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NMR Relaxation Study of Cholesterol Binding with Plant Metabolites

O. Yu. Gluschenko · N. E. Polyakov · T. V. Leshina

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Abstract Nuclear magnetic resonance relaxation technique has been applied to study the interaction of cholesterol and its oxidation products with plant metabolite, glycyrrhizic acid. In all cases, the decay kinetics of echo signal shows bi-exponential behavior. It was demonstrated that the fast component of the decay kinetics belongs to the complex with stoichiometry of 1:2. The stability constants and thermodynamic parameters of these complexes have been measured. We propose that the complexation with glycyrrhizic acid could be an effective approach to regulate the cholesterol level inside and outside of cage membranes. In addition, the importance of this approach as a tool for reducing the cytotoxicity of the oxidation products of cholesterol is under discussion.

1 Introduction

Cholesterol is a natural lipophilic alcohol contained in the cell membranes of most living organisms. It enters the body from two sources: from food and from endogenous synthesis, with the majority (80%) synthesized by the body. Cholesterol (Fig. 1) plays an important role in many biochemical processes, in particular, ensures the stability of cell membranes in a wide range of temperatures, and is needed to produce vitamin D and various steroid hormones. In addition, cholesterol participates in the synthesis of bile acids, and according to some sources, affects the activity of the synapses of the brain and the immune system, including protection against cancer. At the same time, cholesterol earned notoriety for its involvement in the formation of atherosclerotic plaques. To date, it is believed that increased blood levels of cholesterol and low-density lipoprotein (LDL) cholesterol are the main

O. Yu. Gluschenko · N. E. Polyakov (✉) · T. V. Leshina
Institute of Chemical Kinetics and Combustion,
Institutskaya st. 3, Novosibirsk 630090, Russia
e-mail: polyakov@kinetics.nsc.ru

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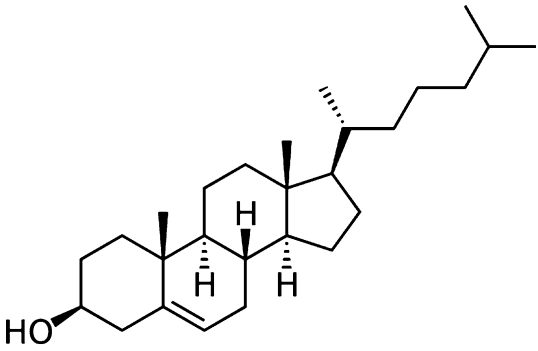
Fig. 1 Structure of cholesterol



factors for risk of atherosclerosis. But there is another insight into the problems of cholesterol: it is like a "repair material" accumulated in the field of microdamage of blood vessels and blocking these lesions. The special attention is paid to oxidation products of cholesterol found in atherosclerotic plaques, which may play a key role in the pathogenesis of atherosclerosis and other diseases [1–4]. In particular, the oxidation of cholesterol is one of the factors of developing heart disease. Oxidation of cholesterol is a more specific problem which would enable cholesterol to become "sticky" and start to form plaque in the walls of the arteries.

The binding of cholesterol and its oxidation products with various complexants and especially with nontoxic plant metabolites can be discussed as an alternative approach to the solution of atherosclerosis problem. At this moment, there are several ways of treatment for atherosclerosis disease. All of them use the drugs which are inhibitors of enzymes responsible for the different stages of cholesterol biosynthesis. They terminate the chain of cholesterol formation, but block also the formation of some other important products of biosynthesis. In addition, these drugs often demonstrate toxic properties. This is why the search for alternative methods of regulation of the cholesterol level is in progress. Up till now, only one successful example of cholesterol complexes is known. It is a complex of cholesterol with cyclodextrins (CDs) which effectively remove cholesterol from membranes [5, 6]. But in this case some negative effects occur. First, this disturbs the structure of membranes, and second, the possibility of crystallization of both the CD and their complexes was detected.

