Lipid Peroxidation *In Vivo*[1]

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Abstract

Under a rather rigidly defined set of empirical conditions, the kinetics of production of a specific tocopherol-deficiency sign, creatinuria, in the rat were comparable to the kinetics of the autoxidation of pure polyunsaturated fatty acids in model systems *in vitro*. The tocopherol requirement of the rat under these conditions was clearly related to the kinetics of lipid peroxidation. Dietary, biologically available selenium decreased the rate of production of creatinuria and the rate of disappearance of tissue polyunsaturated fatty acids (PUFA) equally and, therefore, may have functioned as a lipid antioxidant *in vivo*. A hypothetical model of lipid peroxidation was prepared in an attempt to describe the interaction of PUFA, tocopherol, biologically available selenium, and sulfur amino acids.

Introduction

VITAMIN E, *d*-α-tocopherol, has been generally acknowledged to function mainly, if not solely, as a lipid antioxidant in biological systems (1–4). Tocopherol-deficiency signs are most readily produced in animals fed diets high in polyunsaturated fatty acids (PUFA), and all such signs are readily prevented by several fat-soluble antioxidants differing greatly in structure from α-tocopherol (5–8). In addition, the tissues of tocopherol-deficient animals usually contain ceroid or lipofuscin pigments formed by the interaction of oxidized fats and protein (9–12).

Several nonlipid dietary constituents, specifically sulfur amino acids and biologically available selenium, also greatly affect either the animals' tocopherol requirement or the nature of the tocopherol-deficiency signs produced (13,14). Selenium compounds have been shown to decrease oxygen uptake or formation of thiobarbituric acid reactive materials in tissue preparations from tocopherol-deficient animals (15–17), and to minimize gamma radiation damage to proteins in a model system (18). When pure fats were oxidized in the presence of pure protein, little pigment formation was noted (19). However, if the system contained metals or metalo-proteins, complexes comparable to the biological ceroid or lipofuscin-type pigments were formed (20–21).

The great diversity of tocopherol-deficiency signs plus the complexity of the nonlipid interactions has hindered research in this area. Marked alteration of the animals' tocopherol requirement by the nonlipid dietary constituents, selenium and sulfur amino acids, has led to a reluctance to formulate a universal sequence of reactions occurring in the tissues to produce the various tocopherol-deficiency signs. Development of all tocopherol-deficiency signs, however, must have one point in common, peroxidation of PUFA.

Apart from linoleate stored in neutral lipids, PUFA, those fatty acids which will undergo peroxidation in the antioxidant-deficient animal, are generally characteristic of phospholipids. In the animal, aside from circulating lipoproteins, phospholipids seem to occur principally, if not solely, in the cellular and subcellular membranes (22). On the basis of electron micrographs, Danielli (23) has described the membrane as an oriented bimolecular lipid leaflet. The polar groups of the phospholipid molecules are in contact with the aqueous phase while the long aliphatic chains of the fatty acids form a hydrophobic barrier. The compartmentalization of the aqueous regions of the cell by lipid membranes such as those of the mitochondria and endoplasmic reticulum appears essential to the maintenance of normal metabolic processes (24). These phospholipids normally contain approximately 50% PUFA and Collins (25) has shown that the metabolic activity of the individual phospholipids increases with increasing unsaturation of the fatty acids. It is the purpose of the present paper to attempt to demonstrate by drawing upon known autoxidation reactions (26), our own published nutritional data and tissue lipid analyses (27–30), and on the published data of Tappel (15,18, 31,32) that, under certain empirical conditions and considering only a limited number of variables, it is possible to develop a mathematical treatment of *in vivo* lipid peroxidation directly comparable to the kinetics of *in vitro* lipid autoxidation. A hypothetical reaction scheme is presented to illustrate why such a kinetic treatment may be practical. By relating the action of selenium and sulfur amino acids mainly to secondary rather than to primary lipid peroxidation in this scheme, it has been possible to propose a reaction sequence which appears to be consistent with the complex interactions noted in the description of the animals' tocopherol requirement.

Experimental

The data to be used in analysis of the kinetics of *in vivo* lipid peroxidation are taken largely from a series of papers by Witting and Horwitt (27–30). Since the nutritional aspects of these feeding studies and tissue lipid analyses have been discussed in detail elsewhere, with the exception of a few extremely pertinent figures, they will not be repeated here.

