Biochemical Pharmacology, Vol. 45, No. 11, pp. 2295–2301, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

OXIDATION OF α-TOCOPHEROL IN SUBCELLULAR FRACTIONS FROM RAT BRAIN AND ITS POSSIBLE INVOLVEMENT IN NERVE FUNCTION

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(*Received* 11 *November* 1992; *accepted* 8 *February* 1993)

Abstract—The turnover rate of vitamin E is slow in nerve tissue. Therefore, we have developed *in vitro* techniques to study the biochemical reactions of this nutrient in brain. Subcellular fractions were isolated from the cerebral hemispheres of 4-month-old, male, Fisher 344 rats. Aliquots of fractions (500 μg protein) were suspended in 50 mM phosphate buffer at pH 7.4 and incubated at room temperature (20–22°) or 37° for 2 hr in the presence or absence of the following oxidizing agents: 1 mM tertiary butyl hydroperoxide, 10 μM linoleic acid hydroperoxide, 0.5 to 50 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAPH) or 0.1 to 2 mM 2,2′-azobis (2,4-dimethyl) valeronitrile (ABDVN). The latter two compounds generate free radicals upon heating. After oxidation, the subcellular fractions were sedimented, saponified and assayed for tocopherol by liquid chromatography. Linoleic acid hydroperoxide was the most potent oxidizing agent, suggesting that endogenous fatty acid peroxides (e.g. eicosanoid intermediates) are very powerful oxidizing agents. Vitamin E may play an important role in providing antioxidant protection for membranes against excessive oxidation induced by these peroxides. Tocopherol in mitochondria and microsomes was much more susceptible to oxidation than synaptosomal tocopherol. The possible reasons for this observation are: (a) mitochondria and microsomes may contain less of the other reducing agents such as sulfhydryl compounds than synaptosomes, and/or (b) the electron transport structures in the former two subcellular fractions may be facilitating oxidation of tocopherol induced by free radicals. A portion of tocopherol remained unoxidized in all subcellular fractions even at high concentrations of ABAPH, suggesting that tocopherol exists in labile and nonlabile biochemical compartments or complexes.

Many experimental and clinical studies show that vitamin E (tocopherol) is important for the normal function of the nervous system. For example, Nelson [1] showed that vitamin E deficiency in animals and humans results in degeneration of posterior columns, axonal dystrophy (especially in the gracile and cuneate nuclei), and distal axonapathy in the peripheral (mainly sensory) nerves. The mechanism of pathogenesis of these neuropathological manifestations of vitamin E deficiency is not well understood. A study of the biochemical reactions of tocopherol within brain is essential for an understanding of the neurochemical mechanism of action of vitamin E. Such studies have been difficult to accomplish because of the complex biological interactions between vitamin E and other endogenous antioxidant substances such as sulfhydryl compounds, as well as other nutrients like selenium, β-carotene and vitamin C [2, 3]. Our approach to simplify the problem has been to study the biochemical reactions of tocopherol *in vitro*. Therefore, we have developed conditions for the study of tocopherol oxidation in cellular elements (human platelets) and membranes (human red cell membrane) [3, 4]. Incubations of the latter biological materials with various oxidants, such as hydrogen peroxide, tertiary butyl hydroperoxide and fatty acid hydroperoxide, result in oxidation of vitamin E. Data in this report will show that oxidation of tocopherol present in subcellular fractions from rat brains can also be studied *in vitro* under similar conditions. In addition to hydroperoxides, free radicals generated by the thermal decomposition of 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAPH†; water-soluble) and 2,2′-azobis (2,4-dimethyl) valeronitrile (ABDVN; lipid-soluble) also can induce tocopherol oxidation in subcellular fractions from rat brain.

