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FORMATION OF α-TOCOPHEROL COMPLEXES WITH FATTY ACIDS

A HYPOTHETICAL MECHANISM OF STABILIZATION OF BIOMEMBRANES BY VITAMIN E

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The formation of α-tocopherol complexes with saturated and unsaturated fatty acids in ethanol has been demonstrated. The values of equilibrium constants for α-tocopherol interactions with fatty acids have been determined. These values do not depend in practice on the number of carbon atoms in saturated fatty acid molecules (from 7:0 to 24:0) and are equal to about 40–50 M^{-1}. For unsaturated fatty acids the values of equilibrium constants are increased exponentially with an increase in the number of double bonds in the fatty acid molecule ($1.25 \cdot 10^4$ M^{-1} for arachidonic acid). α-Tocopherol can form complexes with free fatty acids incorporated into phosphatidylcholine liposomes or into skeletal muscle sarcoplasmic reticulum membranes. The formation of α-tocopherol complexes with free fatty acids is regarded as a molecular mechanism of membrane stabilization by vitamin E against the damaging action of free fatty acids.

Introduction

Tocopherols (vitamin E) are known to be indispensable lipid components of biological membranes which provide for their structural and functional stability [1,2]. It has generally been believed that the stabilizing effect of tocopherols is mediated via three main molecular mechanisms, namely: (i) reaction with lipid peroxide radicals; (ii) quenching of singlet molecular oxygen; and (iii) ordering (i.e., restriction of molecular mobility) of the membrane lipid bilayer [3–7]. The first two mechanisms have been investigated in great detail and are commonly accepted [8,9], while the third one, being based on the structural properties of tocopherols, has become the subject of thorough attention only in the last few years.

The structural stabilization of biomembranes by vitamin E can be fulfilled in two ways; (i) by stabilization of the lipid bilayer via a Van der Waals interaction of tocopherols with unsaturated fatty acids (FA) of phospholipids, and (ii) by stabilization of the polypeptide chains of intrinsic proteins. Based on this assumption, the ability of tocopherols to stabilize lipid mono- and bilayers [9–11] and natural (i.e., lysosomal, erythrocyte, sarcoplasmic reticulum) membranes [12–14] has been determined experimentally. Recent studies have demonstrated a high efficiency of the stabilizing effect of α-tocopherol against the damages caused by free fatty acid [15]. This effect can be due either to the direct interaction of α-tocopherol with Ca^{2+}-dependent ATPase or to its interaction with free fatty acid. The aim of present work was to investigate the formation of α-tocopherol-fatty acid complexes as a molecular mechanism responsible for membrane stabilization by vitamin E.

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Materials and Methods

Egg phosphatidylcholine liposomes were prepared by injecting ethanol solution of lipids into 100 mM NaCl/2.5 mM imidazole buffer (pH 7.0) at 37°C. Incorporation of α-tocopherol and fatty acid into the liposomes was performed by injecting solutions into the liposome suspension. The final concentration of ethanol/liposome suspensions and fragments of sarcoplasmic reticulum did not exceed 1%. The fragments of rabbit skeletal muscle sarcoplasmic reticulum were obtained by differential centrifugation as described by Ritov et al. [16]. The ultraviolet absorption spectra were measured on a Beckman 35 spectrophotometer in 1 cm cuvettes. Fluorimetric assays were performed on a Perkin-Elmer MPF 44A spectrofluorimeter in round quartz microcuvettes ($r_{in} = 0.25$ cm). For experimental conditions see legends to the figures. The experimental results were treated statistically by the variation statistics method [17].

(±)-α-Tocopherol and phospholipids were purchased from Serva; fatty acids (95% purity) were from Sigma. Egg phosphatidylcholine was obtained from Reachim. The organic solvents were purified by distillation.