In Vivo Lipid Peroxidation

A hypothetical reaction scheme (Fig. 1) illustrates lipid peroxidation reaction in a small section of a subcellular membrane. Phospholipid molecules are conventionally represented as "tuning forks" (Fig. 1a). The short horizontal line of the "tuning fork" represents the glycerol moiety, while the vertical lines are the fatty acid chains (RH), the center solid circle is the polar portion of the molecule (phosphate, nitrogenous base, etc.), and the long horizontal lines represent the membrane boundary. Upon initiation of peroxidation, a fatty acid free-radical (R·) is formed (Fig. 1b) at the rate K_1. Addition of oxygen produces a peroxy free-radical (ROO·) (Fig. 1c) at the rate K_2. This peroxy free-radical may remove a proton from another molecule of fatty acid to form a hydroperoxide (ROOH) and a new fatty acid free-radical (Fig. 1d) at the rate K_3 thus setting off a chain reaction

$$\begin{aligned} &R\cdot + O_2 \longrightarrow RO_2\cdot \\ &RO_2\cdot + RH \longrightarrow ROOH + R\cdot \end{aligned}$$

(26). In certain photochemically induced free-radical reactions, the quantum yield has been found to be as

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high as 10–50,000 molecules of product per initiation (33). Alternatively, the fatty acid peroxy free-radical may remove a proton from tocopherol or other fat-soluble antioxidant (AH) to form a hydroperoxide (Fig. 1e) at the rate K_4. In this case the antioxidant withdraws free-radicals from the system and terminates the chain reaction (26). Free-radical interactions leading to a net decrease in free-radical concentration (Fig. 1f) may also proceed at the rate K_5 but these reactions are probably of negligible significance during the early stages of peroxidation. All of these reactions involve fatty materials and therefore are depicted as occurring in the hydrophobic phase.

The chain reaction leads to accumulation of lipid hydroperoxides according to the equation $d[ROOH]/dT = (K_1/K_5)^{1/2} K_3[RH]$ *in vitro* (26). Since the polar hydroperoxide group is hydrophilic in character, the peroxidized fatty acid may approach or enter into contact with the external aqueous phase (Fig. 1g). In an area of peroxidation, what might loosely be termed, a hole-in-the-membrane, would thus be formed. The production of such holes-in-the-membrane would account for the high levels of free lysosomal enzymes noted in tocopherol-deficient animals. Tappel (31) has shown that approximately $4–6 \times 10^6$ free-radical initiations are necessary for maximal release into the cytoplasm of the catabolic enzymes contained in the lysosome. Secondary oxidation of fatty acids at the interface in the presence of metals or metalo-protein (Fig. 1i) would be expected to produce protein denaturation and binding of the oxidized fat to protein (ceroid or lipofuscin type pigment) in a manner comparable to similar processes *in vitro* (20,21). However, if the protein contains seleno analogs of the sulfur amino acids (Fig. 1h), Tappel (32) has shown that 50–250 molecules of lipid peroxide may be catalytically destroyed per molecule of selenium. Such a destruction of peroxide would minimize secondary lipid oxidation and protein damage at the interface. Selenium catalyzed reactions which decreased the polarity of the oxygen containing functional group on the fatty acid chain (perhaps from –OOH to a carbonyl group), might permit partial restoration of the hydrophobic barrier (Fig. 1h). The normal sulfur amino acids are less effective in this regard than are their seleno analogs (32). Desai, Calvert and Scott (34) have shown that methionine supplementation is effective in lowering the level of free lysosomal enzymes but not of TBA reactive materials in homogenates of the muscle of the tocopherol-deficient chick showing signs of nutritional muscular dystrophy.

Alternatively, if organo-selenium compounds are in contact with the hydrophobic phase they may function as an antioxidant by terminating the free-radical chain as in Fig. 1e.

According to the above hypothetical mechanism, lipid peroxidation might be divided into two distinct portions. *Primary* lipid peroxidation leading to membrane disruption is characterized as occurring in a relatively isolated hydrophobic environment. All subsequent reactions occurring at the interface or in the aqueous region involve either damage to non-lipid components such as protein by the oxidized fatty acids or rapid *secondary* lipid oxidation reactions occurring in the presence of metals, metalo-proteins, and/or other nonlipid materials. The hypothetical mechanism described is hardly original, probably vastly oversimplified, and may be totally inaccurate. It

