$435^{\dagger}$	564	$-0.085^{\dagger\dagger\dagger***}$	-0.064^{***}
Biceps Femoris	11	11	-0.012	-0.005
Brain	35	38	0.004	-0.001
Heart	29	29	$-0.044^{\dagger\dagger\dagger***}$	-0.037^{**}
Kidney	$12^{\dagger}$	13	$-0.048^{\dagger\dagger\dagger**}$	-0.029^{*}
Liver	13	16	-0.022	-0.015
Lung	33	39	$-0.045^{\dagger\dagger\dagger***}$	-0.059^{***}
Plasma	$4.4^{\dagger\dagger}$	7.0	-0.007	-0.019
RBC	1.2	1.6	-0.019	-0.021
Sciatic Nerve	36	44	$0.027^{\dagger\dagger**}$	0.010
Small Intestine	3.3	3.8	$-0.035^{\dagger\dagger\dagger*}$	-0.045^{*}
Spinal Cord	30	32	-0.002	-0.005
Spleen	22	28	-0.015	-0.012
Testis	24	29	$-0.035^{\dagger\dagger\dagger*}$	-0.040^{***}
Adipose	$51^{\dagger}$	61	0.003	0.007

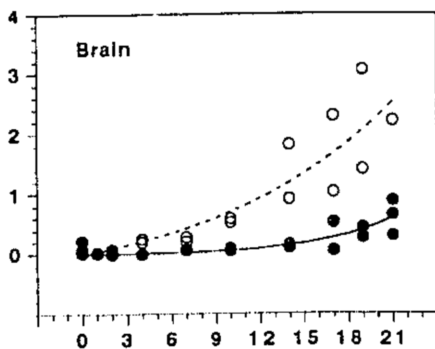
[a] The y-intercept and slope were obtained from the linear least squares fit of log d_0-*RRR*-α-T concentration versus time. The symbols *, **and ***indicate non-zero slopes at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. The symbols †, †† and ††† indicate non-zero common slopes or lower d_0-α-T levels for the LE vs. HE group of animals at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, calculated for combined LE and HE data by analysis of covariance (ANCOVA).

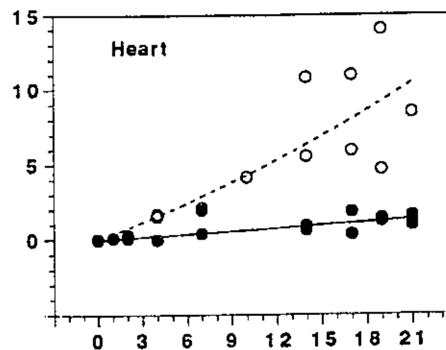
FIGURE 6. Plots of d_6-*RRR*-α-T concentrations vs. time for thirteen tissues, plasma, RBC and feces of guinea pigs.

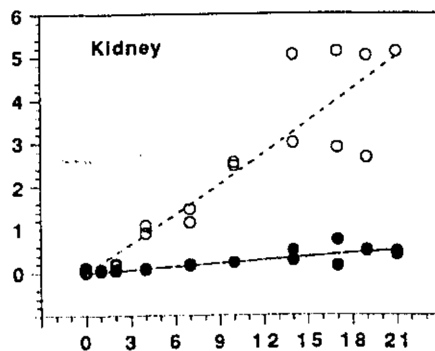
The lines in the plots were obtained by fitting equations of the type
$a = a_0(e^{bt} - 1)$ (concave upwards) or
$a = a_0(1 - e^{-bt})$ (concave downwards)
where $a = d_6$-α-T concentration, t = time, a_0 and b are constants and e = base of natural logarithm.

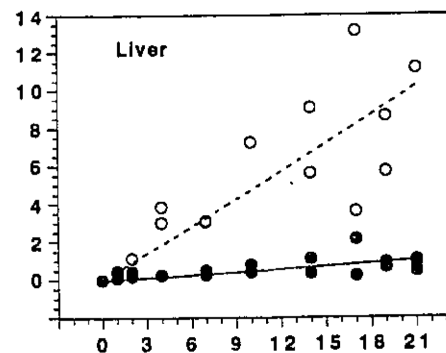


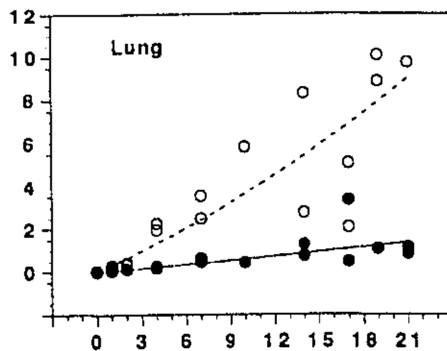


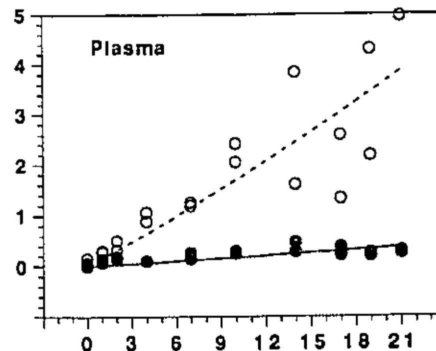






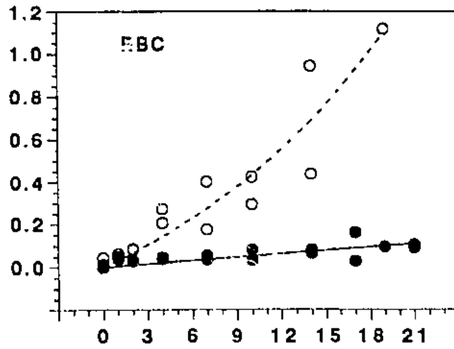


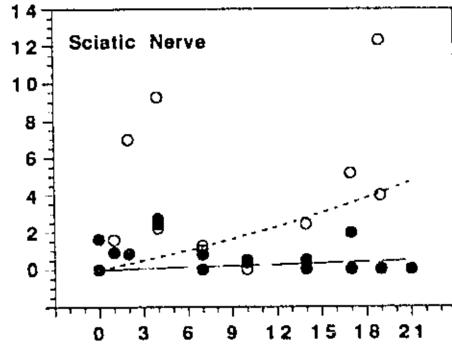


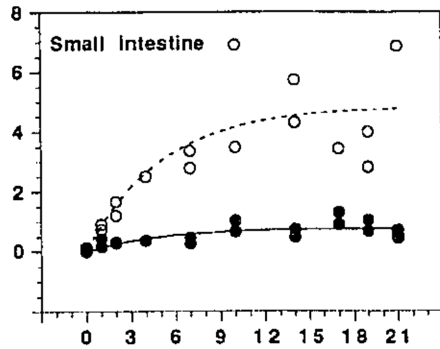


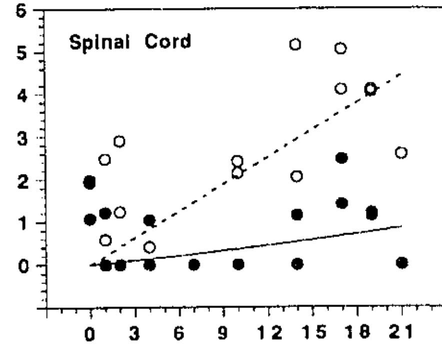
d6-α-T (nmol/g or ml)

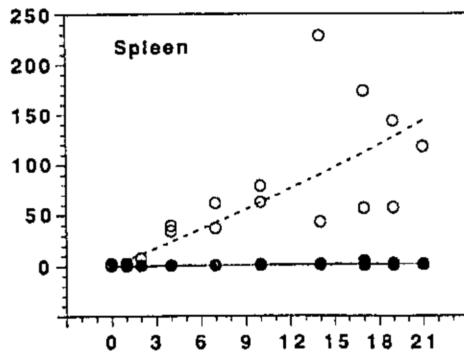
Day

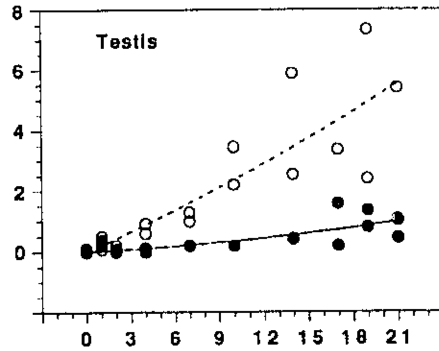


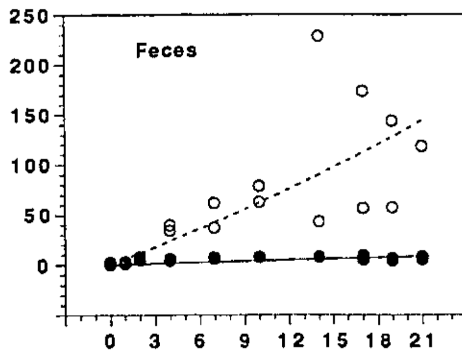


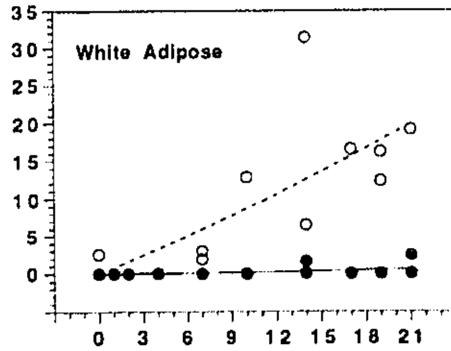












d6-α-T (nmol/g or ml)

Day

Of the tissues that showed no loss of vitamin E over time, only biceps femoris and brain had levels that were the same for both the LE and HE diets. In the remaining tissues and in feces, the levels of vitamin E were higher in the animals on the HE diet.

Sciatic nerve, in contrast to all other tissues, showed an *increase* of total vitamin E with time in the LE group.

Levels of endogenous (d_0-α-T) and newly absorbed (d_6-α-T) vitamin E.

During the 3 week experiment it was expected that the unlabelled α-T would decline and would be be partially replaced by the deuterium-labelled α-T. However, as can be seen in Table 3, loss of d_0-α-T was significant only in adrenal gland, heart, kidney, lung, and testis of the animals maintained on both the LE and HE diets. The levels of d_0-α-T remained virtually constant in adipose tissue, biceps femoris, brain, liver, small intestine, spinal cord, spleen, plasma and RBC, irrespective of the diet employed (as is readily seen in Figure 5). ANCOVA indicated that there was significantly less d_0-α-T in the LE group only in adipose tissue, adrenal gland, kidney and plasma.

It is very noticeable from Appendix 2 and Figures 5 and 6 that the amount of d_6-α-T incorporated into many tissues, as compared to the d_0-α-T already there, represents a very small fraction, e.g., adipose tissue, biceps femoris, brain, liver spinal cord, or spleen. Therefore, in these tissues the total vitamin E is composed mainly of a rather stable pool of d_0-α-T.

Levels of total vitamin E versus levels of vitamin C.

In the 10 tissues for which both vitamins were determined, the changes in vitamin E also were compared as a function of vitamin C concentration. The log-log plots are shown in Figure 7. The lines in each plot are the least

squares fit to the logarithms of the data points. The linear regressions of total α-T versus vitamin C (non-transformed data) were subjected to the same statistical analysis as previously employed for the regressions of total α-T versus time (Table 4).

Treatment of the data in this way showed that the decline of total α-T with declining vitamin C was significant in adrenal gland in both the LE and HE animals and in lung and testis in the HE group. The decline of vitamin E in heart was significant in the LE group.

FIGURE 7. Log-log plots of vitamin E concentrations vs. vitamin C concentrations for ten tissues of guinea pigs.

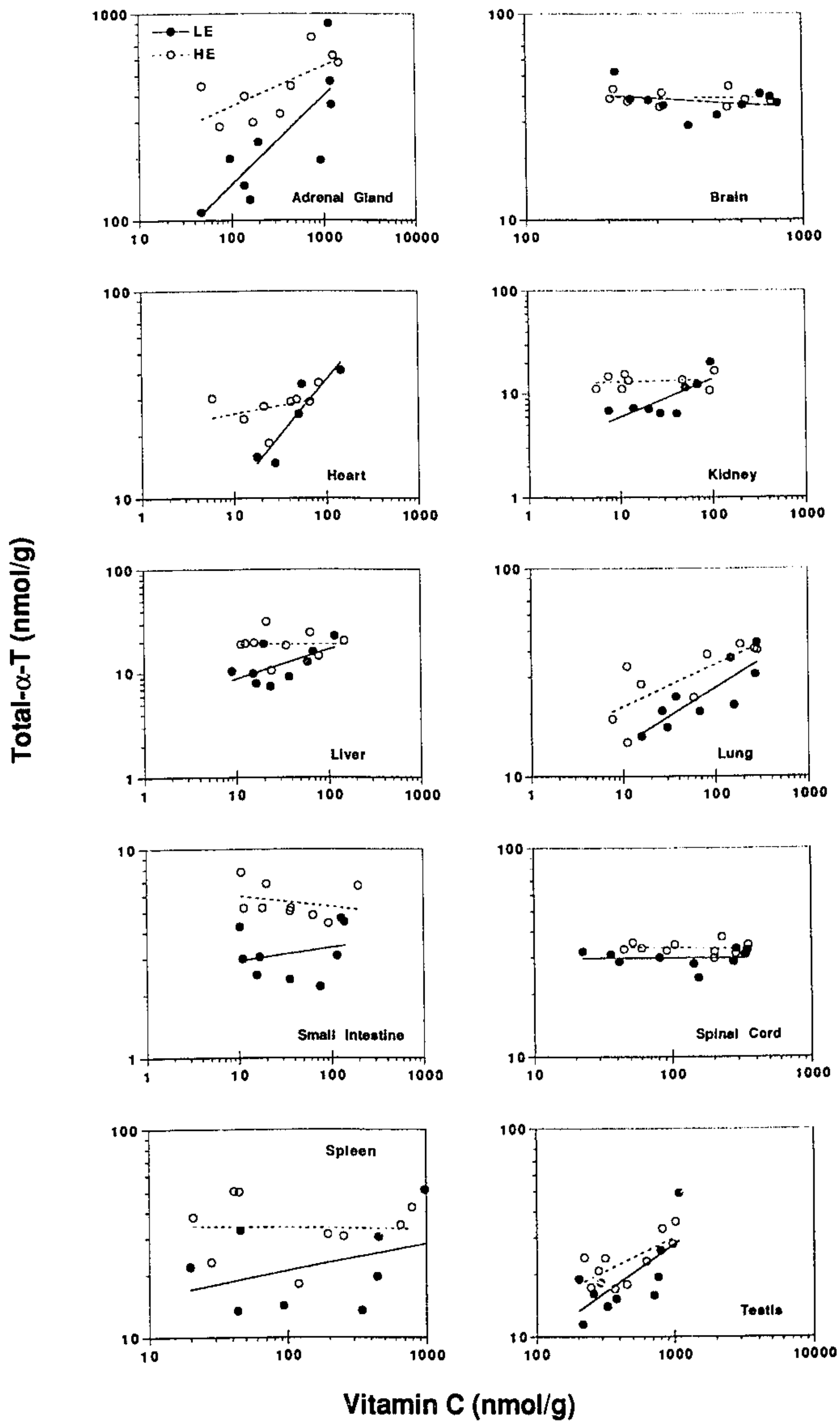

LE
HE
Adrenal Gland
Brain
Heart
Kidney
Liver
Lung
Small Intestine
Spinal Cord
Spleen
Testis
Total-α-T (nmol/g)
Vitamin C (nmol/g)

TABLE 4. Regression and covariance analyses of total *RRR*-α-T concentration vs. vitamin C.

Tissue	Regression Coefficients [a] y-Intercept (nmol/g)		Slope	
	LE	HE	LE	HE
Adrenal	113†	346	0.338*††	0.222*
Brain	41	39	-0.007	-0.001
Heart	13	23	0.219**††	0.160
Kidney	4.1	12	0.141*†	0.032
Liver	10	19	0.065	0.011
Lung	22	24	0.042††	0.062*
Small Intestine	2.8†††	6.8	0.008	-0.007
Spinal Cord	30†	34	0.002	-0.002
Spleen	16	37	0.028*	-0.003
Testis	11	16	0.019	0.016*

[a]The y-intercept and slope were obtained from the linear least squares fit of total *RRR*-α-T concentration versus vitamin C concentration. The superscripts *, ** and *** indicate non-zero slope at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, calculated separately for LE and HE data points by ANOVA. The superscripts †, †† and ††† indicate non-zero common slope and different total *RRR*-α-T levels (effect of LE vs. HE diet) at probability levels of $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, calculated for combined LE and HE data by ANCOVA.