β-Glycyrrhizic acid (GA), a triterpene glycoside produced by widespread plants, *Fabaceae Glycyrrhiza glabra L.*, *Glycyrrhiza uralensis Fisch*, *Glycyrrhiza korshinskyi Grig* (licorice), belongs to plant metabolites combining availability with unique pharmacological activity [7]. GA (Fig. 2), whose molecule consists of hydrophobic (aglycon) and hydrophilic (carbohydrate chain) parts, manifests the properties typical for micelle-forming substances [8–14]. On the other hand, the formation of stable GA complexes with stoichiometry of 1:2 was detected for some hydrophobic molecules even in nonaqueous media [15]. There are several reasons why the interaction of cholesterol with GA may be of interest. First, GA is the natural complexant which forms inclusion compounds with various drugs [16, 17]. Second, unlike the CD, there is no evidence of GA toxicity at low concentrations. Third, there are some data on the influence of GA on the biosynthesis and properties

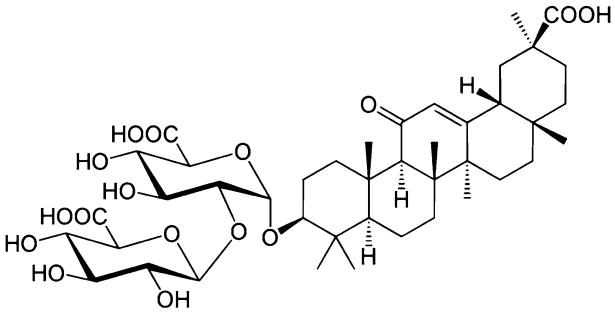


Fig. 2 Structure of glycyrrhizic acid

of cholesterol [7]. In experiments on animals with atherosclerosis, GA and its salts reduce cholesterol, LDL and triglycerides level [18]. Finally, some data indicate the ability of GA to reduce the oxidation of cholesterol [19]. However, the molecular mechanism of these effects is currently unknown. The study of cholesterol interaction with GA may shed light on these facts and open a new way to treatment of atherosclerosis.

The purpose of this study is to investigate the possibility of complexation of cholesterol and some of its oxidation products (ozonolysis) with GA by nuclear magnetic resonance (NMR) relaxation technique. The longitudinal or spin–lattice relaxation rate ($1/T_1$) and the transverse or spin–spin relaxation rate ($1/T_2$) are NMR parameters which are used in supramolecular chemistry to measure different kinds of binding, in particular, to prove the host–guest complexes formation [14, 20–25].

2 Experimental

All NMR experiments (including measurement of relaxation times) were performed on a Bruker DPX-200 NMR spectrometer equipped with a temperature controller. Relaxation times were measured at 300 and 320 K. The T_2 relaxation was measured by means of a Carr–Purcell–Meiboom–Gill (CPMG) sequence from Avance version of Bruker pulse sequence library: $p(90°)$-$(\tau - p(180°) - \tau)_n$-acquisition, where $\tau = 0.6$ ms, and n was varied from 0 to 4,028. Spectrophotometer UV-2401 was used to measure absorption spectra.

The solutions of cholesterol (Aldrich), GA and their complexes were investigated in deuterated solvents CD_3OD (99.5% D) or CD_3CN (99.5% D) (Aldrich). Complexes were prepared by mixing the reagents at different ratios for several hours. While the process of establishing the equilibrium takes a few days at room temperature, it accelerates when the temperature rises up to 60°C. GA was kindly donated by Prof. N. F. Salakhutdinov (NIOC, Novosibirsk). Carotenoid canthaxanthin was kindly donated by Prof. L. D. Kispert (The University of Alabama, Tuscaloosa).

Ozonation of cholesterol was made by the ozonator "The Storm" with a capacity of 300 mg of O_3 in an hour. Ozonolysis products were obtained by ozonation of dry films, as well as of the methanol solution of cholesterol.