Results

In the first experimental series we investigated possible interaction of α-tocopherol with fatty acids in the simplest nonmembrane system, i.e., organic solvent. The ultraviolet spectra of the ethanol solution of α-tocopherol before and after addition of increasing concentrations of fatty acid are shown in Fig. 1. An addition of unsaturated fatty acid to an α-tocopherol solution leads to a decrease of the ultraviolet absorption maxima at 213–214 nm, but does not affect the absorbance at 293 nm, thus indicating an α-tocopherol interaction with fatty acid. Similar spectral changes also take place in isooctane solutions. The absorption changes at 200–220 nm caused by injections of synthetic fatty acid derivatives into an α-tocopherol solution in ethanol have been reported earlier [18]. These changes occur immediately after fatty acid injection and are not eliminated by subsequent incubation for 30–60 min. The absorption changes at 214 nm are also observed when saturated fatty acid

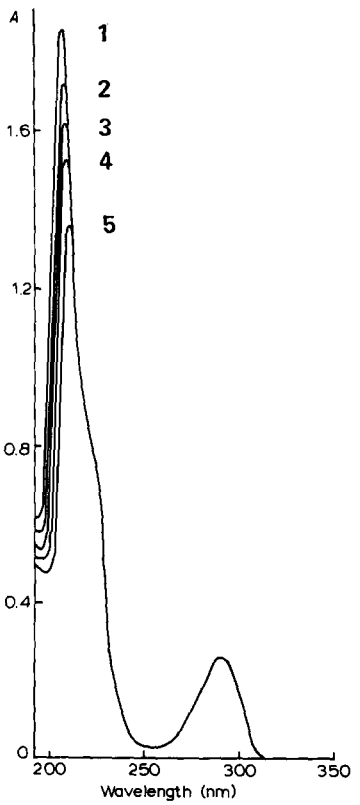

Fig. 1. Ultraviolet absorption spectra of α-tocopherol ($1 \cdot 10^{-4}$ M) in ethanol before (1) and after addition of increasing concentrations of linolenic acid (2–5). The reference sample contained the same amount of fatty acid as the control one.

(from 7:0 to 24:0) are added to an α-tocopherol solution in ethanol; at concentrations up to about $3 \cdot 10^{-2}$ M these changes depend linearly on the fatty acid concentrations used (Fig. 2a). Based on this dependence as well as on the fact that the equilibrium is reached practically instantaneously, we may now calculate the values of the apparent equilibrium constants for α-tocopherol interaction with fatty acid from the changes in the absorption spectra.

Thus, for an equilibrious system we obtain:

$$\alpha\text{-Tocopherol} + \text{fatty acid} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{complex}$$

Hence,

$$K = \frac{k_{+1}}{k_{-1}} = \frac{[\text{complex}]}{[\alpha\text{-tocopherol}] \cdot [\text{fatty acid}]} = \frac{A_0 - A}{A_0 \cdot [\text{fatty acid}]}$$

where A_0 and A are the absorbances at 214 nm in

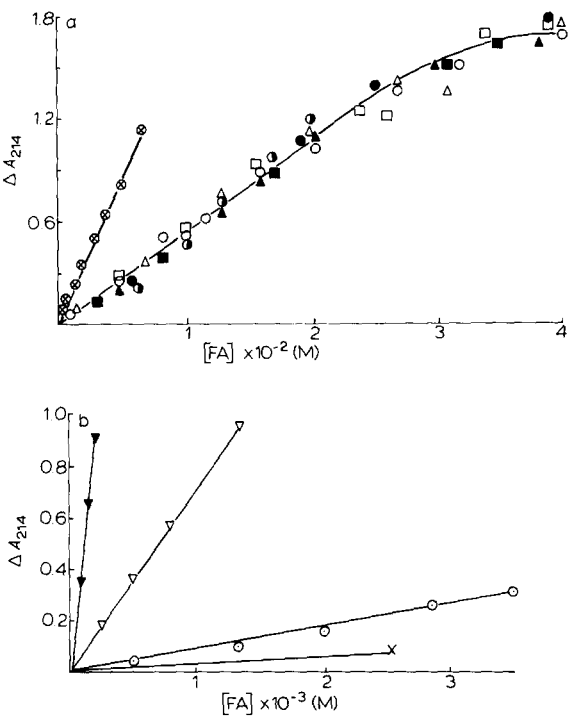

Fig. 2. Dependence of the changes in the absorbance of α-tocopherol ($1 \cdot 10^{-4}$ M) in ethanol at 214 nm on the amount of saturated fatty acid and dipalmitoylphosphatidylcholine (◑, 7:0; △, 8:0; □, 10:0, ■, 12:0; ○, 14:0; ●, 16:0; ▲, 24:0; ⊕, DPPC) (a) or unsaturated fatty acids (⊙, 18:1; ▽ 18:2; ▼, 20:4; ×, saturated fatty acid) (b).