3.3. Discussion

Vitamin C deficiency as a potential model of oxidative stress.

All tissues measured showed a substantial decline of vitamin C over the three-week duration of this experiment. The rate of vitamin C disappearance from each tissue was similar for the LE and HE animals, indicating that the vitamin C behaviour was independent of the level of dietary vitamin E. Adrenal gland, testis and spleen each had similarly high amounts of ascorbic acid at the beginning of the experiment (approx. 1000 nmol/g). Only slightly less vitamin C was initially present in brain. The initial concentrations of vitamin C were about 10 times lower in the other tissues that were measured.

The drop of vitamin C was highly significant in all tissues and was especially sharp in adrenal gland and spleen (an approximately two fold decline over 21 days). In the other tissues a one-fold drop was observed over the same period of time. Although the estimation of vitamin C was limited to 10 tissues, and data for plasma were not obtained, the consistent and dramatic depletion of vitamin C in the tissues examined indicated that the animals rapidly were becoming vitamin C deficient. Indeed, the deficiency was severe enough to cause, eventually, the development of frank scurvy, which made it impossible for the animals to survive much beyond three weeks.

Expectations.

In a tissue "starved" of vitamin C, a decreased level and an increased turnover of vitamin E would be expected if vitamin C indeed contributes to the antioxidant protection of aerobic cells. The contribution by vitamin C may

arise either through the direct interception of water-soluble free radicals or by the efficient regeneration of vitamin E from vitamin E radicals that are formed by reaction of vitamin E with lipid-soluble free radicals. The occurrence of both reactions in vitro has been well documented (22, 23). Thus, a scarcity of vitamin C may expose vitamin E to increased oxidative destruction, even if the rate of free radical generation is small. The increased loss of endogenous ("old") d_0-α-T might exceed the uptake of newly ingested ("new") d_6-α-T. As the total concentration of tissue vitamin E reflects the balance between the loss of "old" and the gain of "new" α-T, a net loss of tissue vitamin E might occur even if there is an abundant supply of tocopherol in the diet.

Preliminary evidence of an effect upon total vitamin E.

It has been clearly demonstrated in the previous biokinetics studies carried out in this laboratory that total (d_0+d_6) vitamin E levels remain steady during "long-term" α-T uptake by rats and guinea pigs maintained on a diet containing 36 mg α-TAc/kg (i.e., the same as in the HE diet) (39, 45). That is, the sum of "new" (deuterium-labelled) and "old" (unlabelled) vitamin E in the tissues and blood of guinea pigs remained essentially constant during the 8 week course of the experiment. On the other hand, the same studies showed that feeding the LE diet, which contains 5 mg α-TAc/kg, caused a depletion of vitamin E in the blood and in all tissues examined, except brain.

In the present study, in which restricted dietary vitamin C produced rapidly declining tissue levels of vitamin C, the total α-T in adrenal gland, lung and testis steadily dropped in animals on the HE diet, a diet which

normally is able to maintain constant levels of vitamin E in these tissues. In this circumstance the decrease of the total pool of tissue vitamin E in adrenal gland, lung, and testis tissue, even in the presence of an abundant supply of dietary tocopherol, clearly reveals the contribution of vitamin C in protecting against autoxidation.

The lower level of total α-T found in plasma, RBC, adipose tissue, liver, spinal cord and spleen of animals placed on the LE diet reflects the early drop related to the reduced intake of d_0-α-T during the 2 week "lead-in" period, since in all these tissues the level of total E was stable during the actual experiment. The lower levels of total α-T in adrenal gland and lung in the LE group also arise from a reduced intake of d_0-α-T during the "lead-in" period. The constant rate of decline of d_0-α-T during the 3 week of feeding the LE and HE scorbutic diets maintained the difference in vitamin E levels in these two tissues.

Non-scorbutic vs. scorbutic levels of dietary ascorbic acid: effect upon d_0- and d_6-α-T in blood and tissues - a comparison with the previous study.

In the previous study, guinea pigs were maintained on three, non-scorbutic levels of dietary ascorbic acid (50, 250 and 5000 mg/kg diet) in both the LE and HE diets. The protocol was very similar to that used in the present study. After a two-week "lead-in" period the animals continued to receive an LE or HE diet in which the d_0-α-T was replaced by d_6-α-T. This made it possible to study the biokinetics of vitamin E (i.e., the rates of disappearance of endogenous d_0-α-T and the incorporation of newly ingested, d_6-α-T) as a function of the different levels of vitamin C over a period of eight weeks (39; Appendix 3).

Unfortunately, plots of d_6-α-T vs. time are not easily "linearized" and therefore the data cannot be subjected to the same statistical analysis that were carried out on d_0-α-T and total α-T. However, since three of the time points at which animals were sacrificed (days 7, 14 and 21) were the same in the two experiments, it is possible to compare the corresponding levels of d_0- and d_6-α-T in blood and tissues at these times (Table 5).

At day 7 the levels of d_0-α-T in the animals placed on the scorbutic diets were approximately 2-3 times lower in kidney and liver (both LE and HE groups) and in RBC (HE group only) and almost five times lower in RBC of LE animals. In the remaining tissues and plasma the levels of d_0-α-T at this time approximately the same in both "normal" and "scorbutic" animals, apparently not being affected by differences in the level of dietary vitamin C.

A dramatic effect of vitamin C deficiency upon levels of d_6-α-T is seen in all tissues. It appears that much of this effects due to impaired absorption of d_6-α-T from the small intestine. At day 7, the level of d_6-α-T in plasma of "scorbutic" animals is about 5 times lower than in plasma of "normal" animals. This result probably reflects a loss of vitamin E during the digestion and absorption of the vitamin from the gut, but could possibly occur also during transportation into the blood via the thoracic lymph. The apparent impairment of delivery of vitamin E to plasma suggests that at this very first step of nutrient uptake, there is a need for the presence of a reducing or radical "repair" agent (e.g., ascorbic acid) to counteract pro-oxidative forces that destroy nutrients by oxidation.

Table 5. **Ratios of d_0- and d_6-*RRR*-α-T concentrations in tissues and blood of "non-scorbutic" (NS) vs. "scorbutic" (SC) guinea pigs[a].**

Tissue	Level of Vitamin E	d_0-α-T (NS/SC)			d_6-α-T (NS/SC)		
		Day			Day		
		7	14	21	7	14	21
Adrenal	HE	1.8	0.63	0.91	8.1	3.4	6.2
	LE	1.1	0.80	0.58	10.4	6.7	6.4
Biceps Femoris	HE	1.2	0.75	0.73	2.9	1.6	1.0
	LE	1.0	1.0	0.81	2.8	5.0	0.56
Brain	HE	0.99	0.55	0.79	7.1	1.2	2.4
	LE	1.0	0.77	0.96	5.7	7.3	2.7
Heart	HE	1.2	0.74	0.78	9.0	2.4	3.1
	LE	0.85	0.73	0.76	5.8	6.8	4.9
Kidney	HE	1.7	0.94	0.92	7.5	3.1	4.0
	LE	1.8	0.93	1.0	8.9	6.7	6.6
Liver	HE	2.9	1.0	0.61	14.7	6.8	4.9
	LE	2.5	1.5	0.82	15.0	12.3	9.1
Lung	HE	1.0	0.98	0.79	4.7	4.5	4.1
	LE	1.3	0.87	1.24	7.1	6.5	9.8
Spleen	HE	1.3	0.26	0.38	6.7	2.1	2.6
	LE	1.2	0.62	0.46	9.4	5.3	4.5
Testis	HE	0.92	0.48	1.1	5.6	2.0	4.3
	LE	0.93	0.87	0.45	6.2	10.2	3.1
Plasma	HE	0.97	0.25	0.23	4.9	1.7	1.7
	LE	1.4	0.31	0.64	4.8	4.2	6.9
RBC	HE	2.8	1.2	0.94	5.3	2.1	-
	LE	4.6	1.9	1.9	27.5	17.1	11

[a]The Concentrations of d_0- and d_6-α-T for the NS group used in the calculation of the ratios are the means of three animals receiving 50, 250 and 5000 mg vitamin C/kg diet, respectively, in the LE and HE groups. (There was no effect of dietary vitamin C on tissue vitamin E (39).) The corresponding values for SC guinea pigs are the means obtained from two animals on the LE and HE diets. The mean tocopherol concentration at day 21 is an approximation that includes the values obtained for two animals at day 19 and for one and three animals for the LE and HE groups at day 21, respectively.

Unfortunately, the levels of vitamin E in the small intestine of the "normal" and "scorbutic" animals can not be compared directly because appropriate measurement was not carried out in the previous study.

Very recently, it has been reported that the free radical, nitric oxide, NO•, may be released at multiple gastrointestinal sites in guinea pigs (149). Nitric oxide is now recognized as a "useful" free radical species. In the brain, for example, it functions as a neural messenger (150) and in central and peripheral blood vessels it is believed to be responsible for the activity of the endothelium-derived relaxant factor (EDRF) (151).

The generation of nitric oxide for these important functions also could cause of initiation of oxidative reactions (including the loss of vitamin E), which may, in part, be controlled by the reducing property of vitamin C (occurring either by transfer of an electron or an H atom to the free radical species). The apparent loss of vitamin E under conditions of vitamin C deficiency could be related to the presence of nitric oxide in the gut, although it is also possible that other catalysts of oxidation, such as dietary iron and copper, could play an important role.

The biggest effect of vitamin C deficiency on the uptake of d_6-α-T is found in RBC of the LE animals (27 times less d_6-α-T in "scorbutic" than in "normal" animals). A possible explanation for this finding is that mammalian erythrocytes are probably exposed to a continuous flux of superoxide radicals arising from the presence of heme iron in an environment with a relatively high partial pressure of oxygen (2). Although the high concentration of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) and the presence of the supporting pentose phosphate pathway

enzymes in erythrocytes could counteract this pro-oxidant condition, the considerable drop in the levels of both d_0- and d_6-α-T in RBC of the "scorbutic" animals, especially those on the LE diet, suggests that the presence of both vitamin C and vitamin E is very important in inhibiting autoxidation in RBC.

The vitamin C-deficient diet appears to exert an additional effect during the uptake of d_6-α-T into tissues. The most remarkable effect is seen in the liver. The level of d_6-α-T is 15 times lower at day 7 in the animals in both the LE and HE groups on the scorbutic diet. The level of d_6-α-T is also lower (5 -10 times) in all other scorbutic tissues, except biceps femoris, where the level is only 3 times lower. These results suggest that a further loss of d_6-α-T occurs during incorporation of vitamin E into most tissues.

Vitamin E, together with all other nutrients, is delivered to tissues via a system of blood capillaries. Being lipid-soluble, vitamin E can diffuse directly through the cell membranes of the capillaries into the interstitial fluids that surround all cells. The oxygen partial pressure on the arterial side of the capillaries is high (95 mm Hg), a condition which can facilitate autoxidation. In the interstitial fluids, the oxygen pressure, although very variable, is on average lower (40 mm Hg), but it can rise when the rate of blood flow is increased, the concentration of hemoglobin is increased and/or the tissue rate of oxygen metabolism is decreased (152). The oxygen partial pressure is thus substantially higher in the interstitial fluids than inside a cell, where the estimated pressure is 23 mm Hg (152).

Superoxide radical can be released into the extracellular spaces by resident macrophages or leukocytes during phagocytosis (145, 146). The

capillaries and the interstitial fluid are thus possible sites where vitamin E may become irreversible oxidized before it can diffuse into the plasma membranes of the individual cells.

With the progress of time, more d_6-α-T reaches and is incorporated into the membranes of cells and organelles, especially in the HE animals. It is possible that substantial losses of vitamin E occur before the vitamin is incorporated into cells. From the d_0-α-T results, it is evident that much of the d_0-α-T is not deleteriously affected by a lack of dietary vitamin C (days 14 and 21, Table 5). It appears that the vitamin E that is already present within the cells either does not turnover rapidly or is well protected by a mechanism that is not vitamin C-dependent.

The data in Table 5 also suggest that beyond day 7 vitamin C deficiency and the apparent associated impairment of d_6-α-T uptake into tissues causes a mobilization or redistribution (perhaps from the liver) of endogenous, d_0-α-T into the circulation. Thus, in the plasma of the animals on the scorbutic diet the level of d_0-α-T is substantially higher in the "normal" animals. Also, in the other tissues there appears to be a tendency to "spare" d_0-α-T as the level of vitamin C declines

It is interesting to qualitatively compare the slopes of the regression lines of d_0-α-T and total α-T vs. time obtained for "scorbutic" (Tables 2 and 3) and "normal" animals (39). The results of this comparison are shown in Table 6.

Table 6. Comparison of slopes of regression lines of d_0-α-T and total α-T concentrations vs. time for "non-scorbutic" (NS) and "scorbutic" (SC) animals.

Tissue	Non-Scorbutic				Scorbutic			
	LE		HE		LE		HE	
	d_0	Total	d_0	Total	d_0	Total	d_0	Total
Adrenal	–	–	–	0	–	–	–	–
Biceps Femoris	–	–	–	0	0	0	0	0
Brain	0	0	0	0	0	0	0	0
Heart	–	–	–	0	–	–	–	0
Kidney	–	–	–	0	–	–	–	0
Liver	–	–	–	0	0	0	0	0
Lung	–	–	–	0	–	–	–	–
Spleen	–	–	–	0	0	0	0	0
Testis	–	–	–	0	–	–	–	–
Plasma	–	–	–	0	0	0	0	0
RBC	–	–	–	0	0	0	0	0
Sciatic Nerve	n.d.	n.d.	n.d.	n.d.	+	+	0	0
Spinal Cord	n.d.	n.d.	n.d.	n.d.	0	0	0	0

The symbols –, 0 and + indicate declining, constant or increasing levels, respectively, of d_0-α-T and total α-T during the 8 week (NS) and 3 week (SC) periods of the experiments. n.d. = not determined.

The remarkable stability of the d_0-α-T levels in muscle, liver, spleen, plasma and RBC in the LE and HE groups of the animals on the scorbutic diet contrasts with the declining levels in the animals receiving vitamin C-sufficient diets. It is tempting to suggest that the vitamin C deficiency and, in turn, the decreased uptake of vitamin E into tissues is sensed by the animals as a general vitamin E deficiency, causing the animals to conserve existing stores of vitamin E. Indeed, a deficiency of vitamin E has been found to lead to a redistribution of vitamin E into neural tissues in rats (Burton and Muller, unpublished data). In this regard, it is interesting to note that the guinea pigs in the present study show a significant increase with time (and with declining vitamin C) of d_0-α-T in sciatic nerve in the LE animals (Table 2). That is, an insufficiency of dietary vitamin C in guinea pigs produces an effect in sciatic nerve tissue which is even more marked than the effect observed during a severe dietary restriction of vitamin E in rats (Burton and Muller, unpublished data).

Biceps femoris is the only tissue where the scorbutic level of vitamin C has a relatively small effect on the uptake of vitamin E. This effect may be related to the fact that, compared to the other tissues examined, in muscle there is relatively less of the cell plasma membranes in which much of the vitamin E normally resides. Muscle fibers are very well innervated. The nerve endings make a junction with each individual muscle fiber (152). Perhaps, the substantial portion of the total pool of vitamin E detected in biceps femoris is of neural origin and the observed changes of vitamin E in muscle reflect, to some extent, the vitamin E behaviour in nerve tissue. There is no data to compare directly the behaviour of vitamin E in the nerve tissues

in "scorbutic" and "normal" animals, but vitamin E in sciatic nerve, as already noted, appears not only to be well protected and independent of vitamin C concentration, it actually increases during the onset of vitamin C deficiency.

Skeletal muscle, on the other hand, is rich in mitochondria that are sites of very intensive ATP production. If, indeed, enzymes of the electron transport chain that are coupled to ATP synthesis do "leak" electrons onto molecular O_2 and generate O_2^-, muscles will required substantial protection against autoxidation. In this scenario, the stability of vitamin E levels in muscle would suggest the existence of a very effective, vitamin C-independent mechanism that is able either to inhibit free radical reactions or to regenerate vitamin E from vitamin E radicals.

Thus, from the biokinetics data it becomes evident that whereas the uptake of vitamin E into cells seems to be vitamin C-dependent, its retention inside the cell appears to remain under the control of a mechanism that is not strongly affected by the level of vitamin C. Furthermore, the persistent decline of vitamin C levels seems to reinforce this unknown protective mechanism in most tissues, and, at the same time, causes the release of increased amounts of endogenous vitamin E (d_0-α-T) in the plasma. This leads to a relatively small variation in total vitamin E levels in some tissues. If this protective mechanism is less efficient in rats than in guinea pigs, it may explain why the vitamin C/vitamin E interaction was readily detected as a decline in total vitamin E in mutant rats (117) but not in guinea pigs (39, 123) fed a low level of vitamin C.