3 Results and Discussion

3.1 Interaction of GA with Cholesterol

Since the NMR relaxation times T_2 of organic molecules are very sensitive to their size and mobility, this technique is often used to study weak intermolecular interaction, in particular, inclusion complexes formation [22]. The change of relaxation time as a result of complexation depends on the change in the rotation correlation time of the molecule to which the nuclei under study belong [21, 24]. The rotation correlation time depends, in turn, on the particle size which can vary substantially upon association of "guest" and "host" molecules. Therefore, in the present study, the dependence of the T_2 relaxation rate of GA and cholesterol protons on their concentration has been studied by pulse NMR methods. Using, in this case, the most popular CPMG pulse sequence, it was revealed that the time dependence of the integral intensity of echo signal of individual compounds, GA and cholesterol, is well described by a mono-exponential function. T_2 relaxation kinetics for both precursors in the non-associate state is mono-exponential with characteristic times of $T_2 \sim 350$ ms for cholesterol and $T_2 \sim 400$ ms for GA protons. On the other hand, the same dependences for the mixture of cholesterol with GA are well described only by the bi-exponential function

$$A(t) = P_1 \times \exp(-t/T_{21}) + P_2 \times \exp(-t/T_{22}). \quad (1)$$

As an example, Fig. 3 shows the kinetics of relaxation of the cholesterol proton at the double bond (see Fig. 1) on a logarithmic scale in the absence and in the presence of GA.

Such bi-exponential behavior of the relaxation kinetics was previously detected for the protons of "host" and "guest" molecules in the micelles formed by GA in aqueous solutions [14]. Usually bi-exponential behavior is assigned to the difference in the relaxation rates of the protons belonging to the same type and located in an associate and in non-associate states. Note that the necessary condition of the observation of bi-exponential relaxation kinetics is slow exchange rate between the associate and non-associate states: $1/\tau < 1/T_2$. In these experiments, we have not observed significant changes in the chemical shifts of the protons investigated in the free and bound forms. Taking into account our previous studies, we assume that this is a common feature of GA complexes [14, 25].

Measurement of the pre-exponential factors allows to determine the fraction of molecules located in the complex and hence to calculate the stability constants and stoichiometry of the complex. In general, the stability constant K for reaction:

$$n\mathrm{Chol} + m\mathrm{GA} \leftrightarrow \mathrm{Chol}_n\mathrm{GA}_m$$

is determined as

$$K = \frac{[\mathrm{Chol}_n\mathrm{GA}_m]}{[\mathrm{Chol}]^n[\mathrm{GA}]^m}, \quad (2)$$

where [Chol] is the free cholesterol concentration, and [GA] is the concentration of free GA. The values of m and n were calculated using the optimization program for

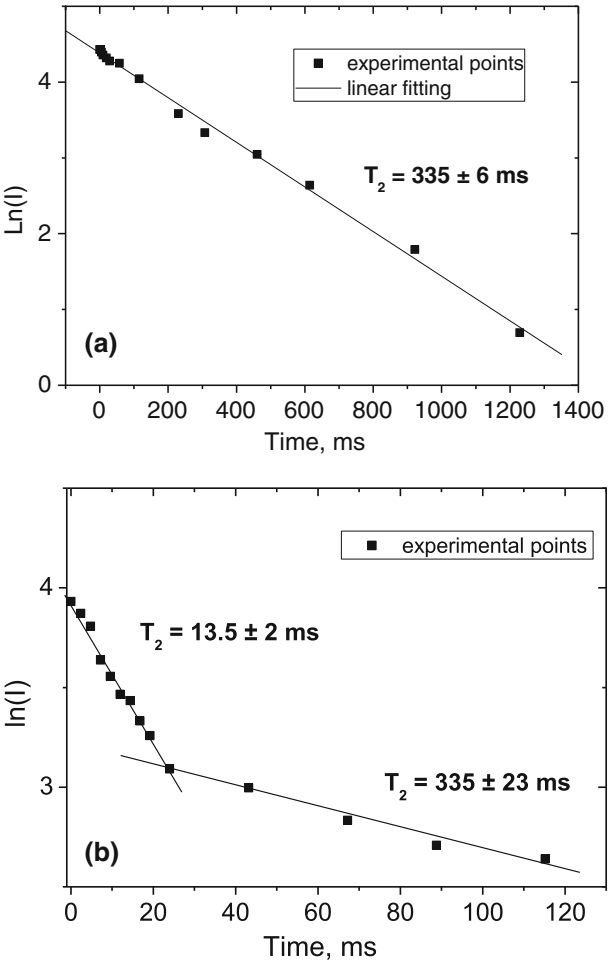


Fig. 3 Example of the decay kinetics of echo signal of cholesterol =CH– protons at the double bond before (**a**) and after (**b**) mixing with GA (1:1, 5 mM) in methanol solution