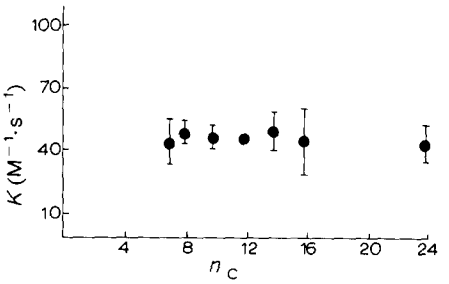

Fig. 3. Dependence of the value of the constant for α-tocopherol interaction with saturated fatty acid (K) in ethanol on the number of carbon atoms in a fatty acid molecule (n_C).

the absence and presence of fatty acid, respectively. The running concentrations of fatty acid depend on the experimental conditions.

Fig. 3 shows that the K value does not depend on the structure of saturated fatty acid when the number of carbons is increased from 7 to 24 and is about 44–50 M^{-1}. At the same time, this value rises drastically with an increase in the degree of unsaturation of fatty acid (Fig. 2b) and in the case of arachidonic acid reaches $1.25 \cdot 10^4$ M^{-1} (Table I). As can be seen from Fig. 4, log K is a linear function of the number of double bonds in a fatty acid molecule. Beside free fatty acid, α-tocopherol can interact with esterified fatty acid. The K value for methyl linoleate is of the same order of magnitude as that for linoleic acid. The data presented in Fig. 2a and Table I suggest that the corresponding value for dipalmitoylphosphatidylcholine is higher than the double K value for palmitic acid. This is indicative of a possible synergistic interaction between two acyl chains in the phospholipid molecule.

In the next experimental series we used a fluorescent technique to study the formation of α-tocopherol-fatty acid complexes. Taking into account that the efficiency of α-tocopherol interaction with fatty acid is sharply increased with an increase in their unsaturation, we used arachidonic acid (20:4) in these experiments. The excitation and emission spectra of α-tocopherol fluorescence in ethanol solution are shown in Fig. 5a. It can be seen that the α-tocopherol fluorescence excitation spectrum has a maximum at 295 nm; the band corresponding to 214 nm is absent. An addition of arachidonic acid to an ethanol solution of α-

TABLE I

VALUES OF THE CONSTANTS (K) AND LOGARITHMS OF THESE CONSTANTS (log K) FOR FATTY ACID INTERACTION WITH α-TOCOPHEROL IN ETHANOL AS CALCULATED FROM THE ULTRAVIOLET SPECTRA

DPPC, dipalmitoylphosphatidylcholine.

Fatty acid	K (M^{-1})	log K
12:0	46.0 ± 2.40	
16:0	45.6 ±18.0	1.62±0.18
18:1	$(1.08 \pm 0.26) \cdot 10^2$	2.03±0.10
18:2	$(9.24 \pm 0.96) \cdot 10^2$	2.93±0.05
20:4	$(1.25 \pm 0.28) \cdot 10^4$	4.10±0.09
20:4 [a]	$(4.79 \pm 2.10) \cdot 10^4$ [b]	
24:0	44.0 ± 9.20	
DPPC	$(1.52 \pm 0.09) \cdot 10^2$	

[a] Data from fluorimetric assays.
[b] $P < 0.05$.

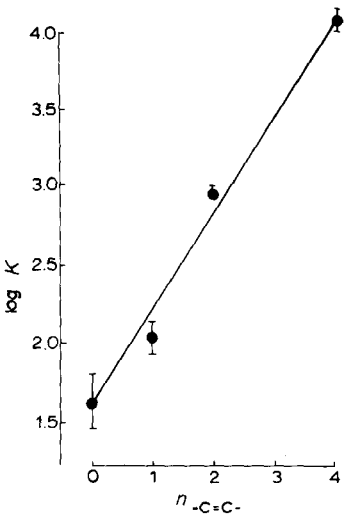

Fig. 4. Dependence of the logarithm of constant for α-tocopherol interaction with fatty acid (log K) in ethanol on the number of double bonds in a fatty acid molecule ($n_{-C=C-}$).