Free radical generation in tissues.

Although uptake of vitamin E into tissues is substantially reduced in animals placed on a scorbutic diet, sufficient vitamin E is available to prevent the total vitamin E level from dropping in the HE group in most of the tissues. However, in the adrenal gland, lung and testis, total vitamin E dropped significantly, indicating that the uptake of d_6-α-T could not keep up with metabolic loss of vitamin E in these tissues (Tables 2, 3, 4 and, for comparison with "normal" animals, Tables 5 and 6).

These three tissues can be considered to be exposed to increased rates of free radical production because they contain substantial quantities of the cytochrome P-450 mixed function oxidases, whose principal function is to introduce oxygen into various endogenous (e.g., cholesterol) and foreign organic substrates (153).

It is believed that the cytochrome P-450 system can "leak" electrons onto oxygen, forming the superoxide radical which disproportionates to hydrogen peroxide that in turn, can lead (through Fenton chemistry) to the production of the very reactive hydroxyl radical (2). All adrenocortical hormones formed in the adrenal cortex are steroids derived from cholesterol. Also, androgens, secreted mainly by testes and in much smaller quantity by the adrenal gland, are steroids synthesized from cholesterol (152).

The fundamental reaction involved in these syntheses is the hydroxylation of cholesterol by the mitochondrial cytochrome P-450 enzymic system. For instance, in the adrenal cortex, electrons required for the steroidogenesis are donated by a non-heme protein, adrenodoxin. A flavoprotein enzyme transfers electrons from NADPH to adrenodoxin. The

reduced form of adrenodoxin can "leak" electrons onto molecular oxygen, reducing it to superoxide radical (2).

Another region of the adrenal glands, the adrenal medulla, may also contribute to the production of free radicals. Adrenaline, the hormone secreted by the adrenal medulla, has been shown to spontaneously oxidize in solution to give superoxide radical.

It is likely, then, that the adrenal gland and testis, which are very active sites of free radical-mediated hydroxylation, are exposed to higher levels of free radical production and, consequently, to an increased risk of autoxidation relative to other tissues.

The adrenal cortex contains higher concentrations of ascorbic acid and α-tocopherol than any other organ, but the detailed physiological role(s) of these vitamins in this tissue has yet to be revealed (154). A regulatory function in steroidogenesis for vitamin E, vitamin C and some products of lipid peroxidation has been proposed (155-157). It is obvious to suggest from the results of the present study that the high concentrations of vitamin C and vitamin E in the adrenal glands reflect, in part, an increased need for protection against the free radicals generated in the normal functioning of this tissue.

Lung, besides containing an active P-450 system (153), also is exposed to a concentration of oxygen that is higher than in any other tissue (152), which enhances the possibility of autoxidation. Thus, in these three tissues vitamin E turnover due to consumption by free radicals may be naturally higher than in most other tissues. This vitamin E turnover apparently can not be totally compensated for by internal mechanisms and therefore requires

a continuous external supply of α-tocopherol to maintain vitamin E status. In this regard, it is surprising that liver, though richly endowed with P-450 enzymes, did not show any overall decline of vitamin E.

Protection of vitamin E inside the cell.

A vitamin C-independent mechanism that may operate in the liver to spare or regenerate vitamin E has been proposed on the basis of in vitro studies. In particular, lipid peroxidation in rat liver microsomes has been reported to be inhibited by glutathione (GSH) (124, 125). The presence of α-tocopherol was found to be essential for this protection (103, 128, 158, 159). Also, if liver microsomes are heated, the inhibitory effect of glutathione on lipid peroxidation is abolished, even when normal levels of tocopherol are present in the microsomes (124). This result, together with the observation that the suppression of lipid peroxidation by glutathione is eliminated by prior treatment of the microsomes with trypsin (125), suggests that the effect of glutathione is mediated by a protein.

It has been proposed that the protection of liver microsomes against lipid peroxidation by GSH proceeds via a regeneration of vitamin E from the vitamin E radical (128) that is catalyzed by a membrane-bound, heat-labile factor. These observations, however, have been confined to the liver and may explain why in this study liver, although a site of high activity of cytochrome P-450 enzymes, did not show a decline of total vitamin E.

Recently, the existence of a similar protective mechanism has been shown to operate in homogenates of human platelets (131). Incubation of a platelet homogenate with arachidonate produced a rapid consumption of tocopherol. Addition of either ascorbic acid or GSH caused significant

regeneration of the endogenous vitamin E. The kinetics of the reaction produced by glutathione (a steady increase of tocopherol, reaching a plateau in 10 min) and the inhibitory effect of denaturating conditions were taken to suggest that the regeneration of tocopherol by glutathione is mediated by an endogenous factor, possibly an enzyme.

Another potential mechanism of vitamin E regeneration may involve biliverdin and conjugated bilirubin (CBR). Recently, these end products of heme catabolism in mammals were shown to be potent antioxidants (160). The water-soluble, conjugated bilirubin (CBR) and its precursor biliverdin, (BV) have been shown to regenerate vitamin E from the vitamin E radical in phosphatidylcholine (PC) liposomes (37). Whether CBR actually contributes to the protection of vitamin E inside living cells remains to be established. The intestine is a likely potential site of CBR activity. However, the obvious impairment of vitamin E absorption from the gut under vitamin C deficient conditions observed in this study indicates that CBR, in contrast to vitamin C, offers little or no protection at all in the intestine.

The remarkable absence of a noticeable decline of vitamin E concentration in brain and spinal cord is related to the very slow loss of d_0-α-T from these tissues. This, together with the enhancement of d_0-α-T in sciatic nerve under vitamin C deficiency (especially in the LE group) suggests that neural vitamin E is under strict protective control. The existence of a mechanism that conserves the levels of vitamin E within the cells is thus strongly implied. The nature of this mechanism, however, remains unknown. It could also be that there is very little metabolism of vitamin E in neural tissues.

Summary.

The use of deuterium labelled α-T as the only source of dietary vitamin E together with dietary control of levels of vitamin C to control tissue vitamin C levels has allowed differentiation to be made between effects of vitamin C levels upon endogenous vitamin E (taken up prior to the experiment) and upon newly ingested, dietary vitamin E (absorbed during the experiment). The biokinetics data have shown that vitamin C seems to be indispensable for the protection and proper uptake of vitamin E from the gut and for delivery into the tissues. Existing levels of vitamin E in tissues other then adrenal gland, lung and testis, in contrast, appear not to depend on vitamin C protection. The stability of the vitamin E levels in these tissues strongly suggests the existence of an alternative, efficient vitamin E conservation mechanism within the cells which vitamin C deficiency even seemed to enhance.

4. CHANGES IN VITAMIN E IN BRAIN FOLLOWING FOREBRAIN ISCHEMIA IN THE TWO VESSEL OCCLUSION MODEL OF STROKE IN RATS

4.1. Experimental procedure

4.1.1. Materials

All reagents and materials used in this section of the study have already been described in section 3.1.1.

4.1.2. Methods

4.1.2.1. Experimental design

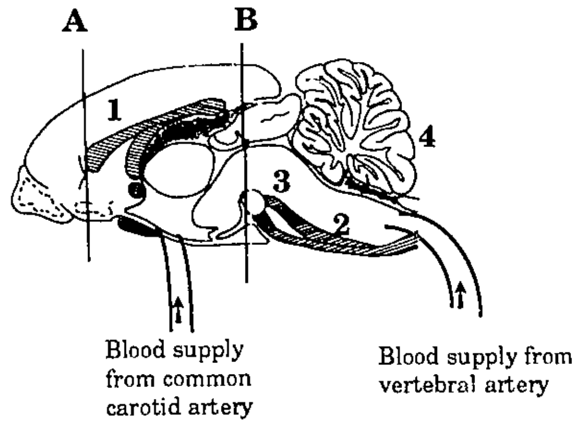
Two-vessel occlusion model of stroke in rat

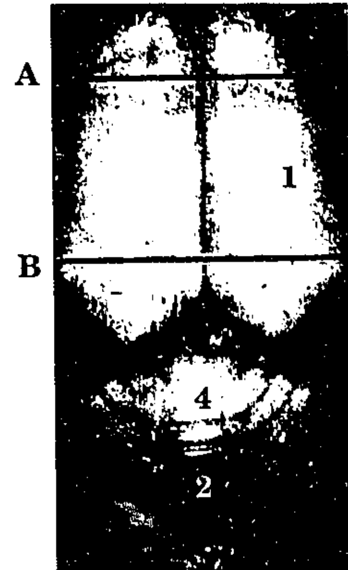
The animals were cared for and operated upon by Dr. E. Preston of the Institute for Biodiagnostics, NRC. The procedure was as follows. Four male Sprague-Dawley rats, 330-400g, were anesthetized with pentobarbital (60 mg/kg i.v.), intubated and mechanically ventilated with 30% oxygen and 70% air. The tail artery was cannulated, blood gases and pH were tested, ventilation was adjusted as required and both carotid arteries were exposed through a neck incision. To induce forebrain ischemia, approximately 7 ml of arterial blood was withdrawn into a heparinized syringe and this volume was then adjusted as necessary to maintain arterial pressure at 42-47 mm Hg for 25 min. During this time both common carotid arteries were occluded using non-traumatizing vascular clamps. At the end of 25 min, blood flow through the carotid arteries was restored and the aspirated blood reinfused. Following reperfusion and wound closure, rats were maintained under normothermic conditions and were permitted to recover for 24 h before being sacrificed by decapitation. Immediately after decapitation, the brain was removed,

bisected down the sagittal midline, and each half of the brain was dissected into 6 components corresponding to the cortex, striatum, hippocampus, diencephalon/mesencephalon, cerebellum and pons-medulla regions (see Fig. 8). Surface membranes and blood vessels were stripped away during dissection.

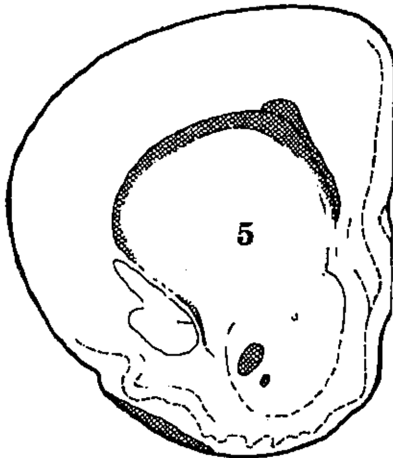
As a control, four rats were subjected to a sham operation which involved all procedures except the induction of ischemia. Brain tissue was removed and dissected in the same manner as in the ischemic animals. All brain samples were stored at -80° C prior to vitamin E analysis.

FIGURE 8. Physical representation of the six anatomical regions of the rat brain analyzed for vitamin E in this study.

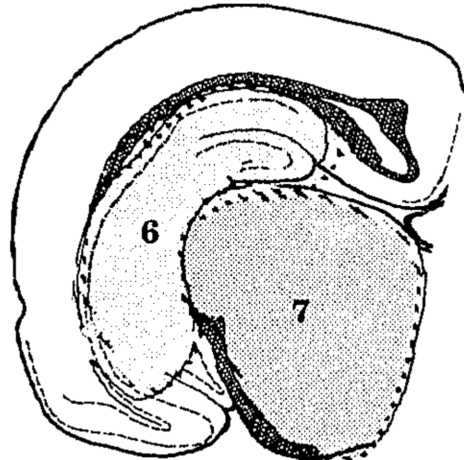




Cross section at **A**



Cross section at **B**



1	Cortex
2	Medulla
3	Pons
4	Cerebellum
5	Striatum
6	Hippocampus
7	Diencephalon-Mesencephalon

4.1.2.2. Analysis of vitamin E

Extraction, purification and measurement of d_0-α-T.

The procedure for the extraction and purification of vitamin E from the different regions of brain was the same as described earlier for the whole half of guinea pig brain. However, the smaller sample sizes required the use of smaller quantities of reagent for extraction. Thus, the frozen sample (approx. 0.1g) was thawed for about 1 min, weighed and placed on ice. The internal standard (50 μl of d_9-α-T in heptane (0.157 mM)) was added immediately and the sample was homogenized in 2 ml of distilled water. SDS (1 ml), ethanol (3 ml) and heptane (3 ml) subsequently were used for extraction of the lipid fraction containing vitamin E. The lipid extracts were purified by HPLC, the vitamin E silylated and the d_0-α-T measured by the GC/MS method as already has been described in section 3.1.2.2.

4.2 Results

Table 7 shows vitamin E concentrations in the six anatomical regions of the brain from the sham-operated rats and from the rats subjected to 25 min of forebrain ischemia followed by reoxygenation and 24 h recovery. The vitamin E levels in the various brain regions have been compared by analysis of variance. The percentage loss of vitamin E in the ischemic tissues relative to the controls is also shown.

In the ischemic rats a significantly lower level of vitamin E is found in all brain regions except the pons-medulla. The losses of vitamin E were very significant in striatum, slightly less pronounced in cortex, hippocampus and diencephalon-mesencephalon, and were least significant in the cerebellum.

Table 7. Effect of controlled ischemia/reoxygenation on the vitamin E concentration in the different regions of rat brain[a].

Brain Regions	Control[b]	Ischemic	Percent Loss
Cerebellum	45.4 ± 2.6	38.7 ± 3.9*	15
Diencephalon–mesencephalon	56.1 ± 4.0	45.4 ± 4.3**	19
Hippocampus	83.0 ± 10.6	60.6 ± 6.4**	27
Pons-medulla	61.5 ± 16.9	45.9 ± 4.0	25
Right frontal cortex	68.6 ± 6.2	44.0 ± 7.2**	36
Striatum	69.6 ± 4.7	48.6 ± 3.4***	30

[a]Concentrations are in nmol/g of tissue. Each result is mean ± standard deviation of data from four animals.
[b]Four control animals were sham operated rats by performing all surgical procedure except the induction of ischemia.
The symbols *, ** and *** indicate that means for control and ischemic tissue are significantly different at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.3. Discussion

A predisposition towards free radical formation induced during ischemia and an enhanced free radical production upon reperfusion are the two common elements in both the xanthine/xanthine oxidase (133-135, 161) and the glutamate (5, 144) theories proposed to explain the pathophysiology of tissue damage following ischemia/reperfusion. An associated event, the attraction of blood leukocytes to the ischemic regions and the subsequent stimulation of the "respiratory burst" in the leukocytes provides an additional source of free radicals in the affected region (145, 146).

Hence, since it is obvious that endogenous antioxidants (e.g., vitamin E, vitamin C, glutathione, urate) would be affected if free radicals contribute to the pathophysiology of ischemic brain tissue damage, their behaviour in *in vitro* models and in different animal models of ischemia has been examined in numerous studies. The results, however, have been equivocal.

When brain tissue is incubated in the presence of iron and ascorbic acid *in vitro*, the ensuing production of free radicals leads to the oxidation of GSH, the appearance of GSSH and to a massive consumption of α-tocopherol (162, 163). Supplementation with α-tocopherol has been shown to inhibit lipid peroxidation in brain homogenates (164).

In vivo, a decrease in the reduced form of ascorbic acid has been reported after focal cerebral ischemia in the cat (165). A protective effect of α-tocopherol administration against ischemic neuronal damage has been observed in the gerbil hippocampus of animals subjected to a short (5 min) ischemia (166).

Pretreatment with α-tocopherol of rats subjected to 1, 3 or 5 hours of incomplete global cerebral ischemia suppressed brain edema and lipid peroxidation, promoted resynthesis of ATP and reduced the appearance of the neurological signs of brain injury (e.g., jumping seizures or coma) in rats (167). Also, in the rat, the occlusion of the middle cerebral artery caused the levels of both α-tocopherol and ascorbate to decline. Simultaneously, the concentration of uric acid, a product of hypoxanthine oxidation, increased significantly (168).

The accumulation of uric acid is consistent with the xanthine/xanthine oxidase theory of the pathophysiology of ischemic tissue injury. A beneficial effect of either oral or intravenous administration of vitamin E on the recovery of brain electrical activity has been observed in dogs subjected to one hr of ischemia (169). Furthermore, alleviation of post-ischemic symptoms of tissue damage upon administration of aminosteroids, purported inhibitors of peroxidation, has been observed in gerbils (170, 171).

However, there are studies which have not given clear evidence that ischemia of brief to moderate duration leads to significant consumption of endogenous antioxidants (ascorbate, α-tocopherol and reduced glutathione) by the presumed free radical mediated mechanism (164, 172-175). In the most recent study, (175) no increase has been detected in the production of hydrogen peroxide and therefore the release of free radicals into cortex or hippocampus of hyperglycemic rats subjected to 15 min of ischemia. (Hyperglycemic rats are presumably at increased risk of acidosis upon ischemia.) Also, levels of glutathione and α-tocopherol were not affected in any brain region at any recovery time (5, 15, and 45 min). These results

contrast with the substantial reduction (15-36 %) of vitamin E found in all brain regions in the present study.