experiments with different ratios of $[Chol]_0$ and $[GA]_0$. Since cholesterol is insoluble in water or water–methanol mixture, in the present study, the complexes were prepared by mixing the starting materials in deuterated methanol. For the calculation of stability constants and thermodynamic parameters of the complex, the samples with different ratios of cholesterol and GA (5 mM/5 mM, 10 mM/5 mM and 5 mM/10 mM) were analyzed at two temperatures: 300 and 320 K. The stoichiometry and stability constants of the complex were determined by fitting the decay kinetics at different concentrations of cholesterol and GA after the establishment of an equilibrium using Eqs. (1) and (2) for concentrations of the complex and free cholesterol: $n \times [Chol_nGA_m] = P_1$ and $[Chol] = P_2$. The resulting stoichiometry was obtained to be 1:2, namely, one molecule of cholesterol binds with two molecules of GA. This result is consistent with existing data on the

complexation of GA with other hydrophobic substances [15, 25]. The calculated stability constant for this stoichiometry is $K_{12} \approx (3 \pm 0.6) \times 10^3$ M^{-2}. The thermodynamic parameters were calculated from the temperature dependence of the stability constant: ΔG (300 K) $= -20 \pm 4$ kJ/mol K, $\Delta H = 0.4 \pm 0.1$ kJ/mol, and ΔS (300 K) $= 68 \pm 14$ J/mol K. It is seen that the entropy factor makes the main contribution to the complex stability. Usually, the increase in an entropy factor is connected with the desolvation of the solutes [26]. We propose that the desolvation of the cholesterol molecule plays an important role in the process of complex formation.

3.2 Interaction of GA with Cholesterol Oxidation Products

The presence of a double bond in the cholesterol molecule provides its susceptibility to oxidation by various reactive oxygen species present in the body. It is well known [27] that when the concentration of toxic oxygen forms exceeds the protecting capability of the antioxidant systems of the organism, the oxidative stress occurs. It plays an important role in the pathogenesis of many serious diseases, including atherosclerosis. The contribution of oxidized forms of cholesterol and LDL cholesterol in the pathogenesis of atherosclerosis is very important. It was found that atherosclerotic plaques contain not only cholesterol but also a series of oxysterols [28, 29]. Atherogenic effect of oxysterols was demonstrated both in vitro and in vivo. In addition, the interaction of cholesterol oxidation products with β-amyloid leads to disruption of the protein metabolism. Alzheimer's disease occurs during the accumulation of β-amyloid in brain tissues, and the presence of oxysterols accelerates this accumulation, and, consequently, accelerates the course of the disease [30]. This is why the development of ways to influence the toxicity of the oxidation products of cholesterol is very important.

Recent studies have identified another active form of oxygen—ozone—in the arteries of a human affected by atherosclerosis [31]. Ozone is a very strong oxidant, commonly used for disinfection, odor removal, water treatment, air pollution and food processing. Since it was established that the primary objectives of ozone are unsaturated lipids in cell membranes, chemical reactions of ozone with lipids were studied in more detail [32]. It was found that ozone is produced by antibodies and cells of the immune system located in the inflammation regions [31]. From all the reactive oxygen species only ozone breaks the double bond of cholesterol to form 5,6-sekosterol (Fig. 4). Subsequent experiments have shown that atherosclerotic tissue contains products formed during the oxidation of cholesterol with ozone [31]. Thus, by oxidizing cholesterol, ozone makes contribution to the formation of atherosclerotic plaques. In addition, the products of ozonolysis are toxic to blood cells, and this may exacerbate the inflammation [31]. In the present study, we have made an attempt to form complexes of ozonolysis products with GA to create a tool of removing oxidizing cholesterol products from the body and atherosclerotic plaques.