The redox properties of α-tocopherol which form the basis of the antioxidant function of this compound have been studied for a number of years. The chemical relationships between tocopherol, tocopheroxide (tocopherone), tocopherolhydroquinone and tocopherolquinone were established by Harrison *et al.* [5]. The structural formulae for the various tocopherol derivatives are illustrated in Fig. 1. The reaction of peroxy compounds with α-tocopherol leads to a number of products. Goodhue and Risley [6] showed that oxidation of *d*-α-tocopherol by benzoyl peroxide in the presence of alcohols resulted in the formation of 8a-alkoxytocopherones. The chemistry and occurrence of such compounds had been reported earlier by

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† Abbreviations: ABAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; and ABDVN, 2,2′azobis (2,4-dimethyl) valeronitrile.

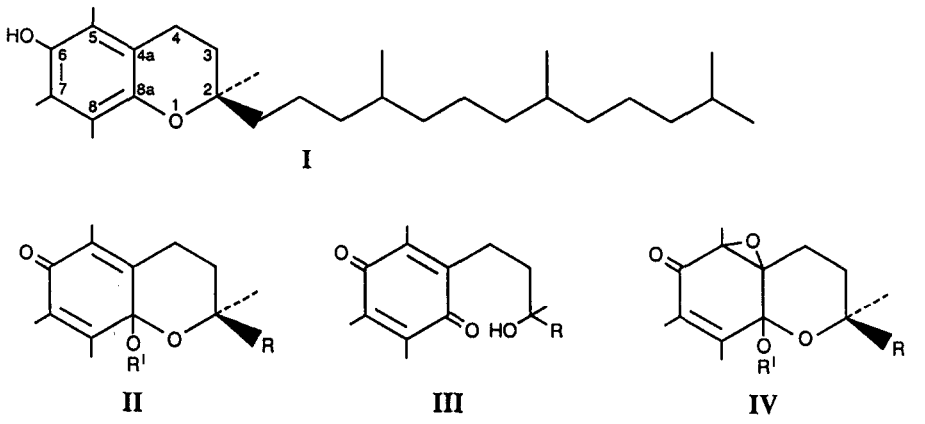

Fig. 1. Structures of α-tocopherol and a few related compounds. The various compounds are: I = α-tocopherol; II = 8a-(alkyldioxy)tocopherone; III = tocopherolquinone; and IV = epoxy derivative of II.

Dürckheimer and Cohen [7]. More recent work by Matsuo *et al.* [8] showed that in the presence of a system that generates *tert*-butylperoxy radical, α-tocopherol is converted, in part, to an epoxy derivative (IV). Liebler *et al.* [9] have confirmed that oxidation of vitamin E by peroxy radicals yields 8a-(alkyldioxy)tocopherones (II), which can then hydrolyze to tocopherolquinone (III). Almost all of the earlier studies deal with vitamin E oxidation in pure chemical medium. The experiments reported in this paper were designed to study the oxidation of vitamin E in a simplified system containing vitamin E in its normal biological environment in nerve tissue membranes. Subcellular fractions were isolated from brain, and the oxidation of vitamin E within the membranes of these fractions was studied.

MATERIALS AND METHODS

Chemicals. All chemicals used were of reagent grade purity from standard sources. Solvents used for chromatography were HPLC grade from Burdick & Jackson Laboratories, Inc., Muskegon, MI. Other special chemicals used were purchased from the following sources: α-tocopherol from Kodak Laboratory Chemicals, Rochester, NY; hydroperoxy linoleic acid (13*S*-hydroperoxy) from Oxford Biomedical Research Inc., Oxford, MI; sucrose (grade 1) from the Sigma Chemical Co., St. Louis, MO; and ABAPH and ABDVN from Polysciences, Inc., Warrington, PA. Absolute ethanol was obtained from the Midwest Solvents Co., Pekin, IL, and was redistilled prior to use.

Biochemical assays. All experiments were repeated and confirmed with brain samples from separate animals. Data from typical experiments are reported. The concentration of total protein was determined by the Lowry technique as modified by Markwell *et al.* [10].