It is possible that the longer duration of ischemia (25 min) and the substantially longer recovery time (24 hrs) employed in our study are responsible for the observed differences. It has been suggested that free radical production may initially occur in small compartments within the tissue, e.g., the microvessels (175). The free radical damage affecting the microvessel "matures" over hours, finally leading to secondary damage that involves a larger portion of the affected tissue and is characterized by edema and infarction.

The involvement of microvessels and the importance of the time factor in the development of post-ischemic damage is supported by observations on vasogenic edema formation. The occurrence of edema is a delayed after-stroke symptom (even 3 days after the stroke) and the involvement of free radicals in edema formation is well documented (136). Another relevant factor is that the xanthine/xanthine oxidase system, which is regarded as one of the major contributors to the production of oxygen radicals, is mainly localized in the microvessels (136).

The smallest depletion of vitamin E was observed in the cerebellum and pons-medulla. This observation may reflect the existence of an alternate route for blood supply, the vertebral artery, resulting in a less extensive ischemia than occurred in the other regions.

It is very interesting to note that the very same animal model of stroke has previously been shown to cause a marked deterioration of the blood brain barrier (BBB) integrity 24 hrs after stroke (44). The effect was confined to the

cortex, striatum, and hippocampus regions. The diencephallon-mesencephalon, cerebellum and pons-medulla regions were not affected. This observation correlates well with the extent of vitamin E loss in the corresponding brain regions found in the present study.

The opening of the BBB has been detected as early as six minutes after stroke although it occurs to a much smaller extent. In rats subjected to only 10 min ischemia this early opening was detected in all regions except pons-medulla and cerebellum. After 24 hours of recovery, the integrity of the BBB was restored (44). However, at the same time as the BBB opening had largely disappeared, NMR imaging indicated edema in the hippocampus and striatum (42, 43). These observations on BBB openings and edema formation are consistent with the assumption that the process of free radicals generation occurs in the microvessels and is not rapid, requiring a certain amount of time to overcome the natural antioxidant defenses. Once this happens, the levels of antioxidants are affected and the symptoms of tissue damage begin to appear. Although further study is certainly needed, this time-course of the development of ischemic injury suggests that the administration of free radical scavengers to victims of stroke may provide benefits when the therapeutic concentrations are maintained over many hours.

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6. APPENDICES

Appendix 1. Mean concentration of vitamin C vs. time in ten guinea pig tissues.

Concentrations are in nmol/g of tissue. The LE and HE groups received 10 mg vitamin C/kg diet and 5 and 36 mg α-TAc/kg diet, respectively. Each result is the mean of values obtained from two animals, except for day 21 at which time the results are from one and three animals for the LE and HE groups, respectively. The results for kidney, lung, spinal cord, testis and feces at day 10 in the LE group and liver and lung at day 10 in the HE group were obtained from one animal only.
p.a. = peak absent, n.d. = not determined.

Tissue	Day	LE	HE
Adrenal	0	1147	1474
	1	1191	1286
	2	1213	759
	4	930	450
	7	196	340
	10	158	1734
	14	139	141
	17	96.2	47.6
	19	46.4	74.8
	21	n.d.	n.d.
Brain	0	706	774
	1	766	705
	2	815	627
	4	611	547
	7	495	539
	10	390	308
	14	317	236
	17	280	313
	19	212	210
	21	241	204
Heart	0	144	82.8
	1	27.5	66.5
	2	50.5	24.1
	4	p.a.	20.7
	7	p.a.	11.8
	10	n.d.	n.d.
	14	27.8	21.1
	17	17.6	2.9
	19	p.a.	12.8
	21	n.d.	n.d.
Kidney	0	94.7	105
	1	68.7	92.7
	2	50.8	47.4
	4	47.1	20.9
	7	p.a.	p.a.
	10	41.0	10.5
	14	7.0	12.4
	17	3.7	7.5
	19	7.5	5.5
	21	n.d.	n.d.

Appendix 2. Mean concentrations of d_0-, d_6- and total-*RRR*-α-T vs. time in blood and tissues of guinea pigs.

Concentrations are in nmol/g of tissue or nmol/ml of plasma or RBC. The LE and HEgroups received 10 mg vitamin C/kg diet and 5 and 36 mg α-TAc/kg diet, respectively. Each result is the mean of values obtained from two animals except for day 21 at which time the results are from one and three animals from the LE and HE groups, respectively. The results for kidney, lung, spinal cord, testis and feces at day 10 in the LE group and for liver and lung at day 10 in the HE group and testis and small intestine at day 17 in the HE group were obtained from one animal only.
p.a. = peak absent and n.d. = not determined.

Appendix 1. (continued)

Tissue	Day	LE	HE
Liver	0	116.4	148.4
	1	68.0	78.3
	2	59.5	63.8
	4	37.5	34.8
	7	16.4	24.0
	10	23.3	12.6
	14	15.3	15.8
	17	19.9	21.3
	19	9.0	11.3
	21	n.d.	n.d.
Lung	0	279	265
	1	147	283
	2	269	188
	4	159	82.1
	7	67.9	58.5
	10	30.1	11.2
	14	26.6	7.7
	17	37.4	11.1
	19	15.6	15.8
	21	n.d.	n.d.
Small Intestine	0	128	197
	1	139	92.6
	2	115	63.6
	4	75.1	36.9
	7	35.4	36.0
	10	16.8	20.1
	14	15.5	10.7
	17	11.0	18.1
	19	10.3	11.4
	21	n.d.	n.d.
Spinal Cord	0	291	352
	1	346	289
	2	336	229
	4	277	203
	7	143	201
	10	155	105
	14	81.1	92.0
	17	41.7	52.4
	19	22.6	60.6
	21	18.6	45.2

Appendix 1. (continued)

Tissue	Day	LE	HE
Spleen	0	982	795
	1	455	655
	2	444	252
	4	345	195
	7	93.2	121
	10	43.6	45.6
	14	p.a.	41.1
	17	45.8	44.9
	19	19.8	20.8
	21	n.d.	n.d.
Testis	0	1078	1016
	1	792	969
	2	760	817
	4	712	627
	7	379	452
	10	328	317
	14	260	223
	17	292	248
	19	203	284
	21	216	371

Tissue	Day	LE d_0	LE d_6	LE Total-α-T	HE d_0	HE d_6	HE Total-α-T
Adipose	0	61.9	p.a.	61.9	62.3	2.6	63.6
	1	66.8	p.a.	66.8	53.4	p.a.	53.4
	2	60.9	p.a.	60.9	100.4	p.a.	100
	4	40.3	p.a.	40.3	70.5	p.a.	70.5
	7	42.3	p.a.	42.3	45.5	2.5	48.0
	10	44.5	p.a.	44.5	61.8	12.9	68.2
	14	48.8	1.6	49.6	101.7	18.9	121
	17	68.5	p.a.	68.5	97.2	16.5	106
	19	56.9	p.a.	56.9	55.0	14.3	69.3
	21	61.0	2.5	61.8	59.5	19.2	78.7
Adrenal	0	896	1.6	898	578	1.1	579
	1	470	1.6	472	624	2.5	627
	2	360	1.9	362	763	7.5	771
	4	192	3.0	195	409	36.9	446
	7	233	5.2	238	277	49.5	327
	10	118	7.2	125	227	70.5	298
	14	138	8.8	147	263	135	398
	17	188	10.2	198	329	115	444
	19	102	7.4	109	183	101	284
	21	91.7	6.0	97.7	159	118	277
Biceps Femoris	0	18.0	0.04	18.0	12.4	0.07	12.4
	1	14.0	0.07	14.1	11.1	0.12	11.2
	2	12.0	0.08	12.1	12.2	0.17	12.4
	4	6.9	p.a.	6.9	11.5	0.48	12.0
	7	9.6	0.18	9.8	8.4	0.41	8.8
	10	6.2	0.17	6.4	8.9	1.0	9.8
	14	7.2	0.24	7.5	12.5	2.9	15.5
	17	13.1	1.7	14.8	10.5	4.7	15.2
	19	14.7	3.2	17.9	9.7	6.5	16.1
	21	7.8	1.8	9.6	9.9	4.7	14.6
Brain	0	40.7	0.11	40.8	37.4	0.05	37.5
	1	39.4	0.02	39.4	40.9	0.02	41.0
	2	36.8	0.05	36.9	38.2	0.06	38.3
	4	36.0	n.d.	36.0	44.2	0.22	44.4
	7	32.1	0.07	32.2	35.0	0.24	35.2
	10	28.6	0.07	28.7	34.8	0.55	35.4
	14	35.8	0.12	35.9	36.1	1.4	37.5
	17	37.9	0.28	38.2	39.7	1.7	41.4
	19	52.1	0.35	52.5	40.8	2.2	43.0
	21	37.8	0.61	38.4	36.5	2.2	38.7

Appendix 2. (continued)

Tissue	Day	LE d_0	LE d_6	LE Total-α-T	HE d_0	HE d_6	HE Total-α-T
Heart	0	41.5	0.05	41.6	36.0	0.05	36.1
	1	35.3	0.12	35.5	28.9	0.13	29.1
	2	25.3	0.10	25.4	29.6	0.28	29.9
	4	15.7	n.d.	15.7	27.5	1.6	29.1
	7	20.0	0.4	20.4	17.1	1.3	18.4
	10	n.d.	n.d.	n.d.	15.7	4.2	19.9
	14	13.9	0.77	14.7	19.4	8.2	27.6
	17	14.6	1.1	15.7	21.7	8.5	30.2
	19	15.1	1.3	16.4	14.6	9.4	24.0
	21	12.6	1.2	13.8	13.5	8.5	22.0
Kidney	0	20.3	0.07	20.4	16.7	0.02	16.8
	1	12.3	0.05	12.4	10.7	0.07	10.8
	2	11.4	0.07	11.5	13.4	0.18	13.6
	4	6.4	0.08	6.5	14.6	1.0	15.6
	7	6.9	0.18	7.0	8.4	1.3	9.7
	10	6.2	0.23	6.4	8.7	2.5	11.2
	14	6.9	0.39	7.3	9.5	4.0	13.5
	17	6.7	0.44	7.1	10.8	4.0	14.8
	19	6.4	0.50	6.9	7.4	3.8	11.3
	21	4.4	0.45	4.9	7.2	5.1	12.3
Liver	0	22.8	0.01	22.8	20.5	0.01	20.5
	1	15.8	0.21	16.1	14.2	0.44	14.6
	2	12.5	0.32	12.9	23.6	1.1	24.7
	4	9.0	0.26	9.3	15.0	3.4	18.5
	7	7.6	0.40	8.0	7.5	3.1	10.6
	10	6.8	0.61	7.4	12.0	7.2	19.2
	14	9.1	0.73	9.9	12.2	7.3	19.5
	17	18.0	1.19	19.2	23.2	8.4	31.6
	19	9.6	0.80	10.4	11.8	7.2	19.0
	21	10.1	0.82	11.0	13.0	11.2	24.1
Lung	0	44.0	0.01	44.0	40.9	0.03	40.9
	1	36.8	0.18	37.0	40.2	0.13	40.3
	2	30.7	0.15	30.9	42.6	0.37	43.0
	4	21.7	0.20	21.9	36.3	2.1	38.4
	7	19.9	0.55	20.5	20.7	3.0	23.7
	10	16.8	0.43	17.2	27.9	5.8	33.7
	14	19.6	1.0	20.6	13.3	5.5	18.8
	17	22.1	1.9	24.0	11.0	3.6	14.5
	19	14.5	1.1	15.5	18.1	9.5	27.6
	21	12.9	0.94	13.9	14.1	9.7	23.9

Appendix 2. (continued)

Tissue	Day	LE d_0	LE d_6	LE Total-α-T	HE d_0	HE d_6	HE Total-α-T
Sciatic Nerve	0	45.4	1.6	46.2	46.2	n.d.	46.2
	1	33.7	0.93	34.6	45.5	1.6	47.1
	2	30.5	0.84	31.3	39.6	7.0	46.6
	4	55.4	2.6	57.9	54.8	5.7	60.5
	7	38.4	0.41	38.8	42.4	1.2	43.5
	10	34.8	0.44	35.2	44.8	n.d	44.8
	14	48.2	0.25	48.4	56.6	1.2	57.8
	17	55.8	1.0	56.8	42.3	2.6	44.8
	19	78.9	n.d.	78.9	53.5	8.1	61.7
	21	67.8	n.d.	67.8	80.4	n.d.	80.4
Small Intestine	0	4.6	0.07	4.7	6.7	0.01	6.7
	1	4.2	0.29	4.5	3.6	0.82	4.4
	2	2.8	0.29	3.1	3.4	1.4	4.8
	4	1.8	0.36	2.2	2.8	2.5	5.2
	7	2.0	0.36	2.4	2.0	3.1	5.1
	10	2.2	0.84	3.1	1.7	5.2	6.9
	14	1.9	0.60	2.5	2.8	5.0	7.8
	17	1.9	1.1	3.0	1.8	3.4	5.2
	19	3.4	0.86	4.3	1.8	3.4	5.2
	21	1.5	0.55	2.1	2.0	6.9	8.9
Spinal Cord	0	31.6	1.5	33.2	32.4	2.0	34.3
	1	31.9	1.2	32.5	29.9	1.5	31.5
	2	31.0	n.d.	31.0	35.6	2.1	37.6
	4	28.1	1.5	28.7	31.7	0.41	31.9
	7	27.9	n.d.	27.9	29.7	n.d.	29.7
	10	23.9	n.d.	23.9	32.2	2.3	34.5
	14	29.3	1.2	29.9	28.7	3.6	32.3
	17	26.7	1.9	28.7	30.8	4.6	35.3
	19	30.9	1.2	32.0	29.2	4.1	33.2
	21	30.9	n.d.	30.9	30.2	2.6	32.8
Spleen	0	51.5	0.05	51.5	41.7	0.56	42.3
	1	30.2	0.25	30.5	34.2	0.53	34.7
	2	19.2	0.49	19.7	29.3	1.35	30.7
	4	13.0	0.45	13.5	26.3	5.1	31.4
	7	13.6	0.63	14.3	13.2	4.9	18.1
	10	12.3	1.1	13.4	15.4	7.6	23.0
	14	15.9	1.3	17.2	31.7	18.9	50.7
	17	29.8	2.8	32.6	39.4	10.9	50.4
	19	20.1	1.8	21.8	23.5	14.4	37.9
	21	19.8	1.5	21.3	22.2	19.9	42.1

Appendix 2. (continued)

Tissue	Day	LE d_0	LE d_6	LE Total-α-T	HE d_0	HE d_6	HE Total-α-T
Testis	0	49.1	0.12	49.2	35.7	0.10	35.7
	1	25.8	0.31	26.1	27.6	0.42	28.0
	2	19.4	0.09	19.4	33.0	0.21	33.1
	4	15.8	0.13	15.9	22.4	0.76	23.1
	7	15.0	0.21	15.3	16.7	1.1	17.9
	10	13.8	0.20	14.0	21.0	2.8	23.8
	14	15.7	0.41	16.2	19.8	4.2	24.1
	17	17.4	0.88	18.3	14.0	3.4	17.4
	19	17.9	1.1	19.0	15.9	4.9	20.8
	21	10.6	0.85	11.5	11.6	5.4	17.0
Plasma	0	6.2	0.06	6.2	8.0	0.15	8.1
	1	5.3	0.11	5.4	7.9	0.29	8.2
	2	5.5	0.17	5.7	11.0	0.41	11.4
	4	2.9	0.11	3.0	5.9	1.0	6.9
	7	2.8	0.21	3.0	3.0	1.2	4.3
	10	3.4	0.28	3.7	5.2	2.2	7.5
	14	5.1	0.38	5.5	5.6	2.7	8.3
	17	4.8	0.31	5.1	6.0	2.0	8.0
	19	2.8	0.24	3.0	5.3	3.2	8.5
	21	5.5	0.28	5.8	6.2	5.0	11.1
RBC	0	2.4	0.01	2.4	2.0	0.04	2.0
	1	1.4	0.04	1.5	1.5	0.05	1.5
	2	1.2	0.03	1.2	2.0	0.08	2.0
	4	0.7	0.04	0.7	1.4	0.24	1.7
	7	0.8	0.04	0.9	0.9	0.29	1.2
	10	0.7	0.06	0.8	1.0	0.36	1.3
	14	0.7	0.07	0.7	1.4	0.69	2.1
	17	1.2	0.09	1.3	1.5	1.1	2.6
	19	1.6	0.10	1.7	n.d.	n.d.	n.d.
	21	1.5	0.10	1.6	n.d.	n.d.	n.d.
Feces	0	12.4	0.80	13.2	39.0	2.2	41.2
	1	5.5	2.0	7.5	27.7	20.5	48.2
	2	6.2	5.1	11.2	13.4	33.0	46.3
	4	2.8	4.9	7.7	8.2	54.0	62.3
	7	2.1	6.4	8.5	3.1	47.2	50.3
	10	3.2	7.3	10.5	5.0	56.1	61.2
	14	3.3	7.6	10.9	4.7	62.1	66.8
	17	3.9	7.0	10.9	4.6	40.9	45.5
	19	3.1	5.2	8.2	1.8	36.7	38.4
	21	4.1	6.3	10.3	6.4	43.3	49.8

Appendix 3. Reference 39.