tocopherol results in a decrease in the intensity of α-tocopherol fluorescence excitable at 295 nm. Arachidonic acid exhibits no fluorescence at the indicated spectral values. The Stern-Folmer plots for the efficiency of fluorescence quenching versus arachidonic acid concentration are non-linear (Fig. 6). This suggests that the decrease of the fluorescence intensity is not due to a dynamical quenching. Taking into account that

$$((F_0/F)-1) = K\cdot[\alpha\text{-tocopherol}]\cdot[\text{fatty acid}],$$

where F_0 and F are the intensities of fluorescence in the absence and at a given concentration of fatty acid, respectively, we may now calculate the value of the constant for the α-tocopherol interaction with fatty acid from the known value of fluorescence quenching. For the initial part of the kinetic curve for α-tocopherol fluorescence quenching by arachidonic acid, K is equal to $(4.79 \pm 2.10)\cdot 10^4$ M^{-1}. This value is well coincident with that for the α-tocopherol interaction with arachidonic acid calculated from the measured absorption spectra within the same concentration range (Table I).

In the last experimental series we investigated the interaction of free fatty acid with α-tocopherol incorporated into artificial and natural membranes

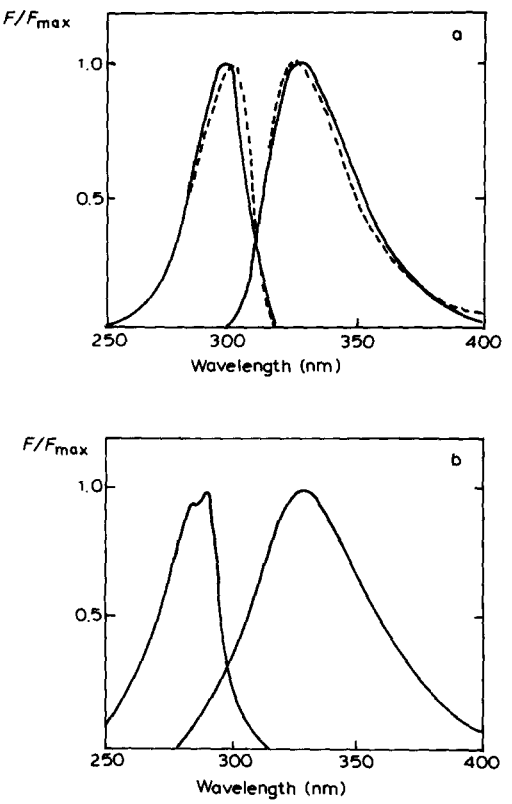

Fig. 5. (a) Normalized excitation and emission spectra of α-tocopherol fluorescence in ethanol (———) and in liposomes (- - - - -). α-Tocopherol concentration in ethanol is $1.25 \cdot 10^{-4}$ M; in liposomes, 50 μg per 0.5 mg of lipid per ml. $\lambda_{ex} = 290$ nm, $\lambda_{em} = 350$ nm. Spectral slit width, 2.5 nm. (b) Normalized excitation and emission fluorescence spectra of sarcoplasmic reticulum membrane suspension (0.5 mg of protein per ml). Spectral slit width, 2.5 nm.

by measuring the α-tocopherol fluorescence spectra. The excitation and emission spectra of α-tocopherol fluorescence in liposomes are shown in Fig. 5a. A comparison of these spectra with those measured in ethanol suggests that both the excitation and emission spectra are identical. A certain narrowing of these spectra in the liposomes is probably due to a lower mobility of the α-tocopherol molecules in the lipid matrix as compared to that in solution. An increase in the α-tocopherol molar ratio in the liposomes enhances the fluorescence intensity. The Stern-Folmer plots for α-tocopherol fluorescence quenching by arachidonic acid both in the liposomes and in ethanol are non-linear (Fig. 7).

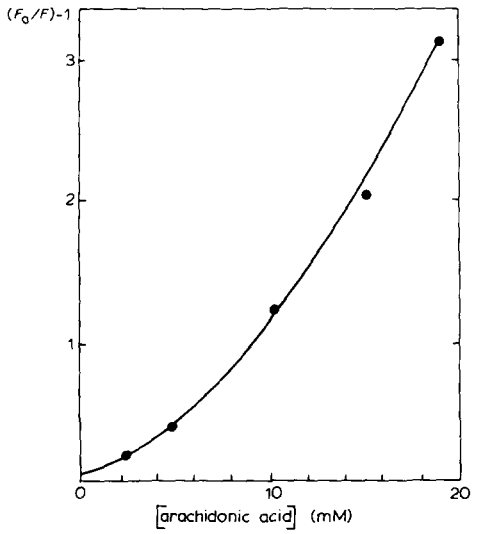

Fig. 6. Dependence of the intensity of α-tocopherol fluorescence quenching in ethanol on arachidonic acid concentration in the Stern-Folmer coordinates. α-Tocopherol concentration, $1.25 \cdot 10^{-4}$ M. $\lambda_{ex} = 295$ nm, $\lambda_{em} = 330$ nm. Spectral slit width, 2.5 nm.