Liquid chromatography of tocopherol and quinone. The liquid chromatographic method for determination of tocopherols was a modification of our published procedure [4]. Briefly, 2 mL ethanol containing 0.025% butylated hydroxytoluene (BHT) and 0.1 mL of 30% ascorbic acid were pipetted into the tubes containing samples for tocopherol analyses. The mixture was saponified at 60° for 30 min after the addition of 1 mL of 10% potassium hydroxide solution. The tubes were cooled and 2 mL of water was added followed by 2 mL of hexane containing 0.025% BHT. Tocopherol and quinone were extracted into the hexane phase by vigorous mixing using a vortexer for 1 min. Hexane phase was separated out and evaporated down under a stream of nitrogen. The residue was redissolved in mobile phase and analyzed by reverse phase liquid chromatography (ultrasphere ODS, 5 μm 4.6 × 250 mm, Beckman Instruments; mobile phase = methanol:water, 98:2). The tocopherols were detected by their native fluorescence (excitation 295 nm, emission 340 nm) and tocopherolquinone by its ultraviolet absorption at 265 nm.

Preparation of subcellular fractions from rat brain. Four-month-old, male Fisher 344 rats were used. The subcellular fractions were isolated from cerebral hemispheres by standard centrifugation methods [11]. Tissue was homogenized in 10 vol. of ice-cold 0.32 M sucrose using a teflon–glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant saved. The pellet was resuspended in 10 mL of 0.32 M sucrose, rehomogenized and centrifuged at 1000 *g* for 10 min. The pooled supernatants were centrifuged at 20,000 *g* for 30 min and the resulting supernatant was saved for the isolation of microsomes. The pellet was washed with 20 mL of 0.32 M sucrose and centrifuged at 20,000 *g* for 30 min. The resulting pellet was resuspended in 12 mL of 0.32 M sucrose, layered over 12 mL each of 0.8 M and 1.2 M sucrose and centrifuged in a Beckman SW 28 rotor at 64,000 *g* for 35 min. The 0.8 to 1.2 M interface was removed, diluted 1 : 2 with cold nanopure water, and centrifuged for 30 min at 27,000 *g* to isolate the synaptosomes. The mitochondrial pellet at the bottom of the

gradient centrifugation tube (under 1.2 M) was taken out and resuspended in 0.32 M sucrose and centrifuged down at 27,000 *g* for 30 min. The washed mitochondria were then used for the oxidation experiments. The earlier supernatant, which was saved for isolation of microsomes, was centrifuged for 60 min at 100,000 *g* to obtain microsomal fraction. The purities of the subcellular fractions were checked using electron microscopy and assays of marker enzymes.

Protocol for incubations. The subcellular fractions (500 μg total protein in a volume of 1 mL) were suspended in 50 mM potassium phosphate, pH 7.4, and incubated at room temperature for 60 min after the addition of hydroperoxide. The reaction mixture was centrifuged for 75 min at 35,000 rpm in a Beckman Ti 50 rotor (81,000 *g*) to sediment the microsomal fraction or for 30 min at 17,000 rpm in Sorvall SS 34, (35,000 *g*) for mitochondria and synaptosomes. The sedimented fractions were then analyzed for tocopherol. The ABAPH and ABDVN incubations were done at 37° for 2 hr and employed the same buffers and the same amount of total protein in each tube. ABDVN was dissolved in ethanol and then added to the medium (final ethanol concentration of 2%). The reaction was stopped by cooling the suspensions in an ice bath. The subcellular fractions were sedimented and analyzed for tocopherol as before.

RESULTS

The experimental procedures of homogenization and isolation of subcellular fractions are lengthy and tocopherol loss by oxidation during this process is possible. However, in a typical experiment the total yields of proteins and tocopherol in all the recovered fractions and washes were 84 and 78%, respectively, suggesting that there was little selective loss of tocopherol during the isolation of the subcellular fractions.