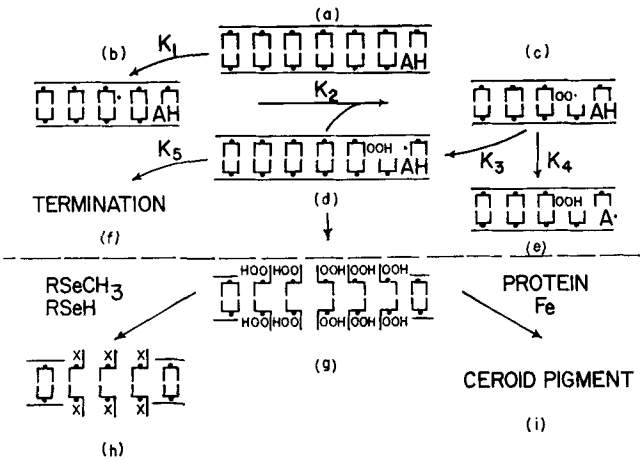

FIG. 1. Hypothetical reaction scheme of *in vivo* lipid peroxidation. Section above broken line describes primary lipid peroxidation and antioxidant action. Section below broken line describes secondary lipid oxidation. (a) cross section of lipid membrane; (b) free-radical formation; (c) uptake of oxygen; (d) formation of hydroperoxide and new free-radical; (e) chain reaction broken by antioxidant; (f) interaction of free-radicals with net loss of free-radicals; (g) "hole-in-the-membrane"; (h) role of seleno amino acids; (i) ceroid pigment formation.

does, however, permit formulation of a working hypothesis, subject to experimental testing.

This hypothesis is based on the knowledge that in the autoxidation of fatty acids, *in vitro*, the slowest and therefore rate-limiting reaction (26) is the removal of a proton from a fatty acid by a fatty acid peroxy free-radical, $RO_2\cdot + RH \xrightarrow{K_3} ROOH + R\cdot$ (Fig. 1c → Fig. 1d). The rate (K_3) of this reaction, and only this reaction, is dependent on the magnitude of n in $CH_3(CH_2)_a(CH{=}CH\,CH_2)_n(CH_2)_b\,CO_2H$ (26). Secondary oxidation *in vivo* and/or damage to non-lipid constituents of the cell, while possibly related to the selenium and sulfur amino acid nutriture of the animal, is still limited by the structurally dependent (magnitude of n in $CH_3(CH_2)_a(CH{=}CH\,CH_2)_n(CH_2)_b\,CO_2H$), rate ($K_3$) of production of the primary products of lipid peroxidation.

The free-radical chain reaction in the isolated hydrophobic region of the membrane may be broken by a fat-soluble antioxidant. The effectiveness of the antioxidant is, in this case, dependent on its relative concentration [RH]/[AH] and on the relative rates K_3/K_4 of the competing reactions, hydroperoxide propagation and antioxidant free-radical chain termination (Fig. 1c, d, and e).

Briefly stated, the working hypothesis is: *In vitro* lipid autoxidation and *in vivo* lipid peroxidation are kinetically comparable, whether uninhibited or antioxidant inhibited, since the rate limiting reaction in both cases depends on the structure of the polyunsaturated fatty acids. Experimentally, therefore, the rate of production of a tocopherol-deficiency sign in an animal and the animal's tocopherol requirement must be related to the fatty acid composition of the animal's tissue lipids as influenced by the fatty acid composition of the dietary fat.

The term antioxidant "requirement" is used in a rather unusual sense in this context. An antioxidant decreases the rate of PUFA peroxidation, $d[O_2]/dT$, by minimizing the number of molecules of lipid peroxide produced per free-radical initiation rather than completely preventing the production of lipid peroxides. Therefore, any statement of requirement for antioxidants must be qualified by a statement of the

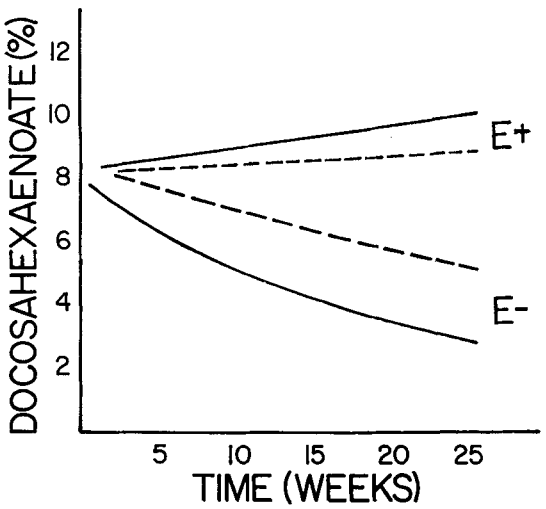

FIG. 2. Docosahexaenoate content of muscle phospholipid fatty acids in tocopherol-supplemented (E+) and tocopherol-deficient (E−) rats. Solid lines: Rats fed tocopherol-deficient basal ration plus 12.5% fat containing 62.2% saturated, 8.2% monoenoic, 5.9% dienoic, and 23.7% trienoic acids. Broken lines: Rats fed tocopherol-deficient basal ration supplemented with 0.13 ppm of selenium as sodium selenite and 0.4% *dl* methionine plus 7.5% fat. Tocopherol supplemented rats received 15 mg *d*-α-tocopheryl acetate/kg rat body weight/week. From top to bottom, the lines (drawn by the method of least squares) are based on 40, 42, 19 and 28 points, respectively.

period of time over which this level of supplementation with the antioxidant will prevent the formation of a harmful quantity of peroxidized lipid. Lipid peroxidation *per se* is inevitable and has been suggested as a specific factor in the generalized process of aging (35).