When studying α-tocopherol interaction with fatty acid in sarcoplasmic reticulum membranes, one should draw a clear-cut boundary between the fluorescence of aromatic amino acids of sarco-

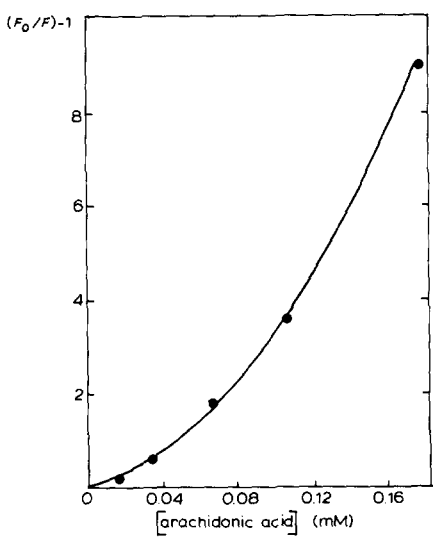

Fig. 7. Dependence of the intensity of α-tocopherol fluorescence quenching in liposomes on arachidonic acid concentration in the Stern-Folmer coordinates. α-Tocopherol concentration, 50 μg per 0.5 mg lipid per ml. $\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 330$ nm. Spectral slit width, 2.5 nm.

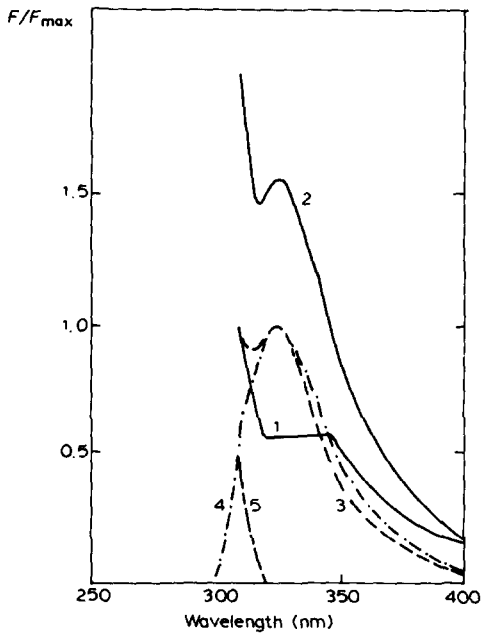

Fig. 8. Emission spectra of fluorescence of: (1) 0.5 mg/ml of sarcoplasmic reticulum protein; (2) 0.5 mg/ml of sarcoplasmic reticulum protein + 100 μg/ml of α-tocopherol; (3) spectral difference (2 − 1); (4) α-tocopherol fluorescence in liposomes (see Fig. 5a); (5) spectral difference (3 − 4); The spectra were normalized according to spectrum 3. $\lambda_{ex.}$ for spectra 1 and 2 = 305 nm. Spectral slit width, 1.5 nm.

plasmic reticulum proteins and that of α-tocopherol. Fig. 5b demonstrates the excitation and emission spectra of sarcoplasmic reticulum fluorescence. Although these spectra of protein fluorescence in sarcoplasmic reticulum membranes partly overlap those of α-tocopherol, the latter can be separated by excitation with a light pencil at and above 305 nm. An addition of exogenous α-tocopherol to a sarcoplasmic reticulum membrane suspension results in a linear increase of the F_{305}^{340} fluorescence intensity. Subtraction of spectrum 2 from spectrum 1 (Fig. 8) gives a spectrum of α-tocopherol fluorescence in sarcoplasmic reticulum membranes. At wavelengths above 320 nm this spectrum coincides well with the fluorescence spectrum of α-tocopherol in liposomes (Fig. 8, curve 3). The difference between the spectra reflects the contribution of light scattering to the measurable fluorescence spectrum of α-tocopherol in sarcoplasmic reticulum membranes. It appears that at wavelengths above 320 nm the light scatter-