Biokinetics of Dietary *RRR*-α-Tocopherol in the Male Guinea Pig at Three Dietary Levels of Vitamin C and Two Levels of Vitamin E. Evidence that Vitamin C Does Not "Spare" Vitamin E *in Vivo*[1]

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The net rates of uptake of "new" and loss of "old" *2R,4'R,8'R*-α-tocopherol (*RRR*-α-TOH, which is natural vitamin E) have been measured in the blood and in nine tissues of male guinea pigs over an eight week period by feeding diets containing deuterium-labelled α-tocopheryl acetate (d_6-*RRR*-α-TOAc). There was an initial two week "lead-in" period during which 24 animals [the "high" vitamin E (HE) group] received diets containing 36 mg of unlabelled (d_0) *RRR*-α-TOAc and 250 mg of ascorbic acid per kg diet, while another 24 animals [the "low" vitamin E (LE) group] received diets containing 5 mg d_0-*RRR*-α-TOAc and 250 mg ascorbic acid per kg diet. The HE group was then divided into three equal subgroups, which were fed diets containing 36 mg d_6-*RRR*-α-TOAc and 5000 mg [the "high" vitamin C (HEHC) subgroup], 250 mg [the "normal" vitamin C (HENC) subgroup] and 50 mg [the "low" vitamin C (HELC) subgroup] ascorbic acid per kg diet. One animal from each group was sacrificed each week and the blood and tissues were analyzed for d_0- and d_6-*RRR*-α-TOH by gas chromatography-mass spectrometry. The LE group was similarly divided into three equal subgroups with animals receiving diets containing 5 mg d_6-*RRR*-α-TOAc and 5,000 mg (LEHC). 250 mg (LENC) and 50 mg (LELC) ascorbic acid per kg diet with a similar protocol being followed for sacrifice and analyses. In the HE group the total (d_0- + d_6-) *RRR*-α-TOH concentrations in blood and tissues remained essentially constant over the eight week experiment, whereas in the LE group the total *RRR*-α-TOH concentrations declined noticeably (except in the brain, an organ with a particularly slow turnover of vitamin E). There were no significant differences in the concentrations of "old" d_0-*RRR*-α-TOH nor in the concentrations of "new" d_6-*RRR*-α-TOH found in any tissue at a particular time between the HEHC, HENC and HELC subgroups, nor between the LEHC, LENC and LELC subgroups. We conclude that the long-postulated "sparing" action of vitamin C on vitamin E, which is well documented *in vitro*, is of negligible importance *in vivo* in guinea pigs that are not oxidatively stressed in comparison with the normal metabolic processes which consume vitamin E (e.g., by oxidizing it irreversibly) or eliminate it from the body. This is true both for guinea pigs with an adequate, well-maintained vitamin E status and for guinea pigs which are receiving insufficient vitamin E to maintain their body stores.

The biokinetics of vitamin E uptake and loss in the HE guinea pigs are compared with analogous data for rats reported previously (*Lipids 22*, 163–172, 1987). For most guinea pig tissues the uptake of vitamin E under "steady-state" conditions was faster than for the comparable rat tissues. However, the brain was an exception with the turnover of vitamin E occurring at only one-third of the rate for the rat.
Lipids 25, 199–210 (1990).

We have recently employed *2R,4'R,8'R*-α-tocopheryl acetate substituted in a metabolically inactive position with three atoms of deuterium (d_3-*RRR*-α-TOAc) to make the first measurements of the net, long-term uptake of (deuterated) natural vitamin E, d_3-*2R,4'R,8'R*-α-tocopheryl (d_3-*RRR*-α-TOH) in the male rat under normal laboratory dietary conditions, using a diet in which the d_3-*RRR*-α-TOAc (36 mg/kg diet) was the only source of vitamin E (1). We discovered that there were dramatic differences in uptake kinetics between tissues. For example, the equalization time, $t_{1:1}$, which is the time required for the new (deuterium-labelled) α-TOH concentration to become equal to that of the old (unlabelled) α-TOH, was estimated to be ca. 9, 18, 40 and 72 days in the lung, heart, brain and spinal cord, respectively (K.U. Ingold, G.W. Burton, and W. Siebrand, 1990, unpublished results).

Since analogous, tissue-sampling experiments on man are almost inconceivable, we decided to examine the biokinetics of natural vitamin E in a different laboratory animal in order to see if the same general pattern of fast-uptake tissues and slow-uptake tissues obtained. We chose the guinea pig as our experimental animal and carried out the biokinetics under conditions similar to those employed in the earlier study on rats. However, in order to increase the sensitivity of the measurement of small amounts of deuterated tocopherol in the presence of a large amount of the unlabelled compound, we employed *2R,4'R,8'R*-α-tocopheryl acetate labelled in metabolically inactive positions with six atoms of deuterium (d_6-*RRR*-α-TOAc). As will be reported elsewhere (K.U. Ingold, G.W. Burton, and W. Siebrand, 1990, unpublished results), guinea pigs and rats have a somewhat similar pattern of fast-uptake and slow-uptake tissues. That is, corresponding tissues could be classified either as "fast" or "slow." However, the fast-uptake tissues of the guinea pig were somewhat "faster" than those of the rat and, while some of the slow-uptake tissues of the guinea pig were "faster" than for the rat, others were dramatically slower, e.g., for the brain, $t_{1:1}$ was 40 and 107 days in the rat and guinea pig, respectively (K.U. Ingold, G.W. Burton, and W. Siebrand, 1990, unpublished results).

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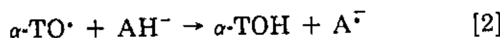
[1] NRCC Publication No. 30775.

Abbreviati ›ns: AH_2, vitamin C; α-TOH, α-tocopherol; *RRR*-α-TOH, *2R,4'R,8'R*-α-tocopherol; *RRR*-α-TOAc, *RRR*-α-tocopheryl acetate; d_0-*RRR*-α-TOH, unlabelled *RRR*-α-tocopherol; d_3-*RRR*-α-TOH, d_3-*RRR*-α-(5-CD_3)tocopherol; d_3-*RRR*-α-TOAc, *RRR*-α-(5-CD_3)tocopheryl acetate; d_6-*RRR*-α-TOH, *RRR*-α-(5,7-$(CD_3)_2$)tocopherol; d_9-*ambo*-α-TOH, *2RS,4'R,8'R*-α-(5,7,8-$(CD_3)_3$)tocopherol; $t_{1:1}$, equalization time; LE, low vitamin E; HE, high vitamin E; LELC, low E, low (vitamin) C; LENC, low E, normal C; LEHC, low E, high C; HELC, high E, low C; HENC, high E, normal C; HEHC, high E, high C; α-TO•, α-tocopheroxyl radical; RBC, red blood cells; ROO•, peroxyl radical; ROOH, hydroperoxide.

The guinea pig was chosen for our second whole animal biokinetic experiment with vitamin E because, unlike a rat but like man, the guinea pig cannot synthesize ascorbic acid (vitamin C, AH_2). The availability of vitamin C depends, therefore, on the dietary level of this compound, which can be manipulated over a wide range. This is important because both vitamin E (2–5) and vitamin C (6–8) are chain-breaking antioxidants and there is a considerable body of evidence which indicates that there is a synergistic antioxidant interaction between these vitamins in a wide variety of *in vitro* model systems (9–39). There is sound experimental evidence that this synergism is due to the "regeneration" of α-TOH by reduction of its initial oxidation product, the tocopheroxyl radical, α-TO•, by the ascorbate anion, AH^-. That is, the lipid-soluble α-TOH traps lipid peroxyl radicals, ROO•, forming α-TO• and lipid hydroperoxide, ROOH, and the α-TO• is then reduced by the water-soluble ascorbate rather than being irreversibly oxidized by reaction with a second peroxyl radical. The overall synergistic interaction between these two radical-trapping antioxidants *in vitro* can be represented by reactions 1–3.

$$ROO^\bullet + \alpha\text{-TOH} \rightarrow ROOH + \alpha\text{-TO}^\bullet \quad [1]$$



$$\alpha\text{-TO}^\bullet + AH^- \rightarrow \alpha\text{-TOH} + A^{\bar{\bullet}} \quad [2]$$

$$A^{\bar{\bullet}} + A^{\bar{\bullet}} \xrightarrow{H^+} A + AH^- \quad [3]$$

There is some much less compelling evidence for an interaction between vitamin E and vitamin C *in vivo* for rats (40–47), guinea pigs (48–57), and premature infants (58). Synergistic E/C interactions have generally been reported, a result which lends support to the hypothesis, drawn from *in vitro* experiments, that vitamin C can "regenerate" vitamin E *in vivo*, or at least "spare" vitamin E by some other *in vivo* mechanism. For example, dietary vitamin C has been reported to enhance plasma levels of vitamin E (40,45,47,50,58), to enhance vitamin E levels in other tissues (52,53,56), and to partially reverse effects due to vitamin E deficiency (47). Antagonistic E/C interactions have also been reported (41–43,48,51). Thus, dietary vitamin C has been reported to lower plasma levels of vitamin E (42,51) and to enhance erythrocyte hemolysis (42,43,51). The difficulties involved in determining whether there is any E/C interaction *in vivo* and, if so, whether the interaction is synergistic or antagonistic can be further illustrated by the pioneering measurements of expired pentane as a measure of lipid peroxidation as described by Tappel and co-workers (41,44,54). These studies demonstrated that the level of lipid peroxidation induced in rats by methyl ethyl ketone peroxide is uninfluenced by vitamin C if the rats have an adequate vitamin E status, but is enhanced by vitamin C when the animals are vitamin E deficient (41), while for iron-loaded rats (44) and CCl_4-intoxicated guinea pigs (54), dosing with vitamin C reduced *in vivo* lipid peroxidation. Overall, the literature indicates that a prooxidant effect of vitamin C has generally, though not always (51), been observed in severely vitamin E deficient animals and might be attributed to a prooxidant (chain-initiating) effect on lipid peroxidation by ascorbate, particularly in the presence of iron (59,60), as well as to the reduced molar effectiveness of ascorbate as a chain-breaking antioxidant at high concentrations (7). With both synergistic and antagonistic E/C interactions being reported it is not surprising to find that slight modifications of the experimental conditions or the use of some different measure of antioxidant status can lead to a failure to detect any kind of E/C interaction *in vivo* (40–42,44–48,50–52,55,56).

Guinea pigs on a diet containing d_6-*RRR*-α-TOAc provide a unique opportunity to search for any *in vivo* protective or destructive effect of vitamin C on vitamin E. An experimental protocol carefully designed to measure vitamin E turnover would be very much more sensitive than any of the earlier whole animal studies in detecting the existence, or otherwise, of an *in vivo* interaction between vitamins E and C. It would therefore provide a far more definitive answer to the important question: Does vitamin C "spare" vitamin E *in vivo*?

We chose essentially the same experimental protocol for the present study of the biokinetics of vitamin E uptake and loss in the guinea pig that we had previously employed with rats (1). However, because far more guinea pigs were employed than in the earlier study on rats (48 vs 9 animals) we limited the number of biological tissues and fluids examined to 11 (vs 23 for the rats). Another difference was that the guinea pigs were given the diet containing d_6-*RRR*-α-TOAc after a two week "lead-in" period, during which the animals were fed a diet containing the same concentration of the unlabelled material, d_0-*RRR*-α-TOAc, whereas for the rats the corresponding "lead-in" time was four weeks. The maximum length of time the animals were on deuterated vitamin E was similar (56 days for guinea pigs vs 65 days for rats), but the guinea pigs were sacrificed for tissue analyses at regular seven day intervals whereas the rats had been sacrificed on days 1, 2, 4, 8, 16, 31 and 65 after being switched to the deuterated tocopherol diet.

The rationale behind the protocol (see Methods section and Fig. 1) is that if vitamin C really does "spare" vitamin E *in vivo* then, at high dietary levels of vitamin C, one would expect a slower than normal loss of "old" vitamin E (d_0-*RRR*-α-TOH) from a tissue and a corresponding slower than normal uptake of "new" vitamin E (d_6-*RRR*-α-TOH). Similarly, at low dietary levels of vitamin C one would expect a faster than normal loss of "old" and a faster than normal uptake of "new" vitamin E.

MATERIALS AND METHODS

Materials. *2R,4′R,8′R*-α-(5,7-$(CD_3)_2$)tocopherol (d_6-*RRR*-α-TOH) was prepared by deuteriomethylation of δ-tocopherol (61) and was then converted to the acetate as previously described (1). *2RS,4′R,8′R*-α-(5,7,8-$(CD_3)_3$)tocopherol (d_9-*ambo*-α-TOH) was prepared for use as an internal standard by condensation of hydroquinone with phytol followed by deuteriomethylation.

Methods. Forty-eight male, two-week-old, specific pathogen free guinea pigs (170–222 g) obtained from Charles River Canada, Inc., P.Q. (St. Constant, Quebec, Canada) were divided into two main groups, a high vitamin E group of 24 animals, HE, and a low vitamin E group of 24 animals, LE. The animals were housed four per plastic cage (1720 cm² floor area) with ground corn cob bedding and were fed a Reid-Briggs Guinea Pig diet modified as follows: corn oil (which contains α-TOH) was omitted as were vitamins E and C; tocopherol-stripped corn oil (7.3%

by weight) containing d_0-*RRR*-α-TOAc or d_6-*RRR*-α-TOAc (5 or 36 mg/kg diet) was added together with ascorbic acid (50, 250 or 5000 mg/kg diet). During these experiments the guinea pigs consumed ca. 15–20 g of diet per day.

All the animals in the HE group were fed a diet containing 36 mg d_0-*RRR*-α-TOAc/kg diet [the same level as for the rats in the earlier study (1)] and 250 mg ascorbic acid/kg diet for two weeks. After this "lead-in" period, i.e., on day 0 of the actual experiment, the HE group of animals were divided into three equal subgroups, all of which received a diet containing 36 mg d_6-*RRR*-α-TOAc but different levels of vitamin C (Fig. 1). The high vitamin C subgroup, HEHC, were fed the megadose (62) level of 5,000 mg ascorbic acid/kg diet; the normal vitamin C subgroup, HENC, continued with 250 mg ascorbic acid/kg diet; and the low vitamin C subgroup, HELC, received the barely antiscorbutic (49–51,53,55,56,63–68) level of 50 mg ascorbic acid/kg diet.

A similar protocol was followed with the LE group, but for these guinea pigs the levels of d_0- and d_6-*RRR*-α-TOAc wre only 5 mg/kg diet. A two week "lead-in" with d_0-α-TOAc and 250 mg ascorbic acid/kg diet was followed by the d_6-*RRR*-α-TOAc and the same three levels of vitamin C as for the HE subgroups. That is, the three LE subgroups, LEHC, LENC, and LELC, received 5,000 mg, 250 mg, and 50 mg ascorbic acid/kg diet, respectively (Fig. 1).

One guinea pig from each of the six sub-groups was sacrificed weekly on days 7, 14, 21, 28, 35, 42, 50 and 56.

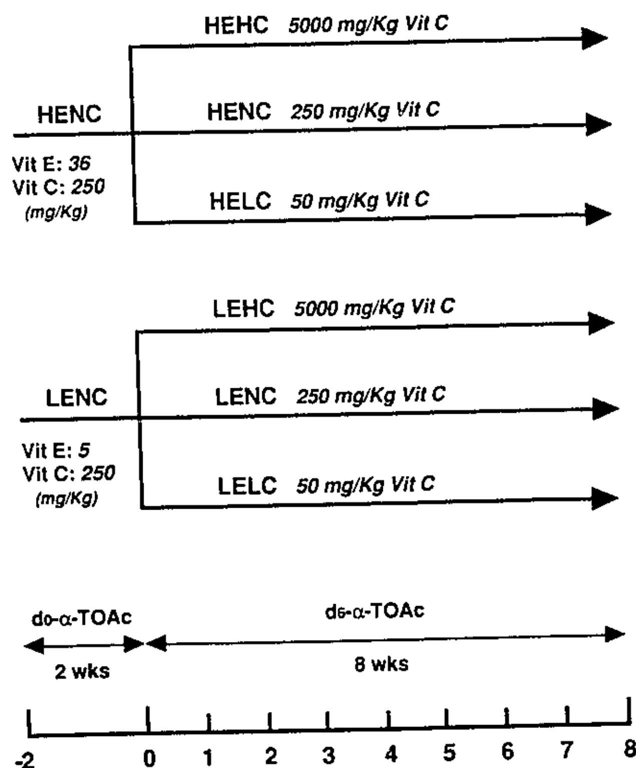


FIG. 1. Dietary regimes for the guinea pigs employed in this study.