It is known that ozonolysis of cholesterol in various solvents results in formation of various oxidation products [33]. In the present study, we have investigated the interaction of GA with three ozonolysis products: ozonide (**II**) formed during

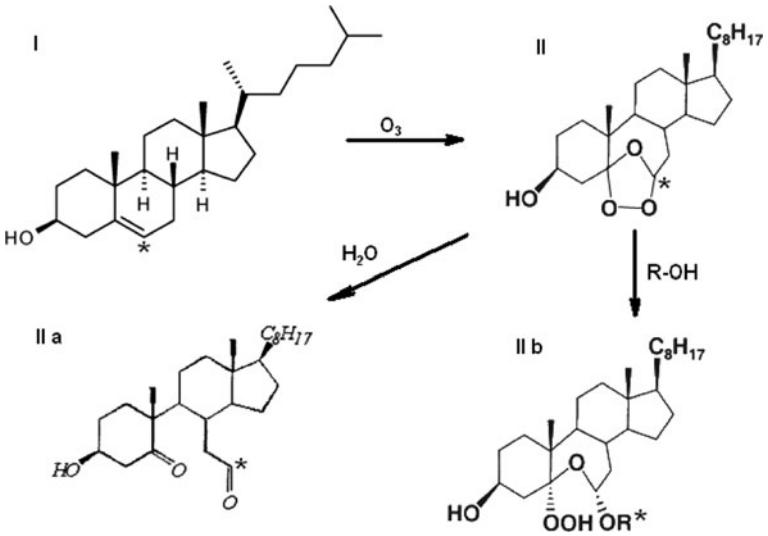


Fig. 4 Cholesterol (**I**) and the products of its ozonolysis. *Asterisk* indicates the protons, for which the relaxation time was measured: δ(CH, **I**) = 5.3 ppm, δ(CH, **II**) = 5.6 ppm, δ(CH, **IIa**) = 9.6 ppm, and δ(OCH_3, **IIb**) = 3.4 ppm in CD_3CN

oxidation of the dry film, product (**IIb**) formed during oxidation of cholesterol in methanol solution, and sekosterol (**IIa**) formed in both cases (Fig. 4). As a result, two products, **II** and **IIb**, show bi-exponential relaxation kinetics in the presence of GA. Figure 5 shows bi-exponential relaxation kinetics detected for oxidation products **IIb**. It was assumed that short relaxation time of the order of 70 ms corresponds to a bound state, and long relaxation time of the order of 700 ms corresponds to the free molecule. The same bi-exponential behavior was observed for ozonide **II**. In the case of sekosterol produced by ozonolysis of dry films or methanol solution of **I**, the relaxation kinetics is mono-exponential with long relaxation time, which may mean the absence of any binding of this product with GA in methanol.

For ozonide the stability constants of the complex with GA were calculated at 300 and at 320 K, assuming stoichiometry of 1:2 determined for the complex of cholesterol. The stability constant for this complex measured at 300 K is $K_{12} = (7 \pm 3) \times 10^7\ M^{-2}$. The calculated thermodynamic parameters of complexation are: $\Delta H = -28 \pm 6$ kJ/mol, ΔG (300 K) $= -45 \pm 9$ kJ/mol, and ΔS (300 K) $= 57 \pm 11$ J/mol K. The negative enthalpy change indicates that, unlike the complexes of cholesterol with GA, for which the stabilization of the complex is exclusively due to the entropy factor, there is an additional contribution to the complex formation. We suppose this might be hydrophilic interaction between OH, C=O and COOH groups of the solutes. This also results in a higher stability constant of this complex.

