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DOES α-TOCOPHEROL INTERACT WITH THE ACTIVE SITE OF CYTOCHROME P-450 IN LIVER MICROSOMES?

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Abundant experimental evidence of the universal role of lipid peroxidation in membrane damage following exposure to extremal factors and the development of various pathological states [4, 6] served as the basis for the use of inhibitors of free-radical oxidation, not only in the laboratory [10], but also in clinical practice [3].

As a result it has now become necessary to look for and screen synthetic antioxidants of low toxicity, with a prolonged stabilizing action in biomembranes [1]. This effect cannot be achieved through an increase in the concentration of antioxidants because of the toxic action which they exhibit [11] and of the oxidative metabolism of nonpolar synthetic inhibitors by the cytochrome P-450 system [5, 8]. Natural membranotropic antioxidants, such as tocopherols and ubiquinones, have a common structural principle: they have a cyclic nucleus with a hydroxy-group and a hydrophobic hydrocarbon "tail." The half-elimination time of natural antioxidants is much longer than that of their analogs without hydrocarbon substituents [9].

To test the hypothesis that hydrocarbon chains in the molecules of natural antioxidants play an essential role as a factor determining the length of their life in the membrane, a comparative investigation was undertaken of interaction of α-tocopherol (TP) and its synthetic derivative 6-hydroxy-2,2,5,7,8-pentamethylchromane (HPMC), which lacks the phytyl chain, with the cytochrome P-450 system, catalyzing the reaction of the first phase of biotransformation of the hydrophobic compounds.

EXPERIMENTAL METHOD

Intact noninbred albino rats weighing 150-180 g were used. The microsomal fraction of rat liver was isolated by differential centrifugation in 1.15% KCl. The concentration of cytochrome P-450 was determined by the method in [12]. Activity of microsomal 7-ethoxycoumarin de-ethylase was estimated from the velocity of the NADPH-dependent reaction of 7-hydroxycoumarin formation by the method in [15]. The spectra of binding of TP and HPMC with microsomes were recorded on a Hitachi-557 spectrophotometer by the method in [7]. TP and HPMC were added to a suspension of microsomes in ethyl alcohol or dimethyl sulfoxide so that the concentration

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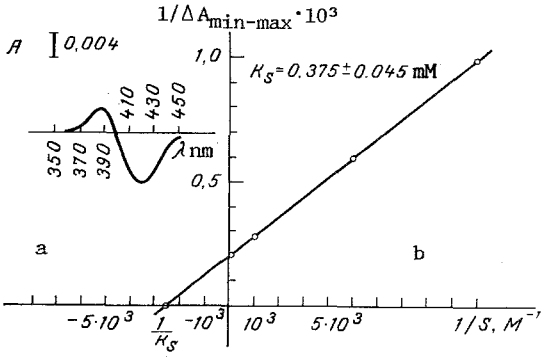

Fig. 1. a) Binding spectrum of HPMC (10^{-2} M) with microsomes (0.5 mg protein in 1 ml) from rat liver in 50 mM phosphate buffer, pH 7.6 (37°C); b) determination of K_S of HPMC with microsomes in double reciprocal coordinates.

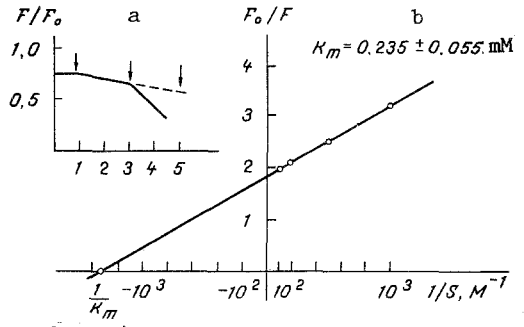

Fig. 2. a) Kinetics of oxidation of NADPH (10^{-4} M) in suspension of microsomes (1 mg protein/ml), incubated in the presence of HPMC (10^{-4} M) or TP (10^{-4} M) in 50 mM phosphate buffer, pH 7.6 (37°C). Arrows indicate additions. From left to right: microsomes, HPMC, TP. Abscissa, incubation time (in min); ordinate, intensity of fluorescence (in relative units); b) determination of K_m of HPMC from rate of oxidation of NADPH by microsomes, in double reciprocal coordinates.

concentration of these solvents did not exceed 0.1%. The protein concentration was determined by the biuret reaction, using bovine serum albumin as the standard.

EXPERIMENTAL RESULTS

Interaction of organic compounds with cytochrome P-450 in liver microsomes causes changes in its spectrum, especially in Cope's band [13]. Depending on the structure of the ligand this interaction can take place either in the protein part of cytochrome P-450, giving rise to differential binding spectra of type I substrates, or in the heme group of cytochrome P-450, giving rise to binding spectra of type II ligands [13]. In the experiments of series I spectra of cytochrome P-450 in microsomes were recorded in the presence of TP or HPMC. HPMC was found to induce type I spectral changes with a characteristic maximum at 385 nm and a minimum at 420 nm (Fig. 1a). The spectral binding constant (K_S) of the cytochrome P-450—complex was 0.375 ± 0.045 mM (Fig. 1b). On incubation of the microsomal suspension with TP no changes took place in the spectrum of cytochrome P-450 in the presence of TP in concentrations up to 10^{-2} M; this probably indicates that cytochrome P-450 does not interact with TP. The absence of effect in this case was not the result of nonincorporation (or of slow incorporation) of TP into the