Kinetics of Uninhibited Lipid Peroxidation. The following simplified equation is frequently used to describe the autoxidation of pure unsaturated fatty acids *in vitro* (26).

$$d\,[ROOH]/dT = (K_1/K_5)^{1/2}\,K_3\,[RH] \qquad (1)$$

Holman (36) found that as n in $CH_3\,(CH_2)_a(CH{=}CHCH_2)_n(CH_2)_bCO_2H$ was increased from 1 to 6, the relative maximum rates of autoxidation of individual pure fatty acid methyl esters *in vitro* at 37C were in the ratios 0.025:1:2:4:–:8. Somewhat more complicated experiments have been necessary to analyze the reaction kinetics in the living animal. In an animal feeding study, the use of individual pure polyunsaturated fatty acids is not entirely practical. The data of Bolland (37) indicates that the equation:

$$K_{3m} = (aK_{3a} + bK_{3b})/m \qquad (2)$$

may be used to describe the rate of propagation of the autoxidation of a mixture (m) of fatty acids (a+b). In the test tube, the autoxidizing system is normally vigorously agitated since the exothermic reaction, $RO_2\cdot + RH \rightarrow ROOH + R\cdot$, (Fig. 1c, d) would otherwise cause local heating and thus distort the kinetic data. Local heating is not a problem in the fluid membrane *in vivo*. In this case, however, the reaction rate is limited by the process of diffusion and the effective rate of propagation of *in vivo* peroxidation must therefore be written as:

$$K_{3m} = [(aK_{3a} + bK_{3b})/m]^{1/2} \qquad (3)$$

The tocopherol-deficient rat develops creatinuria as a sign of the onset of nutritional muscular dystrophy. Typical curves of the ratio of urinary creatine to creatinine during the course of tocopherol-deficiency have been published by Horwitt et al. (38). In general these curves resemble the oxygen uptake curves, a level induction period followed by a rapid exponential rise, recorded in autoxidation studies. When (T_o, time in weeks after start of the experiment) a very mild but significant ($p < 0.005$) creatinuria (creatine to creatinine $\geqq 0.4$ compared to 0.2 in tocopherol supplemented control rats) occurs, it would seem probable that only a limited amount of peroxidation has taken place resulting in slight tissue damage. This has been verified experimentally (30) by the observation that creatinuria occurred after the peroxidative disappearance of approximately 2% of the muscle phospholipid fatty acids or 4×10^{-7} M fatty acid/gram wet weight of muscle. Under these conditions therefore, for practical purposes it is possible to consider:

$$[RH] - [ROOH] = [RH] \qquad (4)$$

Next, it is necessary to establish that at T_o, the time of onset of creatinuria, the same level of lipid peroxidation has taken place in all cases. The net loss of polyunsaturated fatty acids from the muscle phospholipids of the tocopherol-deficient rat has been found (30) to follow first order reaction kinetics (Fig. 2). When creatinuria occurred in three weeks, the docosahexaenoic acid content of the total phospholipids decreased to one-half the level seen in tocopherol or N, N′-diphenyl-p-phenylenediamine (DPPD) supplemented rats in 15 weeks ($D_{½} = 15$ weeks) (Fig. 2—solid line). When the onset of creatinuria was delayed to 7 weeks by supplementation of the previous tocopherol-deficient diet with 0 13 ppm of selenium as sodium selenite and 0.4% *dl* methionine a value of $D_{½} = 32$ weeks was found for docosahexaenoate (Fig. 2—broken line). Interpretation of the rates of disappearance of the other unsaturated fatty acids has been discussed in detail elsewhere (30). These rates are compatible with the relative propagation rates developed below. The similarity of the ratios of rates of production of creatinuria 1:2.3 and rates of docosahexaenoate disappearance 1:2.1 suggests that in both cases the level of tissue damage represented by the specific low level of creatinuria was related to the same quantity of lipid peroxidation. In delaying the formation of a specific quantity of oxidized lipid, selenium and possibly methionine must have acted as antioxidants *in vivo*.