Blood samples were obtained by heart puncture with the animals anesthetized with Innovar-Vet™ (Pitman-Moore Ltd., Don Mills, Ontario). The animals were then sacrificed by arterial perfusion with isotonic NaCl. Blood was separated into plasma and red blood cell (RBC) fractions by centrifugation, and the RBC were washed as described previously (1). The plasma, RBC, and nine weighed tissues (adrenal, brain, heart, kidney, liver, lung, muscle [biceps femoris, b.f.], spleen, and testis) were stored at −80°C prior to analysis. The frozen tissue samples were thawed and 7.95 nmol d_9-*ambo*-α-TOH in 50 μl heptane was immediately added to them; this was followed by tissue homogenization and extraction of α-TOH into heptane, as described previously. The entire brain, the two adrenals, one kidney, and one testis were utilized while weighed portions (0.5–1.0 g) of the heart, spleen, b.f. muscle, liver, and lung were employed. The plasma (500 μl) and RBC (in phosphate buffered saline, 1.3 ml, hematocrit 45%) were mixed with 3.98 nmol and 7.95 nmol d_9-*ambo*-α-TOH in heptane, respectively, as soon as they thawed and the α-TOH was extracted immediately.

The heptane extracts were assayed using a Varian model 5000 high performance liquid chromatography (HPLC) (Varian Associates, Palo Alto, CA) equipped with a 250 × 4 mm Lichrosorb Si 60 (5μ particle size) using 90% hexane/10% butyl methyl ether as the eluent, and the α-TOH fraction in each sample was collected automatically. The relative proportions of d_0-*RRR*-α-TOH, d_6-*RRR*-α-TOH, and d_9-*ambo*-α-TOH in each sample were determined as described previously (1) by gas chromatography-mass spectrometry (GC-MS) analysis following conversion of these tocopherols to their trimethylsilyl ethers. Since the absolute amount of d_9-*ambo*-α-TOH added to each sample was known, the absolute concentrations of "old" d_0-*RRR*-α-TOH and "new" d_6-*RRR*-α-TOH could be readily calculated.

RESULTS

Our complete results are presented in Table 1 which lists the absolute concentrations of d_0-*RRR*-α-TOH and d_6-*RRR*-α-TOH as a function of time in the plasma, RBC, and nine tissues for the six dietary regimes employed in these experiments.

In accordance with our experience with rats (1), the total concentration of vitamin E (i.e., d_0- + d_6-*RRR*-α-TOH) in the tissues of the HE group of guinea pigs remained essentially constant during the eight week experimental period, as is best seen in Figure 2. However, for the LE group of guinea pigs the total concentration of vitamin E declined significantly during the eight weeks in all tissues examined except the brain (where turnover is extremely slow).

DISCUSSION

Does vitamin C "spare" vitamin E in vivo? The rationale for having both the HE and the LE groups of guinea pigs is that at high dietary levels of vitamin E any "sparing" action by vitamin C might be masked from experimental observation because adequate "new" vitamin E would always be available to replace any used, "old" vitamin E. However, with sufficiently low dietary levels of vitamin E the rate of depletion of "old" vitamin E in a tissue

TABLE 1

Time-Dependence of the Absolute Concentrations of d_0- and d_6-*RRR*-α-TOH in Blood and Tissue[a]

		HEHC		HENC		HELC		LEHC		LENC		LELC	
Tissue	Day	d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6	d_0	d_6
Adrenal	7	437	458	401	358	689	383	217	52	217	56	341	54
	14	238	467	117	410	145	518	74	46	152	86	106	44
	21	196	800	108	525	163	726	66	52	58	41	44	36
	28	104	786	61	521	49	561	58	64	129	128	40	34
	35	114	790	64	520	93	617	39	35	22	17	31	59
	42	57	779	41	544	85	755	16	23	28	35	14	14
	49	79	840	—	—	33	437	14	23	20	31	10	16
	56	20	364	33	384	44	672	7.8	22	15	24	27	45
Brain	7	38	2.0	31	1.5	35	1.5	34	0.3	29	0.4	34	0.3
	14	18	1.4	21	1.5	21	2.2	30	0.8	35	1.0	18	0.8
	21	31	4.1	30	5.8	31	6.0	35	1.2	49	1.3	46	1.4
	28	18	4.6	17	4.0	15	3.3	42	1.5	40	1.8	46	1.9
	35	66	18	26	6.0	33	7.4	41	2.3	43	1.3	41	2.6
	42	20	8.1	13	4.2	16	5.8	36	2.3	37	2.4	32	1.4
	49	39	17	—	—	26	10	31	2.5	35	2.4	36	2.1
	56	15	4.9	14	4.8	16	5.8	26	1.4	26	2.2	32	3.8
Heart	7	19	12	21	12	24	10	17	2.5	17	2.5	17	1.9
	14	18	18	11	19	14	23	5.2	2.5	11	5.9	14	7.1
	21	13	27	10	30	10	27	11	6.4	13	7.2	7.4	5.6
	28	7.0	35	6.7	27	3.6	19	7.6	6.3	11	8.3	8.1	6.0
	35	7.2	40	5.7	31	7.7	35	6.9	6.9	3.7	3.2	5.0	10
	42	3.6	34	3.3	28	4.7	33	2.7	3.9	4.7	5.7	4.0	4.0
	49	4.4	44	—	—	3.9	43	3.4	5.3	3.4	4.9	3.1	5.0
	56	1.7	27	2.7	26	2.4	32	0.9	2.5	2.3	3.8	5.0	8.5
Kidney	7	13	11	12	9	18	9	12	1.6	13	1.7	12	1.5
	14	8.0	8.8	8.8	12	10	16	4.5	1.9	9.1	3.6	5.6	2.4
	21	5.7	14	5.7	17	8.6	23	5.1	3.2	7.3	3.8	4.3	2.8
	28	6.8	22	3.7	16	3.6	17	5.0	3.8	8.3	5.9	5.8	4.1
	35	6.6	30	3.9	21	3.8	18	5.0	3.9	2.1	1.7	2.3	4.1
	42	3.1	22	2.1	16	3.7	20	2.3	2.7	3.6	3.8	2.3	2.1
	49	3.5	29	—	—	2.0	20	1.9	2.7	2.1	2.7	2.6	3.7
	56	0.9	12	1.7	14	1.5	16	0.8	1.9	1.5	2.2	2.9	4.6
Liver	7	16	41	17	41	34	55	22	7.0	14	5.5	22	5.6
	14	13	33	11	46	13	67	8.4	5.1	17	13	16	9.0
	21	6.7	42	7.4	41	8.8	53	7.9	7.4	11	9.5	5.3	5.2
	28	8.0	43	4.5	31	6.4	59	7.3	7.5	8.2	7.8	14	12
	35	8.7	67	6.6	54	6.1	48	5.9	6.3	2.1	1.8	4.3	9.4
	42	3.7	55	3.7	43	5.8	51	2.4	3.8	5.4	7.3	4.4	5.0
	49	4.9	55	—	—	3.8	45	3.5	6.7	5.2	8.8	3.9	7.1
	56	1.5	22	2.7	23	2.5	34	0.9	2.6	3.4	5.8	4.6	7.4
Lung	7	25	21	17	12	20	10	20	2.8	28	4.8	34	4.1
	14	11	15	9.5	19	17	39	18	7.3	13	7.2	20	5.0
	21	10	28	14	43	14	49	12	7.3	28	14	11	8.0
	28	11	50	8.6	44	4.5	25	9.3	9.4	15	13	18	13
	35	6.8	40	7.6	47	11	55	12	11	5.7	5.3	6.7	13
	42	4.1	42	6.0	55	9.3	64	5.3	7.1	6.9	8.7	4.7	5.5
	49	5.7	59	—	—	2.9	35	3.4	5.3	4.8	7.3	5.9	10
	56	2.9	44	4.2	44	4.1	53	1.1	3.5	4.0	7.0	8.3	14
Biceps femoris	7	12	3.9	8.9	2.3	12	2.6	9.9	0.5	10	0.6	10	0.4
	14	10	4.5	8.2	4.3	10	5.2	7.2	1.1	7.1	1.2	7.8	1.2
	21	7.3	5.8	7.9	6.3	6.5	4.7	6.8	1.5	6.8	1.6	5.3	1.2
	28	—	—	6.3	7.2	4.2	7.4	5.6	1.5	6.4	2.1	6.5	1.7
	35	8.2	14	5.6	11	5.2	8.0	5.9	2.0	2.5	1.0	2.2	2.0
	42	9.1	17	3.4	9.2	6.2	12	2.8	1.4	4.3	2.1	1.9	1.0
	49	4.2	13	—	—	2.8	9.5	1.7	1.2	2.4	1.4	1.8	1.3
	56	2.3	9.4	2.6	8.7	2.6	12	0.7	0.8	1.5	1.0	2.8	2.4
Plasma	7	2.2	5.1	1.7	4.0	4.8	8.5	2.9	0.7	4.2	1.4	4.3	1.0
	14	1.5	4.1	1.4	3.8	1.4	6.2	1.2	1.1	1.9	2.0	—	—
	21	1.0	7.1	1.5	7.0	1.5	6.9	2.2	1.8	4.3	2.3	1.5	1.3
	28	1.2	7.6	0.5	3.7	0.4	3.9	1.3	3.7	1.5	1.4	1.4	1.1
	35	1.4	10	0.7	4.9	0.9	6.7	1.9	1.7	1.2	0.7	1.5	2.1
	42	0.6	8.6	0.5	5.6	0.7	5.4	1.5	1.8	2.5	1.9	1.0	0.9
	49	0.9	9.7	—	—	0.4	4.9	1.7	1.2	0.8	1.5	0.6	1.1
	56	0.4	3.4	0.6	4.9	0.3	4.6	0.4	1.2	0.5	0.9	0.6	1.2

(Continued next page)

TABLE 1 *(Continued)*

Tissue	Day	HEHC d_0	HEHC d_6	HENC d_0	HENC d_6	HELC d_0	HELC d_6	LEHC d_0	LEHC d_6	LENC d_0	LENC d_6	LELC d_0	LELC d_6
Red cells	7	2.5	5.5	2.1	4.1	3.3	4.8	3.1	1.2	3.9	1.2	4.1	0.9
	14	1.4	3.3	0.3	1.2	1.0	4.3	1.2	1.1	1.3	1.2	—	—
	21	1.1	5.0	2.7	6.0	1.1	6.2	2.5	1.5	1.2	0.6	1.9	1.1
	28	1.2	7.5	0.7	4.8	0.5	4.0	1.8	1.4	1.7	1.5	1.8	1.6
	35	1.1	7.6	0.8	4.3	0.8	6.1	2.3	1.2	1.0	0.5	1.4	1.9
	42	0.5	5.8	0.4	3.9	0.6	4.6	1.3	1.2	1.6	1.3	1.2	0.8
	49	0.6	6.5	—	—	0.5	5.3	0.7	1.2	0.8	1.1	0.8	1.4
	56	0.0	4.8	1.1	4.7	0.0	6.7	0.2	0.7	0.6	1.0	0.8	1.3
Spleen	7	17	38	14	28	22	32	15	6.9	17	6.4	17	4.5
	14	—	—	8.8	40	7.7	40	6.3	4.7	11	8.7	12	7.4
	21	12	45	7.3	44	7.2	46	10	8.5	11	8.2	6.3	6.1
	28	6.9	56	4.3	39	3.3	39	6.3	6.9	11	11	11	10
	35	6.7	56	5.0	40	4.7	38	6.6	7.0	4.4	3.6	3.4	7.6
	42	3.1	47	2.5	34	4.9	43	2.1	3.6	4.6	7.0	3.4	4.2
	49	4.8	56	—	—	3.1	41	3.3	6.2	3.9	7.2	3.5	6.7
	56	1.7	26	2.4	24	2.2	32	0.9	2.8	2.8	5.0	5.5	9.2
Testis	7	14	7.7	15	6.0	17	5.0	14	1.2	12	1.4	16	1.3
	14	14	11	5	5.5	9.5	9.2	13	5.1	15	4.9	13	2.6
	21	4.9	9.2	10	16	31	42	7.1	3.3	5.9	2.9	6.4	2.8
	28	11	29	6.2	16	5.6	15	4.7	2.7	6.6	3.7	6.8	3.0
	35	6.4	18	3.6	11	4.8	13	4.2	2.5	2.5	1.5	2.5	4.0
	42	4.0	22	3.7	18	3.1	18	3.3	2.8	3.5	2.9	2.6	1.9
	49	3.6	24	—	—	2.1	15	2.3	2.7	2.5	2.8	2.2	2.4
	56	3.2	16	2.4	13	2.3	16	0.6	1.2	1.4	1.6	2.5	3.5

[a]Concentrations are in nmol/ml of plasma or packed red cells or nmol/g of tissue. HE and LE, 36 and 5 mg α-TOAc/kg diet; HC, NC and LC, 5,000, 250 and 50 mg ascorbic acid/kg diet, respectively.

should exceed its rate of replacement by "new" vitamin E. Under such dietary conditions, any *in vivo* "sparing" action of vitamin C on vitamin E should become obvious as a fairly dramatic difference in the rates of loss of "old" vitamin E (and, indeed, of total vitamin E) between the LEHC, LENC, and LELC subgroups of animals, with this rate being least for the LEHC subgroup and greatest for the LELC subgroup. Thus, the LEHC, LENC, and LELC subgroups should provide an even more sensitive probe for any *in vivo* vitamin C/vitamin E interaction than would the three HE subgroups.

We have recently analyzed the biokinetics of vitamin E in rats, guinea pigs and man under "steady-state" conditions (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results), i.e., under conditions where the total concentration of "new" (deuterium-labelled) and "old" (unlabelled) vitamin E in a tissue remained essentially constant during the experiment. In Figure 2, we show the total concentration of *RRR*-α-TOH (i.e., d_0 + d_6) for plasma and eight tissues from the HEHC, HENC, and HELC subgroups of guinea pigs at each time point. Not surprisingly, the data show considerable scatter because these comparisons of the total tocopherol concentration in a tissue at any particular time are based on samples obtained from three different animals. Furthermore, "identical" guinea pigs unfortunately show more individual variations in vitamin E levels than do "identical" rats in similar experiments. Nevertheless, despite the scatter two things are immediately obvious: First, in each of the subgroups the total concentration of vitamin E in a given tissue remained approximately constant for the eight week experiment. Second, the total concentration of vitamin E (given as its eight week average in Table 2) does not differ significantly in any tissue between the HEHC, HENC, and HELC subgroups. Hence, it appears that any "sparing" or "regenerating" action by vitamin C on the rate at which vitamin E is consumed (by all metabolic routes) is negligible in comparison with the normal turn-over of vitamin E in guinea pigs under the conditions of the HE experiment.

This conclusion was somewhat unexpected in view of the extensive and conclusive evidence that vitamin C "spares" and/or "regenerates" vitamin E in a wide variety of *in vitro* model systems (9-37) and the extensive, though less conclusive, evidence for the same phenomenon *in vivo* (40,45-47,50,52,53,56,58). We have therefore confirmed this conclusion by reanalyzing the data in Table 1 to demonstrate that the rate of loss of "old" vitamin E from a particular tissue is not significantly different between the HEHC, HENC, and HELC subgroups of guinea pigs. Figure 3 shows plots for plasma and eight tissues of the ratio of "old" vitamin E/total vitamin E, i.e., d_0-*RRR*-α-TOH/[d_0- + d_6-*RRR*-α-TOH]. We use "old" vitamin E/total vitamin E ratios in order to minimize the effect of differences between individual animals and temporal fluctuations within an animal. Figure 3 provides convincing proof that the rate of loss of "old" vitamin E from a tissue is quite uninfluenced by the level of vitamin C in the diet of the HE group of guinea pigs. Indeed, plots of log[d_0-*RRR*-α-TOH] vs time, which were found to be approximately linear (vide infra), showed no statistically significant differences between the HEHC, HENC, and HELC slopes for each tissue.