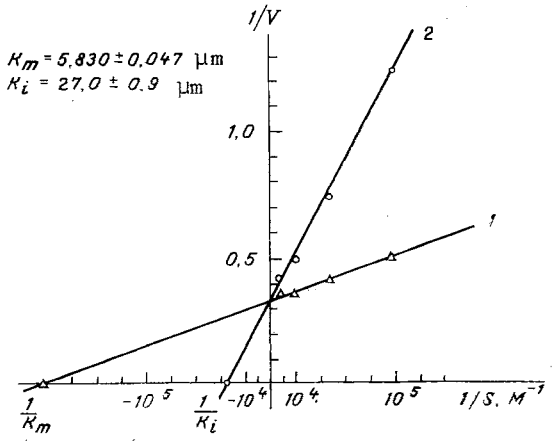

Fig. 3. Inhibition of 7-ethoxycoumarin de-ethylase by HPMC in rat liver microsomes. 1) Determination of K_m for 7-ethoxycoumarin de-ethylase; 2) determination of K_i for 7-ethoxycoumarin de-ethylase by HPMC. Abscissa, reciprocal of concentration of 7-ethoxycoumarin (in M^{-1}); ordinate, reciprocal of 7-hydroxycoumarin formation (in mmoles/min/mg protein).

microsomal membranes: after incubation the characteristic fluorescence of TP, with maxima in the excitation spectrum at 295 nm and in the emission spectrum at 325 nm, could not be recorded in the supernatant.

Substrates inducing the appearance of type I spectral changes are known to undergo NADPH-dependent oxidative hydroxylation in the mono-oxygenase reaction [13]. In the next series of experiments the kinetics of NADPH oxidation was therefore measured in a suspension of microsomes incubated in the presence of TP or HPMC. It will be clear from Fig. 2a, which gives typical kinetic curves of the change in intensity of fluorescence of reduced NADPH in the microsomal suspension, that addition of HPMC accelerates NADPH oxidation whereas TP does not affect the kinetics of the change in intensity of fluorescence of reduced NADPH. Considering that NADPH is the source of reducing equivalents for mono-oxygenase reactions catalyzed by cytochrome P-450, it can be concluded that a reaction of oxidative metabolism of HPMC, which does not take place with TP, occurs in microsomes. The value of K_m for HPMC (Fig. 2b), estimated from the change in the intensity of fluorescence of NADPH, was 0.23 ± 0.055 mM, in good agreement with the value of K_s for HPMC. The closely similar values of the K_m and K_s for HPMC probably reflect the affinity of cytochrome P-450 for this susbstrate.

7-Ethoxycoumarin, HPMC, and TP are compounds with a γ-pyran ring. 7-Ethoxycoumarin is a good substrate of cytochrome P-450 [15] and induces type I spectral changes in microsomes. The value of K_m in the 7-ethoxycoumarin de-ethylase reaction is 5.830 ± 0.047 μM. Data showing (Fig. 3) that HPMC is an effective competitive inhibitor of NADPH-dependent O-de-ethylation of 7-ethoxycoumarin, with $K_i = (2.70 \pm 0.09) \times 10^{-5}$ M, are therefore interesting. TP has no inhibitory action on the 7-ethoxycoumarin de-ethylase reaction.

It can be concluded from the results given above that TP is not a substrate for cytochrome P-450, whereas its derivative which lacks the phytyl chain (HPMC) binds effectively with and is metabolized by cytochrome P-450. Considering that cytochrome P-450 catalyzes oxidative hydroxylation of various aliphatic hydrocarbons [14], it must be accepted that steric hindrances exist for the interaction of TP with cytochrome P-450, due to the low mobility of TP in the membrane and presence of a hydrogen bond between the chromanoxyl group of TP and the carbonyl oxygen atom of the phospholipids [2]. As a result the first phase of biotransformation of TP with the participation of cytochrome P-450 beocme impossible, and it is probably this which determines the duration of its stay in the membrane.

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QUANTITATIVE CHANGES IN Cu,Zn-ACTIVATED SUPEROXIDE DISMUTASE AND CATALASE IN THE LIVER OF RATS WITH ALLOXAN DIABETES

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In rats with alloxan diabetes (AD) activity of superoxide dismutase (SOD) and catalase in the tissues is reduced, along with activity of other enzymes involved in antiradical protection of the cell [1, 9, 11]. On the other hand, elevation of the glucose level leads to an increase in the concentration of superoxide anions ($O_2^{\cdot-}$) in experiments not only *in vitro* [5], but also *in vivo* [12].

Administration of exogenous SOD has a protective effect in AD, for it lowers the blood glucose concentration to normal and inhibits excessive lipid peroxidation [4, 8].

The aim of this investigation was to study the spectral properties and activity of Cu, Zn-activated SOD (Cu,Zn-SOD) and catalase, isolated from rat liver and purified in order to discover the causes of their inactivation in AD.

EXPERIMENTAL METHOD

Experimental AD was induced in Wistar rats weighing 150-200 g by a single intraperitoneal injection of alloxan in a dose of 15 mg/100 g body weight. The rats were decapitated on the 5th day.

Cu,Zn-SOD and catalase were isolated from 60 g of rat liver by treatment with acetone [13]. The proteins were purified by ion-exchange chromatography of DE-52 cellulose (Whatman, England) and by gel-filtration on Toyopearl-55 (Japan).

SOD activity was studied by the method in [14], with determination of the quantity of $O_2^{\cdot-}$ undergoing dismutation in a certain time. As the source of $O_2^{\cdot-}$ generation an aqueous solution of H_2O_2 (0.5 M), pH 10.5, was used; the reaction mixture was incubated at 90°C for 2 min.

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