Equation 1 has therefore been rewritten in the form:

$$1/T_o = [(aK_{3a} + bK_{3b})/m]^{1/2}\,(K_1/K_5)^{1/2} \qquad (5)$$

for use *in vivo*. By using young, rapidly growing animals whose tissue lipids rapidly equilibrate with the fatty acid composition of the dietary fat, the constants a, b, etc. may be expressed in terms of the g/g dietary fat of each fatty acid type, i.e. monoenoic, dienoic, etc. Witting and Horwitt (27) fed a variety of specially prepared fats which were, with one exception, of constant total unsaturation, iodine value 82, but differed in the source of unsaturation (i.e. monoenoic, dienoic, trienoic, or higher polyenoic fatty acids) to tocopherol-deficient rats and recorded

TABLE I

Calculated Rate of Propagation of Fatty Acid Peroxidation *In Vivo*

Number of double bonds in fatty acid	1	2	3	4	5	6
Series B						
$K_3/K_5 \times 10^2$	0.05	2	4	8	12	16
Series A						
$K_3/K_5 \times 10^2$	0.1	4	8	(16)[a]	(24)	(32)
Relative rate *in vivo*	0.025	1	2	4	6	8
Relative rate *in vitro*	0.025	1	2	4	6	8

[a] Only two equations containing terms with n = 4, 5, and 6 were available in the series. The tabulated values, obtained by analogy to Series B, satisfy these equations.

the time of onset of creatinuria. From this data it has been possible to generate the following series of simultaneous equations which are useful in evaluating the contribution of each fatty acid type, i.e. monoenoic, dienoic, etc. to the rate of production of creatinuria. The rate of free radical initiation (K_1) is assumed to be constant and variation in $K_{3m}/K_5^{1/2}$ to be due entirely to the effect of variation of n in $CH_3(CH_2)_a(CH{=}CHCH_2)_nCH_2)_bCO_2H$ on K_3.

		Series A	*Series B*
$K_5^{-1/2}(0.096\,K_{3a} + 0.030\,K_{3b})^{1/2}$	=	1/28	.....
$K_5^{-1/2}(0.078\,K_{3a} + 0.022\,K_{3b})^{1/2}$	=	.....	1/49
$K_5^{-1/2}(0.811\,K_{3a} + 0.072\,K_{3b})^{1/2}$	=	1/17	1/18
$K_5^{-1/2}(0.212\,K_{3a} + 0.330\,K_{3b})^{1/2}$	=	1/8	1/12
$K_5^{-1/2}(0.100\,K_{3a} + 0.084\,K_{3b} + 0.231\,K_{3c})^{1/2}$	=	1/7	1/9
$K_5^{-1/2}(0.120\,K_{3a} + 0.046\,K_{3b} + 0.121\,K_{3c} + 0.014\,K_{3d} + 0.032\,K_{3e} + 0.029\,K_{3f})^{1/2}$	=	1/6	1/8
$K_5^{-1/2}(0.173\,K_{3a} + 0.131\,K_{3b} + 0.001\,K_{3c} + 0.019\,K_{3d} + 0.041\,K_{3e} + 0.051\,K_{3f})^{1/2}$	=	.....	1/7
$K_5^{-1/2}(0.206\,K_{3a} + 0.022\,K_{3b} + 0.017\,K_{3c} + 0.023\,K_{3d} + 0.056\,K_{3e} + 0.052\,K_{3f})^{1/2}$	=	1/5	1/7

In series B the diet fed in series A was supplemented with 0.13 ppm of selenium as sodium selenite and 0.4% *dl* methionine. Numerical values of the various contributions to the rate of production of creatinuria calculated in this manner are tabulated in terms of the number of double bonds in the fatty acid molecules (Table I). These numerical values are in the same ratios as the relative maximum rates of propagation of the autoxidation of these same fatty acids *in vitro* as determined by Holman (36). While the numerical values found for the contributions of the various fatty acids to the rates of propagation of the peroxidation were halved by supplementing the diet with selenium and methionine, their ratios remained the same (Table I). Plotting the rate of production of creatinuria ($1/T_o$) versus a calculated description:

$$EP_o = (\%\ \text{monoenoate} \times 0.025) + (\%\ \text{dienoate} \times 1) + (\%\ \text{trienoate} \times 2) + \%\ \text{tetraenoate} \times 4) + (\%\ \text{pentaenoate} \times 6) + (\%\ \text{hexaenoate} \times 8) \quad (6)$$

of the estimated peroxidizability (EP_o) dietary fat (Fig. 3) illustrates graphically the level of experimental scatter encountered. Note that selenium and methionine supplementation effected the slope of the curve without altering the direct relation between $1/T_o$ and EP_o.

Although equation 5 does not consider the quantities K_1 and $[O_2]$ as variables, it appears to reasonably describe *in vivo* lipid peroxidation kinetics. Experiments involving hypo or hyperoxia would, however, necessitate consideration of $[O_2]$ as a variable. Similarly, exposure to ionizing radiation, to materials which altered membrane permeability, or to actual physical injury resulting in tissue damage would necessitate consideration of K_1, the rate of free-radical initiation, as experimental variable.