Confirmation that increased levels of vitamin C in the

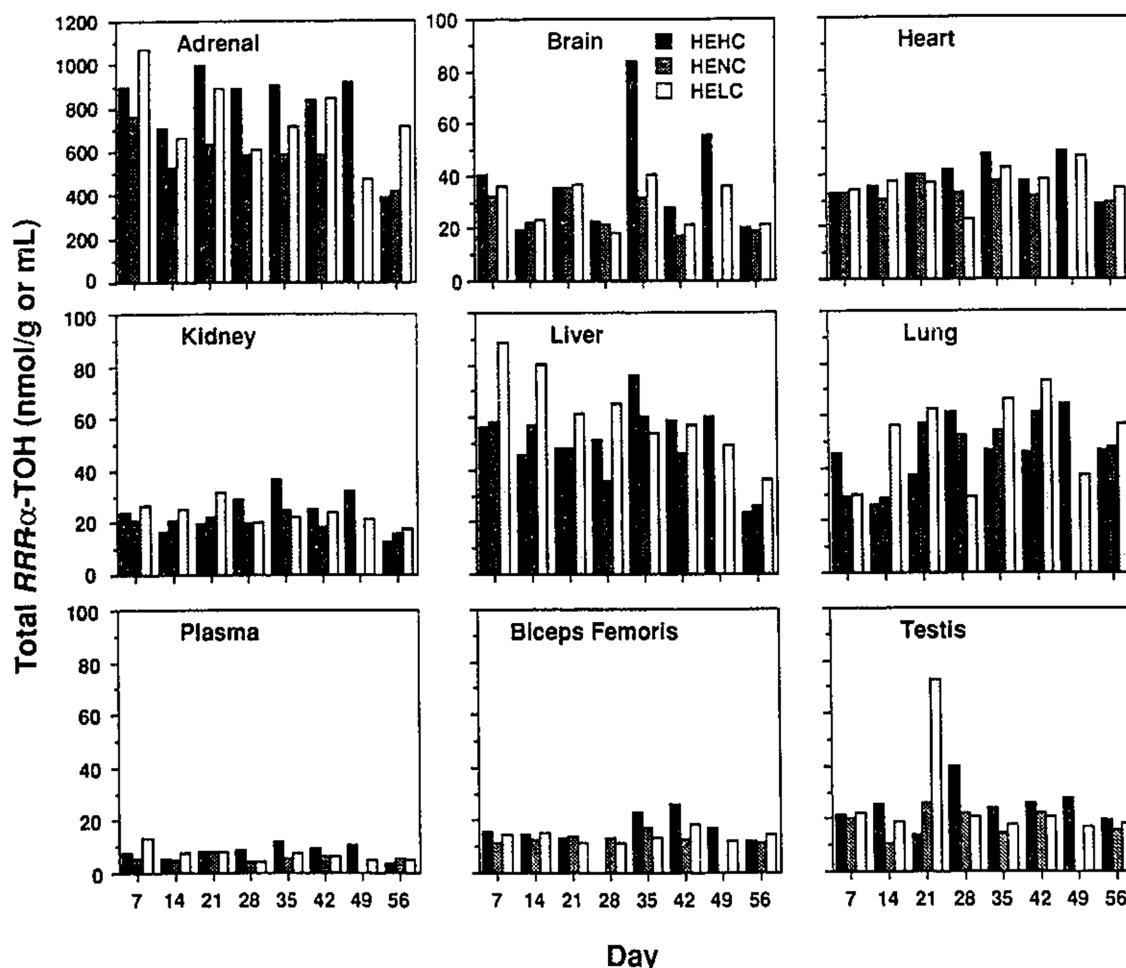


FIG. 2. Total (i.e., d_0- + d_6-) *RRR*-α-TOH concentrations for plasma and eight tissues from the HEHC, HENC, and HELC subgroups of guinea pigs have been plotted at weekly intervals. Note the different vertical scale for adrenal.

TABLE 2

Effect of Vitamin C on Total α-Tocopherol Concentrations in Blood and Tissues of Guinea Pigs[a]

	HEHC	HENC	HELC
Adrenal	816 ± 194	584 ± 103	746 ± 185
Brain	38 ± 22	26 ± 7	29 ± 9
Heart	39 ± 7	33 ± 4	37 ± 7
Kidney	25 ± 8	20 ± 3	24 ± 4
Liver	53 ± 15	47 ± 13	62 ± 17
Lung	47 ± 12	47 ± 13	51 ± 17
Biceps femoris	17 ± 5	13 ± 2	14 ± 2
Plasma	8 ± 3	6 ± 1	7 ± 3
Red cells	7 ± 2	5 ± 2	6 ± 1
Spleen	47 ± 22	42 ± 8	46 ± 7
Testis	25 ± 7	19 ± 5	26 ± 19

[a]Guinea pigs were maintained on high vitamin E (HE) diets (36 mg acetate/kg diet) containing vitamin C at high (HEHC, 5000 mg/kg), normal (HENC, 250 mg/kg) or low (HELC, 50 mg/kg) levels. Concentrations (nmol/g or ml), are the mean and standard deviation of values obtained from eight animals killed at the rate of one per week over an eight week period. The only statistically significant difference found was between adrenal HEHC and HENC ($p < 0.05$).

diet did, indeed, lead to increased levels in the animals was obtained by measuring, using an HPLC method (69), the ascorbate levels in extracts of homogenized samples of a selection of frozen tissues from animals in the HE group. The concentrations of ascorbate in the livers of each of the LC, NC, and HC animals were found to be 42, 237, and 750 nmol/g, respectively, at two weeks, and 100, 179 and 788 nmol/g, respectively, at five weeks. Values obtained for lung in the LC and HC groups at two weeks were 47 and 199 nmol/g, respectively, and the corresponding values for testis were 156 and 749 nmol/g, respectively.

In the LE group of guinea pigs the total concentration of vitamin E decreased substantially over eight weeks in all tissues except the brain (Fig. 4). [The absence of a noticeable decline in the brain is due to the very slow loss of "old (and gain of "new") vitamin E by this organ (Table 1). The literature suggests that had we examined adipose tissue it also would have shown a very slow turnover of vitamin E (70).] The LE group certainly does not have a "steady-state" vitamin E status but is instead progressing towards deficiency or a very much lower steady-

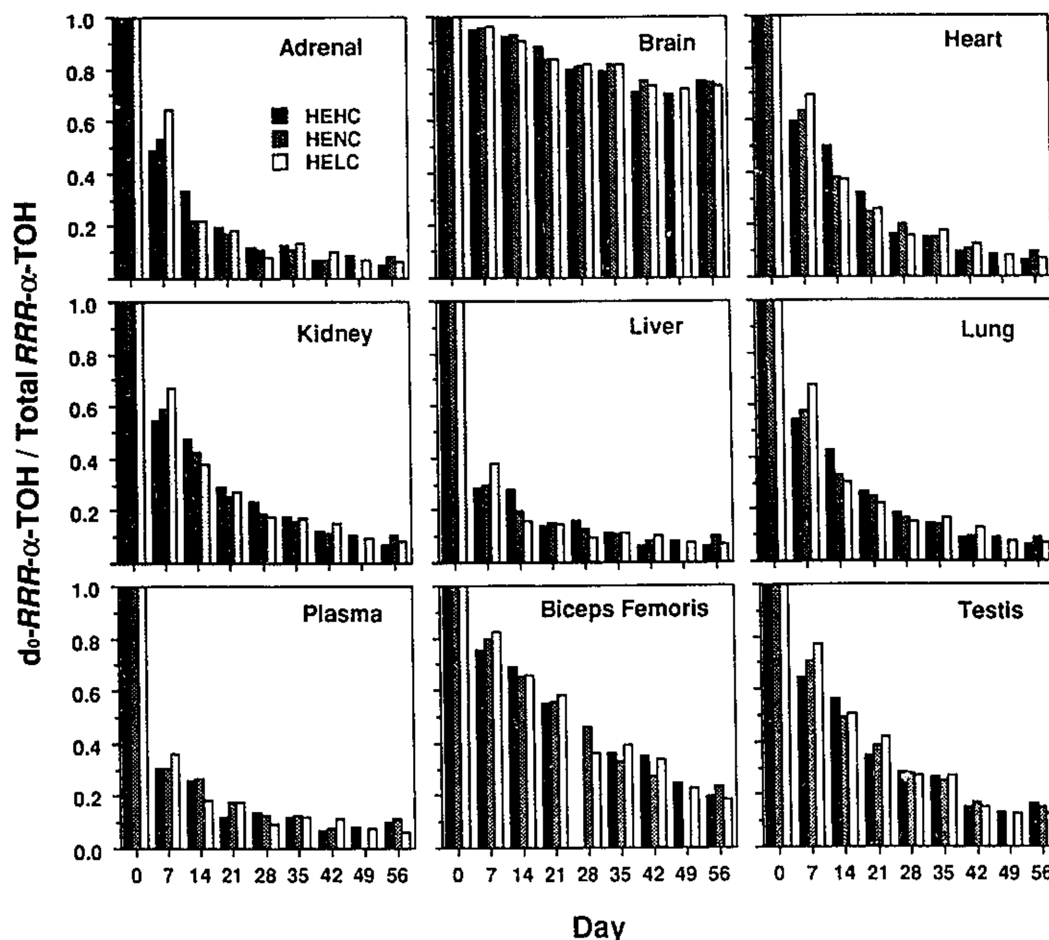


FIG. 3. "Old" d_0-*RRR*-α-TOH/total (d_0- + d_6-) *RRR*-α-TOH ratios for plasma and eight tissues from the HEHC, HENC, and HELC subgroups of guinea pigs have been plotted at weekly intervals.

state level of vitamin E, during the eight week experiment. Nevertheless, the data shown in Figure 4 demonstrate that there is no statistically significant difference in the total concentration of vitamin E in a particular tissue between the LEHC, LENC, and LELC subgroups of guinea pigs over the eight week course of this experiment. We conclude that any "sparing" or "regenerating" of vitamin E by vitamin C is negligible in comparison with those metabolic processes which consume vitamin E or eliminate it from the body, even in a guinea pig which is receiving insufficient vitamin E in its diet to maintain its body stores.

This conclusion was also unexpected. Therefore, in Figure 5 we present plots of the ratio of "old" vitamin E/total vitamin E for plasma and eight tissues. Again, differences between the slopes of plots of log[d_0-*RRR*-α-TOH] vs time for the LEHC, LENC, and LELC subgroups for each tissue were not statistically different. Obviously, the rate of loss of "old" vitamin E from a tissue was quite uninfluenced by the level of vitamin C in the diet of the LE group just as was the case for the HE group of guinea pigs.

In summary, we can find no evidence for an interaction between vitamin C and vitamin E *in vivo* despite a careful, sensitive, and sophisticated search using an appropriate animal model, i.e., an animal which does not synthesize ascorbic acid. We conclude that any synergistic (i.e., "sparing") or antagonistic interaction between these two vitamins *in vivo* in animals not subject to enhanced oxidative stress is negligible in comparison to other metabolic processes. Strictly speaking, this conclusion applies only to guinea pigs. However, we can see no reason why it should not also apply to other animals, including man.

To conclude this section we note that our present results demonstrate that even the most carefully modeled *in vitro* system may fail to reproduce the *in vivo* reality. In this case, the most careful models have involved α-tocopherol dissolved in dilinoleoylphosphatidylcholine (26) or soybean phosphatidylcholine (27) multilamellar liposomes dispersed in water containing ascorbic acid which was subjected to attack at 37°C by thermally-generated, water- or lipid-soluble peroxyl radicals (71). On addition of vitamin C, the water-soluble peroxyl radicals were efficiently trapped which prevented them from attacking the phospholipid bilayer and hence "spared" the vitamin E until all the vitamin C had been consumed (26,27). This result has been confirmed not only

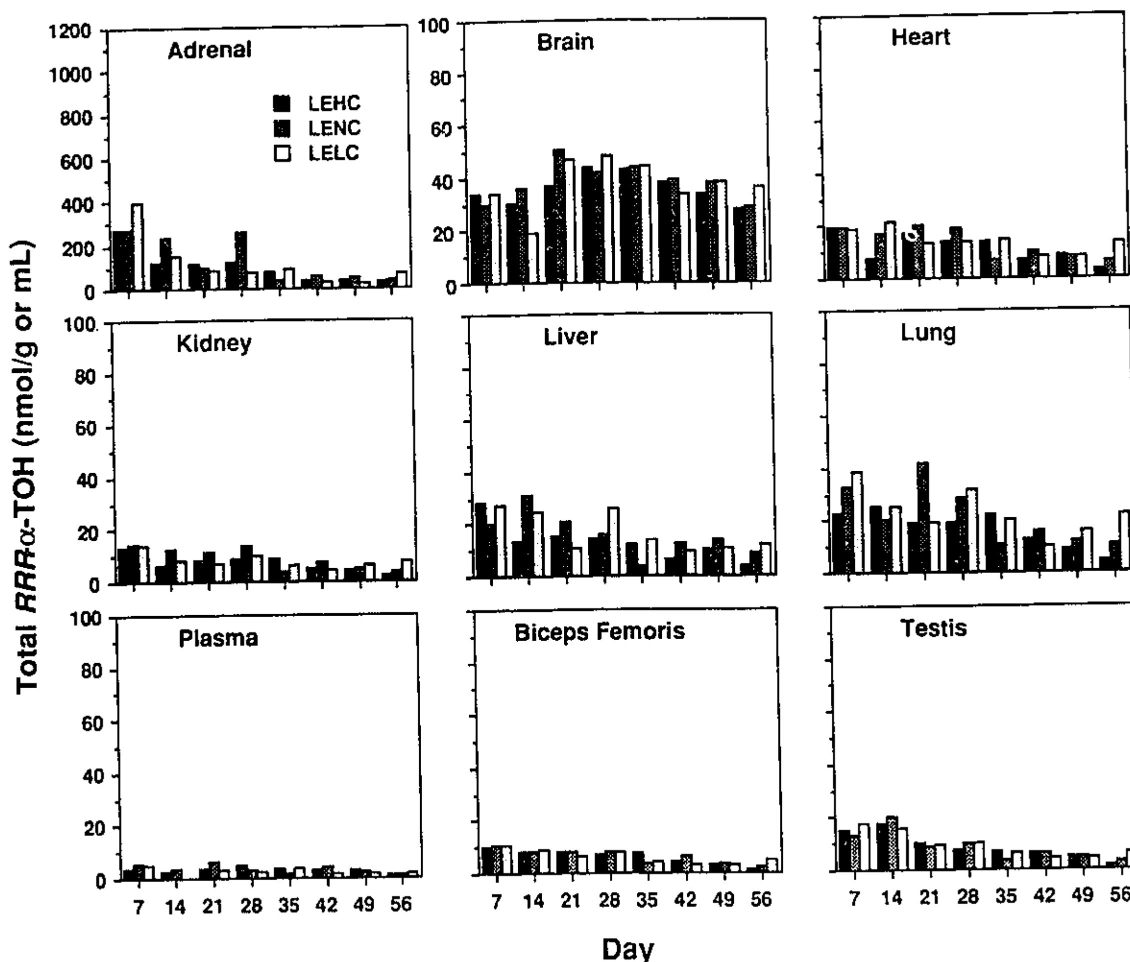


FIG. 4. Total (i.e., d_0- + d_6-) *RRR*-α-TOH concentrations for plasma and eight tissues from the LEHC, LENC and LELC subgroups of guinea pigs have been plotted at weekly intervals. Note that the vertical scales in this figure (including that for adrenal) have been made the same as those in Figure 2 for comparative purposes.

in analogous liposomal and micellar model systems (24,25,28,33) but also in plasma when the plasma was subjected to attack by water-soluble peroxyl radicals (69,72, 73). However, vitamin C is not unique in such systems since other water-soluble, radical-trapping antioxidants (e.g., plasma proteins, glutathione, cysteine, urate, bilirubin, etc.)—both in plasma (69,72,73) and in liposomal model systems (74-77)—also "spare" vitamin E from attack by water-soluble peroxyl radicals. More interesting are the liposomal systems in which lipid-soluble peroxyl radicals are generated from a lipid-soluble initiator (71). In such systems ascorbate by itself was ineffective at protecting the phospholipid from peroxidation (26,27,78). The same is true for most other potentially available water-soluble physiological antioxidants [except for conjugated bilirubin and biliverdin (78)] both by themselves and [except for cysteine (77)] in the presence of vitamin E (74,76,78). Ascorbate is virtually unique in that it regained its antioxidant capabilities in the presence of vitamin E (26,27). That is, when oxidation is initiated in the lipid phase, vitamin C is the only water-soluble antioxidant which becomes active when vitamin E is present. Presumably this "sparing" by vitamin C of vitamin E occurs via the "regenerating" reaction [2], as was suggested over 40 years ago by Golumbic (79).