The α-tocopherol content per unit protein for each fraction was characteristic for the particular subcellular fraction. These values were consistent between experiments. The concentrations of α-tocopherol per mg protein in a typical experiment were: 0.34, 0.65, and 0.37 nmol for mitochondria, microsomes and synaptosomes, respectively. Control incubations for 2 hr with no oxidizing agent resulted typically in the oxidation of 25, 14 and 15% of the total tocopherol in mitochondria, microsomes, and synaptosomes, respectively. It should be noted that this loss also includes physical loss of material during processing of fractions. Peroxides and free radical generators induce oxidation above this baseline oxidation. The extent of such induced oxidations was calculated by subtracting baseline oxidations from the observed total oxidation.

The spontaneous oxidation of α-tocopherol in mitochondrial and synaptosomal fractions was compared. Aliquots of these fractions were incubated at 37° for various times extending up to 2 hr. The results (Fig. 2) show that tocopherol in mitochondria was much more susceptible to oxidation than that in synaptosomes.

The oxidation of tocopherol within membranes of subcellular particles was studied using linoleic acid hydroperoxide (10 μM final concentration), tertiary butyl hydroperoxide (1 mM) and ABAPH (1 mM). When challenged with any of these oxidants, tocopherol in mitochondria or microsomes underwent much more oxidation compared with synaptosomal tocopherol (Fig. 3). Thus, the mitochondrial tocopherol was more labile under mild (spontaneous, Fig. 2) or strong (free radical-induced, Fig. 3) conditions of oxidation. Linoleic acid hydroperoxide was a much more potent oxidizing agent compared with the exogenous agents, tertiary butyl hydroperoxide and ABAPH.

The amount of tocopherolquinone produced from the oxidations was also estimated simultaneously in all experiments. The percent conversion of α-tocopherolquinone tended to be quite variable and ranged between 12 and 45%. Some tocopherolquinone was always produced after incubation, suggesting that an oxidative reaction occurred within the membranes under the *in vitro* conditions used. In an experiment where the different subcellular organelles were incubated with various concentrations (2, 20 and 40 mM) of ABAPH, the percent conversion of tocopherol to the quinone decreased as the ABAPH concentrations were increased. However, incubations of α-tocopherolquinone with ABAPH in pure solutions showed no direct reaction between the two compounds. Therefore, it is possible that an increased concentration of free radicals from higher ABAPH concentrations may result in more of the tocopheroxyl radicals combining directly with the free radicals from ABAPH. This would be expected to be associated with a decrease in yield of quinone with increasing ABAPH concentration.

Since the liberation of free radical species from ABAPH can be precisely controlled, further experiments were done with this oxidizing agent. The isolated subcellular fractions were incubated with ABAPH over a wide range of concentrations (0.5 to 50 mM). These experiments involving a number of ABAPH concentrations and three subcellular fractions were lengthy and, therefore, the data from oxidations done on different days had to be pooled. To ensure that data were collected under very similar conditions, all subcellular fractions were adjusted to contain 500 μg of protein and animals from one individual batch raised on the same batch of diet were used. The dependence of the percent of tocopherol oxidized upon ABAPH concentration in the medium is illustrated in Fig. 4A for the synaptosomal fraction. Similar curves were observed for the microsomal and mitochondrial fractions. The curves exhibited logarithmic relationships. For example, the equation for the curve in Fig. 4A was $y = 22.64 + 29.66 \log X$ ($R^2 = 0.908$). More than 50% of the α-tocopherol was oxidized at ABAPH concentrations of less than 10 mM and an increase in concentration of ABAPH to 20 and 50 mM did not increase tocopherol oxidation in proportion to the increase in ABAPH concentration. Furthermore, a substantial amount of tocopherol (about 35%) tended to remain unoxidized at the higher concentrations. With mitochondria and