On the basis of Figure 3, it might be argued that tocopherol does not participate in any enzymatic reactions. Extrapolation of the curves to $EP_o = 0$ and $T_o = \infty$ would seem to indicate that as the potential peroxidizability of the dietary fat approaches zero, the animal shows no evidence of a tocopherol requirement by the criterion of creatinuria. Such an extrapolation is not valid in any animal having an essential fatty acid requirement since *de novo* synthesis of eicosatrienoates from acetate via oleate and palmitoleate (39) would render the curve discontinuous.

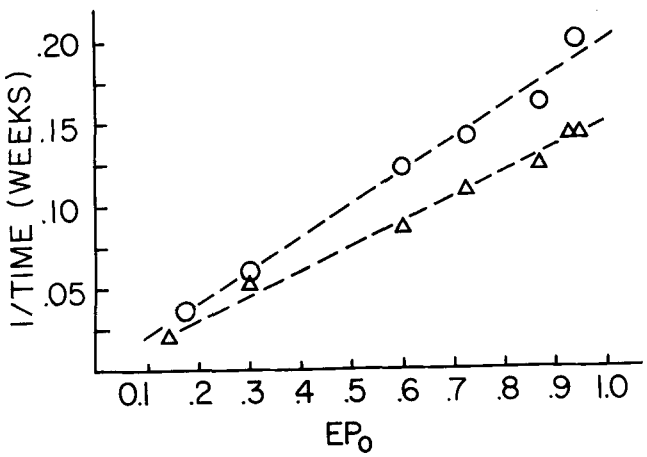

FIG. 3. Rate of production of creatinuria versus calculated description of potential maximum rate of peroxidation of the dietary *in vivo*. Circles—group fed basal ration; triangles—group fed basal ration plus 0.13 ppm selenium and 0.4% *dl* methionine. $EP_o = [(\%\ \text{monoenoate} \times 0.025) + (\%\ \text{dienoate} \times 1) + (\%\ \text{trienoate} \times 2) + (\%\ \text{tetraenoate} \times 4) + (\%\ \text{pentaenoate} \times 6) + (\%\ \text{hexaenoate} \times 8)]^{1/2}$.

Kinetics of Competitive Inhibition of Lipid Peroxidation

The equation:

$$d[O_2]/dT = K_1K_3[RH]/K_4[AH] \quad (7)$$

is usually used to express the rate of the antioxidant inhibited autoxidation *in vitro* (26). Lips (40) has studied the relative efficacy of tocopherol as an antioxidant for pure methyl oleate, linoleate, and linolenate *in vitro*. Those quantities of α-tocopherol found to be just sufficient to prevent rapid peroxidation were in the approximate ratios 1:5:9 for methyl oleate, linoleate and linolenate, respectively. Since K_4 (Fig. 1c, d, e) is a constant for a given antioxidant under a given set of conditions, the tocopherol requirement, [RH]/[AH] generated by the various fatty acids, i.e. monoenoic, dienoic, etc. may be evaluated from the ratio, K_3/K_4, of the rates of the competing reactions (Fig. 1c, d, e).

The quantities of peroxidized lipid produced in the tissues at the time of onset of creatinuria, T_o, in the unsupplemented rat and T_E in the animal supplemented with α-tocopherol at the dietary level E_c in (mg *d*-α-tocopheryl acetate/kg rat body weight/week), are thought to be identical. The antioxidant does not actually prevent lipid peroxidation, but rather minimizes the number of molecules of fatty acid hydroperoxide formed per free-radical initiation. If K_1 (Fig. 1a, b), the rate of free-radical initiation, is constant, it follows that $T_E - T_o$ is a measure of the number of additional free-radical initiations required to produce this quantity of peroxidized lipid in the presence of a quantity of antioxidant [AH] proportional to E_c. It should be possible to maintain the tissue level of antioxidant [AH] relatively constant by continuous tocopherol supplementation in the animal experiments Thus it would seem possible to preclude the argument that $T_E - T_o$ is a measure of the number of additional free-radical initiations required to destroy, via the reaction with fatty acid peroxy free-radicals (Fig. 1c, e), the tocopherol in the tissues and that thereafter the majority of the peroxidized

lipid is then produced according to the kinetics of uninhibited peroxidation.