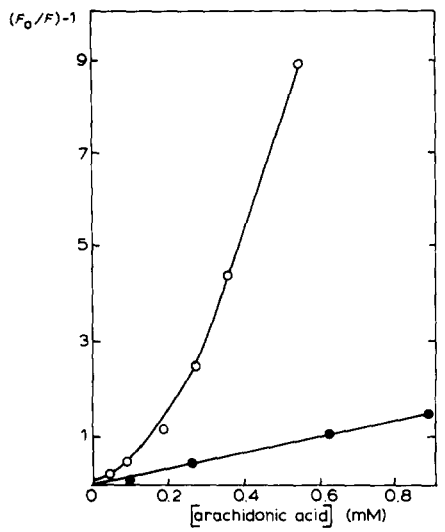

Fig. 9. Dependence of the intensity of α-tocopherol (○———○) and sarcoplasmic reticulum proteins (●———●) fluorescence quenching on arachidonic acid concentration in the Stern-Folmer coordinates. The suspension contained 0.5 mg/ml of sarcoplasmic reticulum protein + 100 μg/ml of α-tocopherol. $\lambda_{ex.}$ for sarcoplasmic reticulum proteins and α-tocopherol, 280 and 305 nm, respectively. Spectral slit width, 1.5 nm.

ing does not affect the fluorescence intensity. Thus, F_{305}^{340} is a reflection of the real α-tocopherol content in sarcoplasmic reticulum membranes.

An addition of arachidonic acid to sarcoplasmic reticulum membranes results in quenching of α-tocopherol fluorescence (Fig. 9). The efficiency of this process in sarcoplasmic reticulum membranes as well as in ethanol and liposomes is non-linear in the Stern-Folmer coordinates. The Stern-Folmer plots for protein fluorescence quenching linearly depend on arachidonic acid concentration.

Discussion

The data obtained suggest that α-tocopherol forms complexes with free fatty acid both in solution and in membrane systems (e.g., liposomes and sarcoplasmic reticulum membrane suspensions). The formation of these complexes has been confirmed experimentally by modification of α-tocopherol absorption spectra in the presence of saturated and unsaturated fatty acid (e.g., decrease in absorption around 214 nm in the absence of spectral changes at 293 nm) and by a reduction of the α-tocopherol fluorescence intensity (F_{290}^{320}), which is not accompanied by the changes in the position of excitation and emission maxima.

The interaction between α-tocopherol and fatty acid is non-covalent, since the complex formation occurs almost instantaneously after the mixing of the reagents. The thin-layer chromatograms of α-tocopherol-fatty acid mixtures reveal two spots with R_F values coinciding with those for α-tocopherol and fatty acid, respectively. Besides, it is known that the α-tocopherol adducts formed via fatty acid interaction with its chromanol nucleus do not emit fluorescence within the indicated spectral region [19].

Our experimental results on α-tocopherol interaction with fatty acid in solutions suggest that: (i) the formation of the complexes is quite intensive; (ii) the values of the interaction constants, K, for saturated fatty acids do not in practice depend on the number of carbon atoms within the range 7 : 0–24 : 0; and (iii) the values of equilibrium constants for unsaturated fatty acid are much higher than those for saturated fatty acid and within the range of 0–4, log K is linearly dependent on the number of double bonds in the fatty acid molecule.

The role of α-tocopherols as biological membrane stabilizers can become extremely important when free fatty acids take part in the destabilizing effects. Indeed, as was demonstrated earlier [15], the marked protective effect of α-tocopherol on the enzymatic system of Ca^{2+} transport in sarcoplasmic reticulum membranes manifests itself only in the presence of the products of phospholipid hydrolysis by phospholipases of A_2 type or by exogenous free fatty acid. The protective effect of α-tocopherol is practically absent in fresh preparations of sarcoplasmic reticulum. This assumption is quantitatively in good agreement with our experimental evidence on the fluorescence quenching of α-tocopherol incorporated into sarcoplasmic reticulum membranes after addition of arachidonic acid to the sarcoplasmic reticulum suspension.

According to the present-day concepts, the sharp elevation of free fatty acid content is considered to be the key pathogenic factor causing heart and skeletal muscle lesions under some pathologi-

cal conditions, such as ischemia, stress damage, hypoxia, etc. [20–22].

Thus, we may conclude that the formation of α-tocopherol complexes with fatty acid can be regarded as a molecular mechanism responsible for the stabilization of biomembranes exposed to the damaging action of free fatty acid.

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