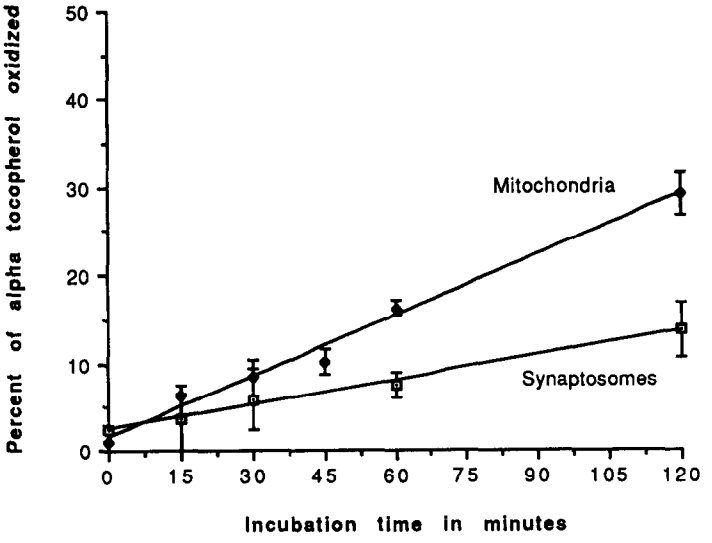

Fig. 2. Comparison of the rates of spontaneous oxidation of α-tocopherol upon incubation of mitochondrial and synaptosomal fractions from rat brains. Rat brain synaptosomal and mitochondrial fractions were isolated by standard centrifugation techniques and were incubated at 37°. The individual points shown are means ± SD (N = 4).

microsomes, the unoxidized portions of tocopherol were roughly 30 and 25%, respectively.

In a separate experiment the subcellular fractions isolated were incubated with 1, 3 and 10 mM ABAPH in the presence of sodium dodecyl sulfate. At the highest concentration of ABAPH, nearly all (93–95%) of the tocopherol was oxidized with the three fractions. Thus, the tocopherol becomes available for oxidation upon solubilization of membrane components with a detergent.

The initial portion of the curve in Fig. 4A was examined in more detail. If the logarithms of ABAPH concentration (mM) were plotted against the percent of tocopherol oxidized, a straight line relationship [formula $y = 19.61 + 44.71\,X$ ($R^2 = 0.969$)] was observed (see Fig. 4B). Similar straight line relationships were seen with microsomes and mitochondria also. The ABAPH concentrations that would cause the oxidation of 50% of α-tocopherol in each fraction were calculated to be 4.78, 1.48 and 1.89 mM for synaptosomes, mitochondria and microsomes, respectively. The concentrations of α-tocopherol in these fractions were different, as expected. To account for these differences in the amounts of tocopherol in each fraction, the concentration of ABAPH required to achieve 50% oxidation was divided by the respective tocopherol contents of the fraction. The concentrations of ABAPH required to oxidize 50% of tocopherol in synaptosomes, mitochondria and microsomes were then calculated to be 30.6, 11.0 and 9.2 mM ABAPH per nmol α-tocopherol, respectively. By either calculation, α-tocopherol in mitochondria and microsomes tended to be much more susceptible to oxidation by ABAPH when compared with synaptosomes.

The subcellular fractions were also incubated with the lipid-soluble initiator ABDVN. In a typical experiment with rat brain synaptosomes, the concentration of ABDVN required to oxidize 50% of the total synaptosomal α-tocopherol was 0.85 mM, which was considerably less than that for ABAPH (4.78 mM, see above paragraph).