The question as to why water- and lipid-soluble peroxyl radicals in the presence of phospholipid bilayers and water-soluble peroxyl radicals in plasma do not reflect the situation in guinea pigs with steady or declining vitamin E status is intriguing. The simplest answer (Occam's answer) would be that under normal conditions the flux of peroxyl radicals which enters the lipids of a healthy animal is not nearly as high as has frequently been supposed. This answer receives support from Tappel's finding that expired pentane levels from animals are extremely low, even for animals that are receiving inadequate or no vitamin E, relative to the levels reached for animals that are oxidatively stressed in various ways (41,44,54,80) (A. L. Tappel, 1989, private communication). This would mean that for oxidatively unstressed animals, only a very small fraction of the available vitamin E is actually destroyed by trapping the peroxyl radicals. A much more interesting possibility is that the tocopheroxyl radical is formed extensively even in a healthy animal, but is reduced *in vivo* not by ascorbate but by some other, possibly enzymic, process. There is, in fact, considerable

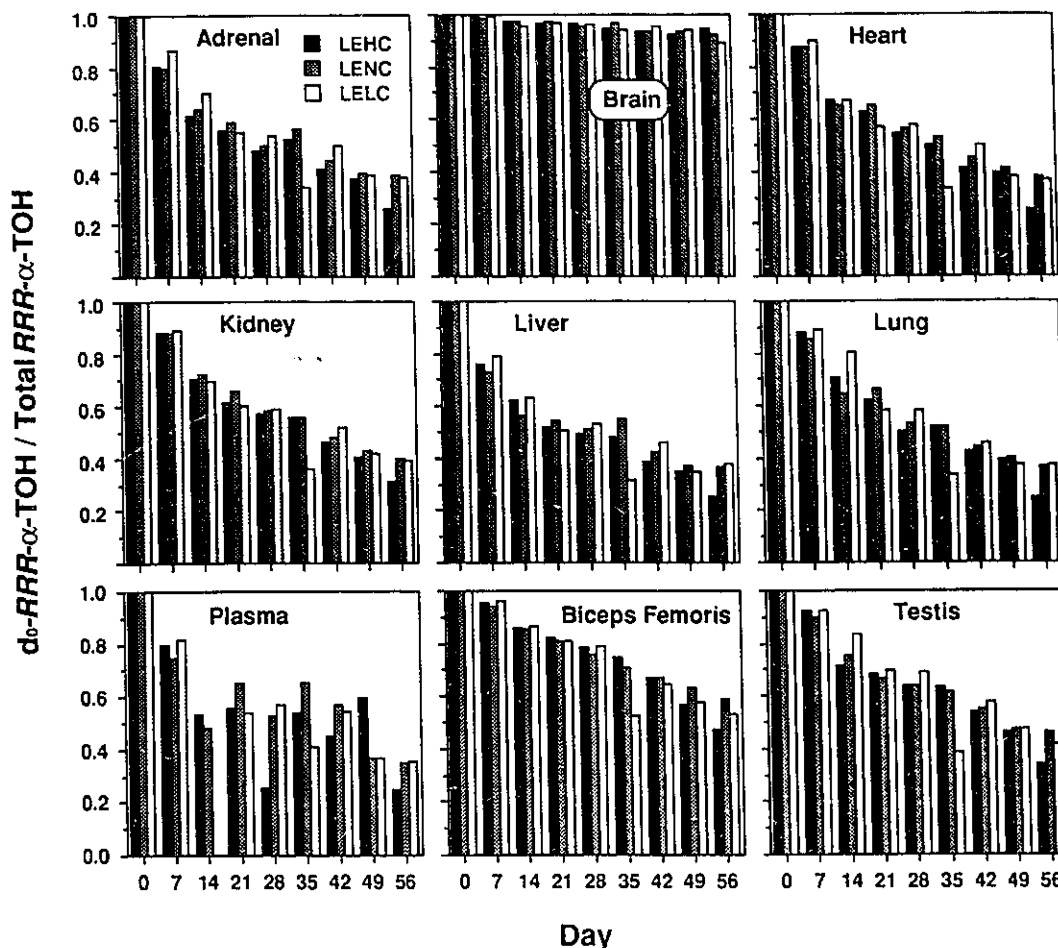


FIG. 5. "Old" d_0-*RRR*-α-TOH/total (d_0- + d_6-) *RRR*-α-TOH ratios for plasma and eight tissues from the LEHC, LENC, and LELC subgroups of guinea pigs have been plotted at weekly intervals.

evidence that rat liver microsomes and other organelles and tissues contain a membrane-bound, heat-labile, glutathione-dependent, free-radical reductase which probably acts by converting the tocopheroxyl radical to tocopherol (38,81–90) and which therefore participates in the *in vivo* protective system against lipid peroxidation. Free radical reductase activity in microsomes may also be NADPH dependent (91). A quantitative and unequivocal determination of the peroxyl radical flux in the lipids of healthy animals would add enormously to our understanding of free radical biology.

Biokinetics of vitamin E in guinea pigs. Comparison with rats. There are no significant differences in the biokinetics of vitamin E between the HEHC, HENC, and HELC subgroups of animals and between the LEHC, LENC, and LELC subgroups. This allows us to combine the biokinetic data for all the HE and all the LE guinea pigs, which simplifies biokinetic comparisons between these groups. Moreover, the HE biokinetic data can be compared with the analogous data for the same tissues obtained from the HE rats (1).

Under "steady-state" conditions of vitamin E the tissues of an animal can be divided broadly into two kinetic groups (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results). The first group, which includes brain, heart, muscle, and testes, shows slow, first order (i.e., exponential) loss of "old" and gain of "new" vitamin E. The second group, which includes plasma, liver, lung, adrenal gland, and kidneys, shows non-exponential behavior with an initial, rapid change in vitamin E concentration compared with later stages. Formally, the behavior of the fast tissues can be represented as the sum of two (or more) first-order processes (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results).

Since this detailed kinetic analysis of the HE ("steady-state") group of guinea pigs will be reported elsewhere (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results), though without the supporting raw data which is given in Table 1, we will not repeat it here. For present purposes it is sufficient to give the time required under "steady-state" conditions for the concentration of deuterium-labelled, "new" vitamin E in a tissue to become equal to the concentration of unlabelled, "old" vitamin E. These equalization times, $t_{1:1}$, give a simple measure of the speed with which vitamin E turns over in different tissues under "steady-state" conditions. Equalization times can be estimated by inspection of the

TABLE 3

Comparison of Tissue Equalization Times ($t_{1:1}$; days) in Young Guinea Pigs and Rats[a]

Tissue	Guinea pig	Rat
Plasma	3.7	6.2
Liver	3.0	6.9
Lung	9.9	8.8
Kidney	9.8	13
Heart	14	18
Testis	17	40
Biceps femoris	24	23
Brain	107	40

[a]Values for all tissues, except guinea pig brain, were obtained by interpolation of concentration data plotted in single- or multiexponential form vs time. Because of a very slow rate of turnover, the value for guinea pig brain was estimated by extrapolation.

raw data but can be determined more reliably from exponential (slow tissues) or multiexponential (fast tissues) plots (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results). Values of $t_{1:1}$ for the HE guinea pigs' tissues, obtained in this way after combining the HEHC, HENC, and HELC data, are given in Table 3. For comparison this table also includes the $t_{1:1}$ values for the same classes of tissue obtained from HE rats, i.e., from rats fed a diet containing 36 mg d_3-*RRR*-α-TOAc/kg diet and having essentially constant concentrations of vitamin E in their tissues. Some of the rat $t_{1:1}$ values differ from those previously reported (1) because of our current application of a more sophisticated kinetic treatment (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results).

Inspection of Table 3 reveals that all but one of the fast (multiexponential kinetics) tissues from guinea pigs exchange vitamin E more rapidly than the corresponding rat tissues, whereas the slow (monoexponential kinetics) tissues from the guinea pig may exchange vitamin E more, or less, rapidly than the corresponding rat tissues. The most striking difference in the tissue biokinetics of vitamin E between guinea pigs and rats lies in the brain [and probably in adipose tissue (cf., 1,70)]; under "steady-state" conditions the transport of vitamin E into or out of the guinea pigs' brain occurs at only ca. 40% of the rate for the rat. It would be extremely interesting to have analogous data for humans.

There appears to be only one earlier "comparative" study of vitamin E uptake by rats and guinea pigs and only blood, adrenals, heart, and liver were examined (92). On diets containing 30 mg *all-racemic*-α-TOAc per kg of diet the rats after 46 weeks had from 1.4 to 2.0 times as much α-TOH (per ml or per g tissue) as did the guinea pigs in the same tissues after 32 weeks (92). The relevance of this observation to our own measurements is not obvious.

Equalization times for the LE group of guinea pigs have not been calculated because the vitamin E status of these animals is not at a "steady-state," but declines in all tissues except the brain (Table 1 and Fig. 4). This fact is especially evident in plots of the logarithm of total tissue vitamin E vs time (Fig. 6).

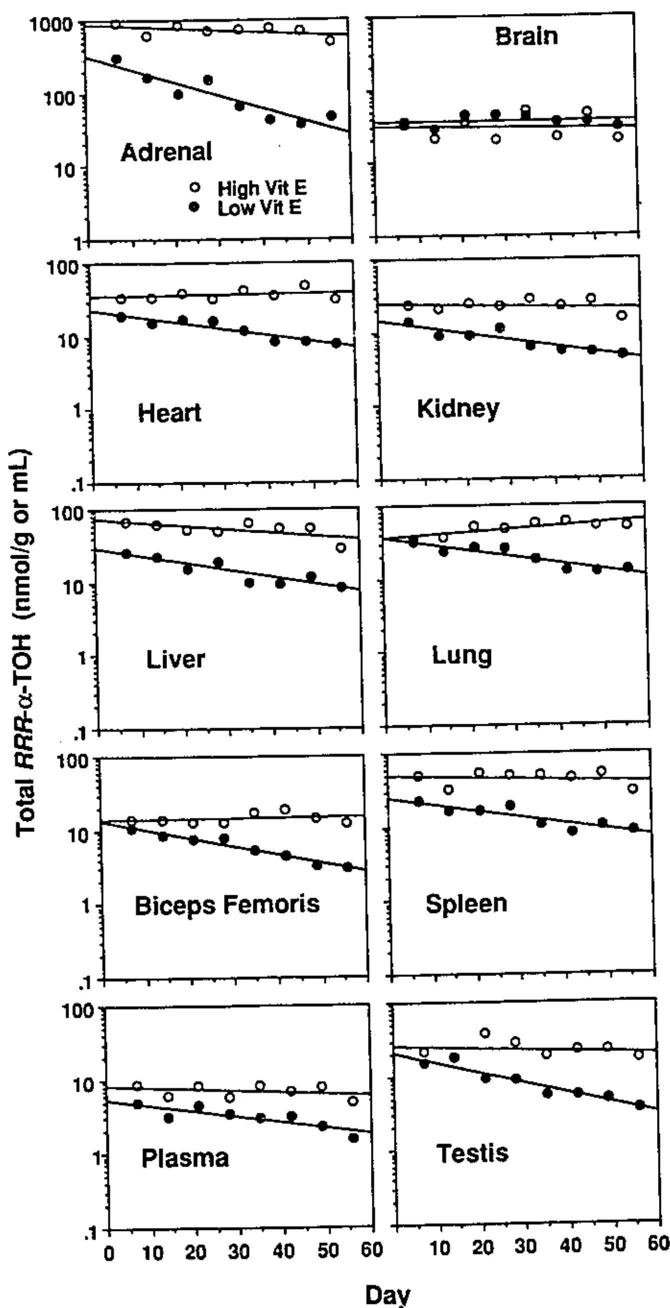


FIG. 6. Semi-logarithmic plots of total (d_0- + d_6-) *RRR*-α-TOH concentrations vs time for plasma and nine tissues from the HE (○) and LE (●) groups of guinea pigs. The individual points which are displayed are the averaged values found for the HC, NC and LC subroups of animals. However each line is the least squares fit to all of the HC, NC and LC data points. Note the different vertical scale for adrenal and brain.

Interestingly, the rates of loss of "old" vitamin E from the tissues of the LE animals were not different from the corresponding rates of the HE animals. This is shown in Figure 7 in which we have plotted log[d_0-*RRR*-α-TOH] vs time for both fast and slow tissues. Although, under "steady-state" conditions, the biokinetics for the fast tissues can be somewhat better described in terms of a

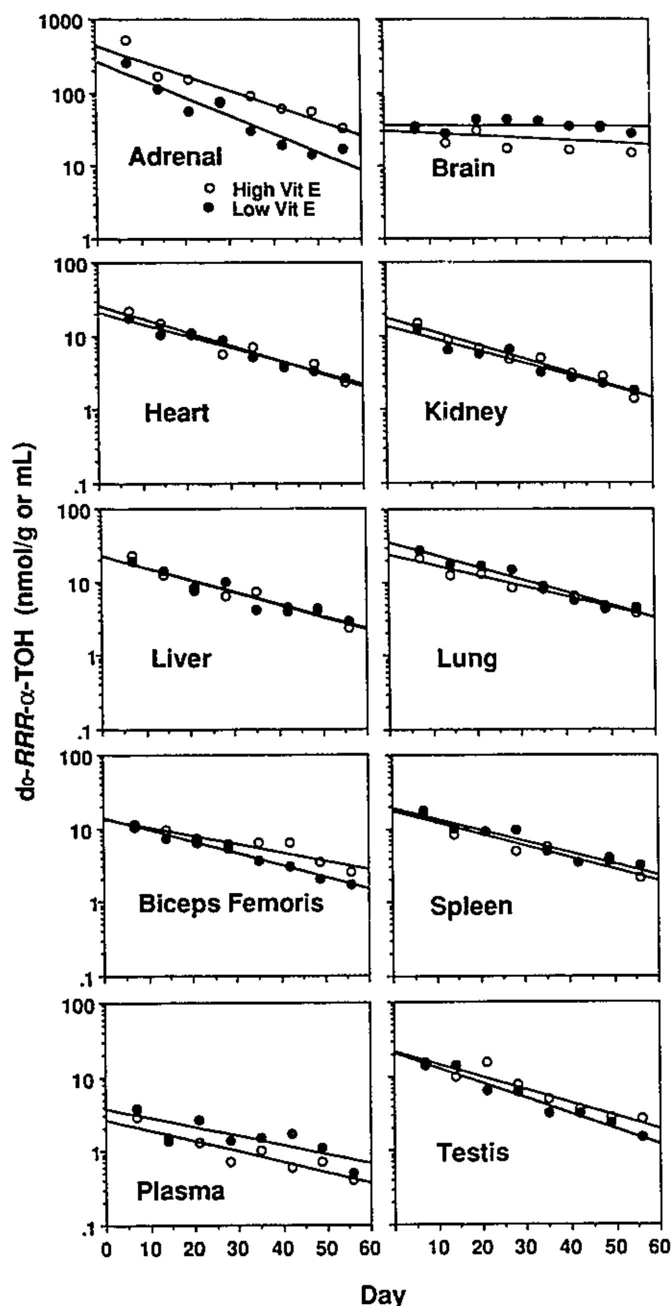


FIG. 7. Semi-logarithmic plots of "old" d_0-*RRR*-α-TOH concentrations vs time for plasma and nine tissues from the HE (O) and LE (●) groups of guinea pigs. The individual points which are displayed are the averaged values found for the HC, NC and LC subgroups of animals. However each line is the least squares fit to all of the HC, NC and LC data points. Note the different vertical scale for adrenal and brain.

multiexponential (K. U. Ingold, G. W. Burton, and W. Siebrand, 1990, unpublished results), it is reasonable to use a single exponential because the data in Figure 7 do not include the initial rapid decline that occurs in the first few days.

It is not unreasonable that "old" vitamin E should be lost from a specific tissue of the LE and HE groups of animals at the same rate, since different rates would imply the existence of some physiological "feed-back" mechanism which could "sense" the vitamin E status of the animal. We hypothesize that such a physiological "feed-back" process may be present in certain "critical" tissues which have very slow rates of uptake and loss of vitamin E. The brain would appear to be a prime candidate for possession of a control mechanism which would ensure that the rate at which it lost vitamin E would depend on the animal's overall vitamin E status, i.e., the rate of loss would be lower when the animal was put on a diet containing an inadequate (or no) vitamin E compared with an animal on a normal diet. Unfortunately, our eight-week-long study with the guinea pigs was of too short a duration for any such effect (or lack of such effect) to demonstrate itself. We therefore plan to carry out further experiments to determine whether certain critical tissues do or do not possess a "feed-back" mechanism.

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