In vitro the expression:

$$T_o^2/T_E = K_5/K_3\,[RH]\,K_4\,[AH] \qquad (8)$$

produces a constant ratio for a given antioxidant (26). Diffusion in the fluid membrane and the necessity of considering fatty acid mixtures has been discussed in the previous section. In the present study, it is also necessary to consider the competition of each fatty acid individually with tocopherol as a proton donor to a peroxy free-radical. Therefore:

$$\frac{K_3\,[RH]}{K_4\,[AH]} = \frac{\left(\frac{a}{m}K_{3a}\right)^{1/2}\left(\frac{a}{m}\right)^{1/2}}{(E_c\,K_4)^{1/2}(E_c)^{1/2}} + \frac{\left(\frac{b}{m}K_{3b}\right)^{1/2}\left(\frac{b}{m}\right)^{1/2}}{(E_c\,K_4)^{1/2}(E_c)^{1/2}} \qquad (9)$$

Witting and Horwitt (27) found that $E_c/(T_E-T_o)$ was constant for each dietary fat, if the diet (Series B) also contained added selenium (0.13 ppm as sodium selenite) and methionine (0.4% *dl*). The experimental data are described by the equation:

$$E_c/T_E-T_o = \frac{\frac{a}{m}\left(\frac{K_{3a}}{K_5}\right)^{1/2} + \frac{b}{m}\left(\frac{K_{3b}}{K_5}\right)^{1/2}}{(K_4)^{1/2}} \qquad (10)$$

From the data in reference 27, it is possible to generate the following series of simultaneous equations.

$$K_5^{-1/2}\,[0.811\,(K_{3a})^{1/2} + 0.072\,(K_{3b})^{1/2}] = E_c/(T_E-T_o) = 0.023$$

$$K_5^{-1/2}\,[0.212\,(K_{3a})^{1/2} + 0.360\,(K_{3b})^{1/2}] = 0.056$$

$$K_5^{-1/2}\,[0.100\,(K_{3a})^{1/2} + 0.084\,(K_{3b})^{1/2} + 0.231\,(K_{3c})^{1/2}] = 0.060$$

$$K_5^{-1/2}\,[0.206\,(K_{3a})^{1/2} + 0.022\,(K_{3b})^{1/2} + 0.017\,(K_{3c})^{1/2} + 0.023\,(K_{3d})^{1/2} + 0.056\,(K_{3e})^{1/2} + 0.052\,(K_{3f})^{1/2}] = 0.054$$

Values cited for $E_c/(T_E-T_o)$ are the averages of 1, 5, 8 and 5 supplemented groups of rats, respectively. The original description of this nutritional study stated that the value of T_o obtained by feeding the fat described in the first equation above was probably somewhat low (27). A better value for $E_c/(T_E-T_o)$ in this case would be 0.029.

It is neither necessary nor possible to solve directly for the last 3 unknowns in the above equations. Values obtained previously for K_3/K_5 in the uninhibited peroxidation studies, series B, may be used directly in these equations. If it is arbitrarily stated that a unit of dietary tocopherol will *delay* for a unit period of time the peroxidation *in vivo* of a unit of dietary linoleate, the relative quantities of tocopherol needed to delay for the same period of time the peroxidation of the same quantity of fatty acids containing 1 to 6 double bonds fall into the ratios $(.025)^{1/2}:(1)^{1/2}:(2)^{1/2}:(4)^{1/2}:(6)^{1/2}:(8)^{1/2}$ or 0.16:1:1.4:2:2.4:2.8, respectively. An interesting simplification is apparent if all of the values are multiplied by two. The series may then be rounded off to 0.3:2:3:4:5:6. In other words, the tocopherol requirement generated by feeding a given fatty acid, other than monoenes, is proportional to the number of double bonds in the fatty acid molecule.

Since K_4 is not a variable, it may be assigned the arbitrary value of 1. Any other antioxidant would then have a value greater or less than one depending on whether it was a better or worse antioxidant than α-tocopherol. For other antioxidants where penetration into the tissues and retention therein differed from α-tocopherol, the relation between E_c and [AH] would be effected. This is particularly true in the case of antioxidant such as N, N'-diphenylparaphenylenediamine (DPPD) that is distributed rather evenly throughout the body lipid rather than concentrated in mitochondrial and microsomal membrane lipid in the manner of α-tocopherol.

The calculations in terms of dietary fat composition described herein are possible because each dietary fat fed at a level of 7.5% or higher to weanling rats, rapidly produced a distinct, but constant, equilibrium tissue lipid composition (27). Since adipose tissue may contain a sizeable reservoir of PUFA, usually linoleate which is slow to turn over, the kinetic treatment described herein is not applicable to non-equilibrium states or to older animals having large stores of adipose tissue.