DISCUSSION

Tocopherol is localized within the membranes of biological tissues. The major factor responsible for this localization is that the molecule is very hydrophobic and has an affinity for the lipid bilayer portion of membranes. Various attempts have been made to study the turnover and metabolism of this compound. The experimental techniques involve monitoring the fate of tocopherol with labeled molecules containing radioactive or stable isotopes. The latter technique was pioneered by Burton, Ingold and colleagues who estimate that the half-life of tocopherol is approximately one month in rat brain and more than three and a half months in guinea pig brain [12]. The half-lives reported by these authors are defined as the times required for the tissue to pick up or exchange half of its tocopherol with the ingested deuterium-labeled tocopherol. It is conceivable that the metabolic half-life of tocopherol in brain could be even longer in view of the well known protective interactions of tocopherol with other nutrients. For example, many of the symptoms of vitamin E deficiency in animals can be prevented by administration of selenium, synthetic antioxidants and sulfur amino acids [13], and vitamin C stabilizes tissue vitamin E concentrations [14]. Such interactions make it difficult to study the metabolism of tocopherols in brain. The experiments reported in this paper using *in vitro* techniques

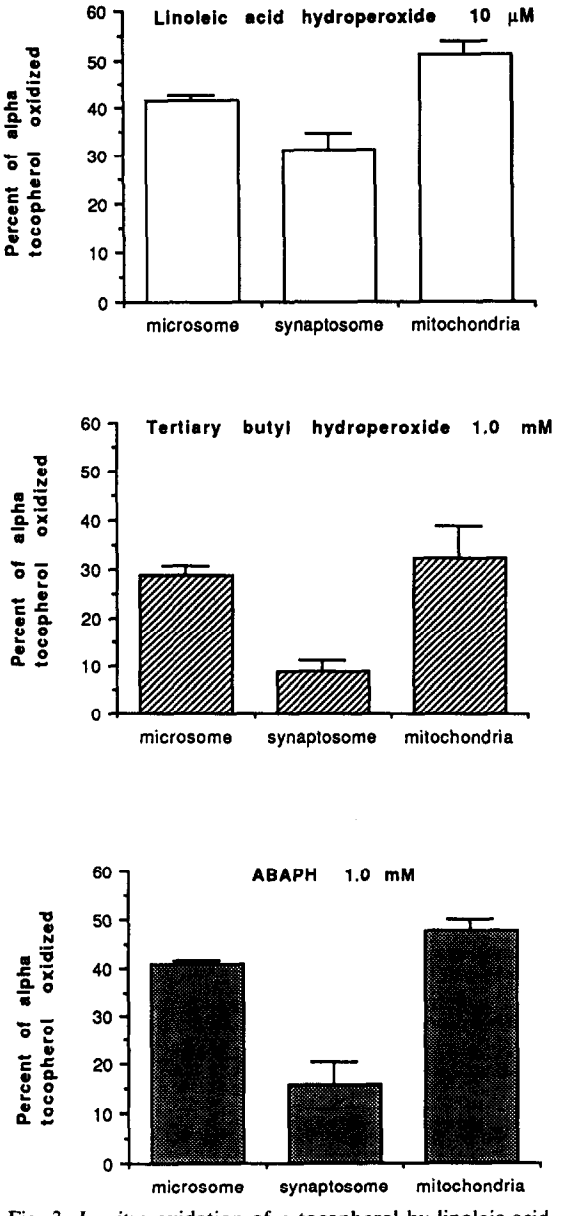

Fig. 3. *In vitro* oxidation of α-tocopherol by linoleic acid hydroperoxide (10 μM final concentration), tertiary butyl hydroperoxide (1 mM) and ABAPH (1 mM). Rat brain subcellular fractions were isolated by standard centrifugation techniques, and aliquots containing 500 μg protein were incubated at room temperature (hydroperoxides) or 37° (ABAPH) for 2 hr. α-Tocopherol concentrations in the fractions were determined by HPLC. The individual points shown are means ± SD (N = 4).

provide another approach to study the biochemical reactions of tocopherol in brain membranes. Oxidation of tocopherol and its modification by other endogenous antioxidants can be conveniently studied using this procedure in hours or days rather than weeks or months.

The tocopherol within the membranes of the subcellular fractions is oxidized upon incubation with tertiary butyl or linoleic hydroperoxides and ABAPH. We have demonstrated previously that tocopherol in blood platelets, red blood cell membranes and cerebrospinal fluid can also be oxidized under similar conditions [3, 4, 15]. The occurrence of an oxidative reaction was established by showing the production of tocopherolquinone in all experiments. The percent conversion of tocopherol to its quinone turned out to be quite variable and accounted for less than 45% of tocopherol oxidized. In most cases, the conversion yield was in the range of only 5–25%. Thus, most of the tocopherol oxidized is converted to other compounds that may include dimers and trimers of tocopherol [16].

Functionally, it is of great interest that linoleic acid hydroperoxide is the most potent of all oxidizing agents studied. Endogenous peroxides of unsaturated fatty acids like linoleic and arachidonic acids are produced under normal physiological conditions during eicosanoid metabolism. α-Tocopherol is present in membranes where the eicosanoid peroxides are formed. Therefore, tocopherol could play a significant role in controlling the level of these endogenous peroxides and prevent their accumulation to levels high enough to cause oxidative damage to membranes.

The calculated concentrations of ABAPH in the incubation medium that would result in 50% oxidation of tocopherol were much lower for microsomes and mitochondria compared to synaptosomes (see Results). Therefore, microsomes and mitochondria are more susceptible to oxidation by ABAPH than synaptosomes. The reasons for the enhanced oxidizability of tocopherol in mitochondria and microsomes could be that these two subcellular fractions may contain less endogenous reducing agents and/or that the electron transport enzymes and cofactors present may be facilitating oxidations induced by free radicals.

A somewhat unexpected finding was that a significant portion of the total tocopherol within the subcellular fractions was not oxidized even in the presence of very high concentrations of ABAPH (Fig. 4A). Melhorn *et al.* [17] have also found that when liver microsomes and mitochondria are oxidized by enzymatically generated free radicals, a portion of the tocopherol remained unoxidized. One of the most likely explanations of this phenomenon could be that tocopherol within the membrane occurs in at least two separate biochemical compartments with different turnover rates. It is interesting to note that the presence of different pools of tocopherol within tissues was reported by Bieri [18] who examined the rate of depletion of α-tocopherol from plasma, liver, heart, testis and muscle tissues of the rat. The conclusion was that tocopherol existed in labile and nonlabile compartments in these tissues. Our experiments show that different compartments of tocopherol occur at the subcellular membrane level as well. These compartments are obliterated by treatment with sodium dodecyl sulfate (see Results), which is known to have complex interactions with membranes including solubilization of membrane proteins [19]. Analogous to our results on tocopherol, it has been reported that hydrophobic