The response to tocopherol supplementation, delay in onset of creatinuria, was not the same when the diet contained low levels of selenium and sulfur amino acids. At a series of suboptimum levels of selenium addition (28), low levels of tocopherol supplementation stimulated growth but did not delay the onset of creatinuria. Between 1.2–2.4 mg *d*-α-tocopherol acetate/kg rat/week, there was a sudden dramatic delay in the onset of creatinuria, and the dose response curves approached those seen at relatively optimum levels of selenium supplementation. Apparently then, the tissue damage producing creatinuria is to some measure distinct, via organo-selenium, from other tissue damage restricting growth.

Discussion

The working hypothesis derived from the hypothetical reaction scheme describing the interrelationship of PUFA, α-tocopherol, biologically available selenium and sulfur amino acids in the living animal successfully describes only a portion of the experimental data and therefore should not be taken too literally. It does, however, tend to support the antioxidant function of vitamin E. Under certain specific, empirical conditions the rate of production of a specific tocopherol-deficiency sign, creatinuria in the rat, and the rate of "disappearance" of PUFA from the muscle phospholipids may be related to the magnitude of n in $CH_3(CH_2)_a(CH=CHCH_2)_n(CH_2)_bCO_2H$ in either the tissue lipid fatty acids or the dietary lipid fatty acids in a rather simple fashion. Under these conditions, dietary, biologically available selenium acts, at least in part, in a manner similar to a lipid antioxidant *in vivo*. Using a diet containing specific levels of added selenium and methionine, the tocopherol requirement of the rat, in terms of delay in onset of creatinuria, may be expressed in terms of the magnitude of n in $CH_3(CH_2)_a(CH=CHCH_2)_n(CH_2)_bCO_2H$ in the dietary lipid fatty acids. Data obtained at suboptimum levels of dietary selenium and sulfur amino acids and low levels of α-tocopherol supplementation, however, indicate a potentiation of tocopherol activity by these non-lipid dietary constituents. Furthermore, selenium and sulfur amino acids enhance a growth response at low levels of tocopherol supplementation, an effect which is, in some manner, distinct from the tissue damage which produces creatinuria. Here, therefore, lies the area of interaction requiring elucidation.

Only by separating what is known from what is not known according to some tentative overall scheme is it possible to design experiments applicable to the solution of this extremely complex interrelationship. Since relatively few of the myriad biological variables have been considered herein, the conclusions may be of only academic interest. Any experimental condition or dietary alteration which in any way changes membrane permeability (exposure to ionizing radiation, hyper-or hypoxia, various toxic substances such as carbon tetrachloride or certain organo-phosphorus compounds, choline deficiency, various dietary trace metals or necrosis of unspecified origin) might effect lipid peroxidation *in vivo*.

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Analytical Separation of Nonlipid Water Soluble Substances and Gangliosides from Other Lipids by Dextran Gel Column Chromatography

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Abstract

A column chromatographic procedure is reported utilizing a dextran gel (Sephadex) for the complete separation of the major lipid classes from water-soluble nonlipids. Lipids other than gangliosides are eluted first with chloroform/methanol 19/1 saturated with water, gangliosides with chloroform/methanol/water containing acetic acid, and water-soluble nonlipids with methanol/water 1/1. Results for adult human whole brain, grey and white matter, and normal infant whole brain lipids are presented. With beef brain lipid as sample the ganglioside fraction is essentially pure, but with human brain lipid samples only about 70% of the second fraction is ganglioside. All ganglioside and water soluble nonlipid of a human spleen chloroform/methanol extract was separated from lipids with the procedure. Control studies with $P^{32}O_4\equiv$ and C_{14} labelled glucose showed that all counts were present in fraction 3. Similar studies with C_{14} labelled amino acids (glycine, serine, alanine, phenylalanine) showed that only phenylalanine counts were eluted in fraction 2 along with the gangliosides. The procedure was applied for removal of large amounts of ammonium acetate from DEAE cellulose column fractions and for complete removal of adsorbent and salts from lipids eluted from thin-layer chromatograms. After passage through the dextran gel columns, lipids eluted from thin-layer chromatograms were found to give infrared spectra identical to those of pure samples obtained by other procedures.

Introduction

WATER-SOLUBLE, NONLIPID substances (sugars, amino acids, and inorganic salts, etc.) are invariably extracted from tissues along with lipids. It is necessary to remove nonlipids from lipids for determination of total lipid by weighing and to prevent contamination of column chromatographic fractions with nonlipids. A convenient, rapid, quantitative means is also desirable for removal of salts introduced into lipid preparations by various laboratory procedures. Gangliosides, the complex group of water soluble lipids, occur in some organs (particularly brain) and present an additional problem for it is desirable to isolate gangliosides free of other lipids and nonlipids for quantitative determination of total gangliosides.

Dialysis, solvent partition, cellulose column chromatography, and DEAE cellulose column chromatography have all been used for separation of lipids from nonlipids (4-8). The recent report of Wells