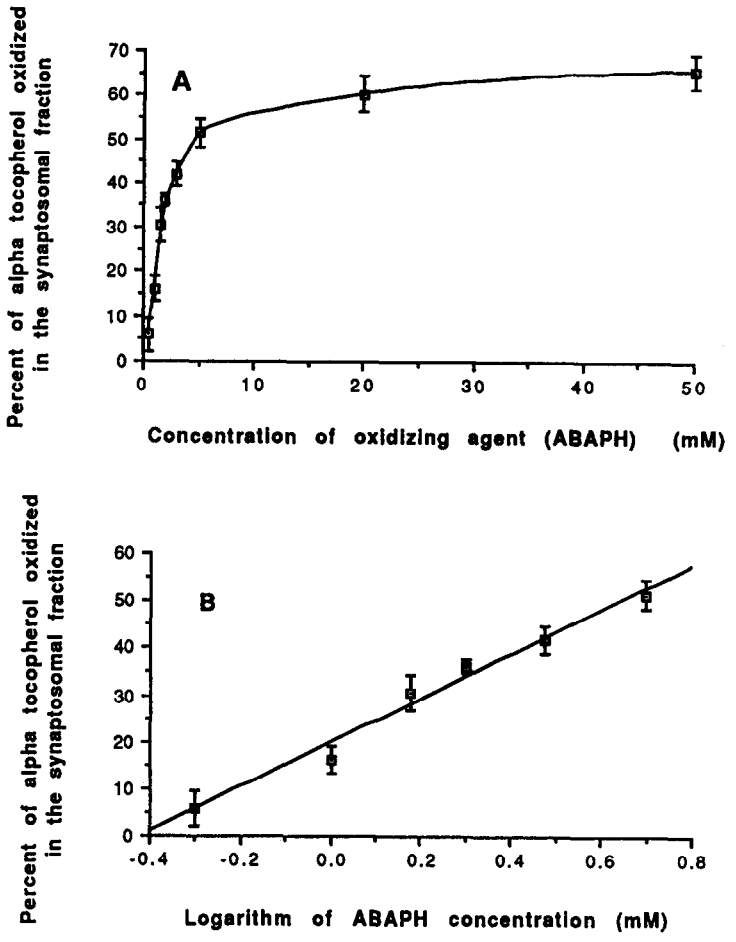

Fig. 4. (A) Dependence of the extent of oxidation of synaptosomal α-tocopherol upon the concentration of ABAPH in the incubation medium. Rat brain synaptosomal fractions were isolated by standard centrifugation techniques. Aliquots (500 μg protein) were incubated at 37° with ABAPH (0.5 to 50 mM) for 2 hr and the reaction was stopped by cooling. Synaptosomes were centrifuged down and analyzed for α-tocopherol by HPLC. (B) Oxidation of synaptosomal α-tocopherol by ABAPH in 0.5 to 10 mM final concentration. The individual points shown are means ± SD (N = 4).

molecules in the lipid bilayer of membranes are not homogeneously distributed. For example, individual lipids have been known to partition into discrete pools within biological membranes. Lipids show asymmetric distribution in a plane transverse to the bilayer, and asymmetric localization within the lateral plane of the bilayer results in lateral phase separation or formation of lipid domains [20]. Using fluorescent analogs of sterols, Nemecz *et al.* [21] have observed that cholesterol exists in three pools of widely differing half-lives in unilamellar liposomal vesicles. It is quite likely that tocopherol molecules are interacting with the lipid bilayer in a manner similar to cholesterol and partition into compartments of different half-lives. Some of the tocopherol may be found within a protein–lipid complex inside the membrane. Alternately, tocopherol may be sequestered within inaccessible portions of the membranes such as the inner mitochondrial membrane. Whatever the mechanism may be, our observations show that tocopherol occurs in labile and nonlabile compartments within membranes of subcellular organelles from brain. This observation could be true for membranes from other tissues as well.

In conclusion, the experiments reported here show that the *in vitro* incubation of membranes or subcellular fractions is a convenient and fast method for studying the oxidation of vitamin E in brain. The high potency of linoleic acid hydroperoxide in oxidizing vitamin E suggests that endogenous eicosanoid peroxides could cause membrane damage if their levels are not kept under tight control. Vitamin E could play a major role in buffering the concentrations of membrane peroxides. Finally, the special susceptibility of mitochondrial tocopherol to oxidation indicates that vitamin E may be crucial for maintenance of mitochondrial function and thus is essential for normal maintenance of nerve tissue that is critically dependent upon energy supply from mitochondria. It is possible that deficient antioxidant protection by compounds such as vitamin E may

play a role in the pathogenesis of degenerative brain diseases such as Parkinson's disease where abnormal peroxidation and mitochondrial function have been reported [22, 23].

Acknowledgements—This work was supported by research funds from the Department of Veterans Affairs and the Parkinson's Disease Foundation. The author thanks Mr. W. Ed Smith and Mr. Hung T. Quach for expert technical assistance.

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