It should be emphasized that the calculated changes in enthalpy and entropy of the complex formation with GA are higher or comparable with the corresponding values measured for the dimerization of cholesterol and its interaction with major components of cage membranes [34]. The calculated dimerization enthalpy and

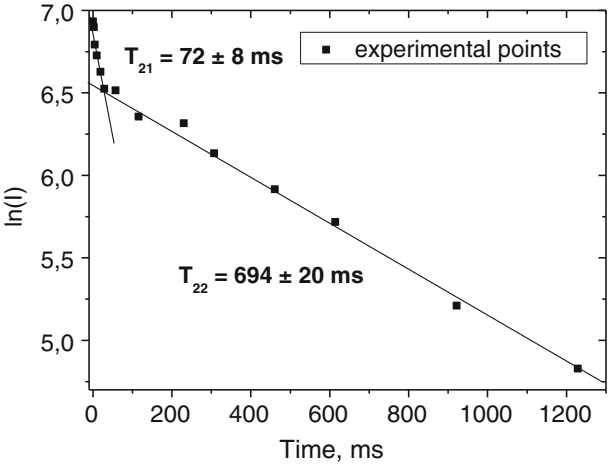


Fig. 5 Experimental decay kinetics of the echo signal of cholesterol oxidation product **IIb** in the presence of GA in methanol solution

entropy for cholesterol were found equal to 8.8 kJ/mol and 15 J/mol K, respectively. A spectroscopic study of the association of cholesterol with lecithin, the major component of biomembranes, allowed to determine the enthalpy of formation of a hydrogen-bonded complex, $\Delta H = 28$ kJ/mol. Since it is known that triglycerides also take part in the formation of atherosclerotic lesions, such measurements were carried out on the intermolecular interaction between cholesterol and tributyrin (a triglyceride). The enthalpy for the formation of the cholesterol–tributyrin complex was found to be 21.7 kJ/mol, while the entropy was 50.6 J/mol K. We can see that the thermodynamic parameters for the self-associates of cholesterol and for associates with the components of cage membranes measured in Ref. [34] are close to those (or less) obtained in the present study for GA complexes with cholesterol and its oxidation products. It should be kept in mind that all measurements in this study were made in methanol solution to get high concentration of the complexes for NMR measurements. One can expect that in aqueous solution the binding of cholesterol with GA will be significantly enhanced by the hydrophobic interaction. This gives hope to the possibility of using the GA to extract cholesterol and its oxidation products from lipid membranes and LDL complexes. Note that the complexes of cholesterol with CD are already being used for this purpose in practice [5, 6].

3.3 Cholesterol–GA Aggregates as a Drug Delivery System

Next part of our study of cholesterol–GA interaction was the investigation of the ability of cholesterol–GA aggregates to absorb small molecules, for example, some drug molecules. Recently, a novel self-assembling hydrogel system based on inclusion complexes between β-cyclodextrin and cholesterol was described [35]. Due to the assumed biocompatibility and expected physiological clearance, the

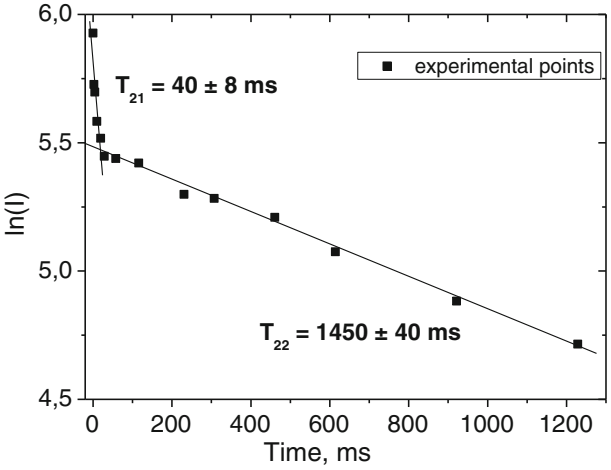


Fig. 6 Decay kinetics of the echo signal of nifedipine aromatic protons in CPMG experiment in methanol solution in the presence of cholesterol and GA (both 5 mM). Concentration of nifedipine is 1 mM

authors propose that these hydrogels offer excellent opportunities as drug delivery matrices and for other pharmaceutical and biomedical applications.

From this point of view, as an example, we have taken nifedipine, which is able to form 1:2 complexes with GA in aqueous as well as in methanol solutions [25]. It was demonstrated in Ref. [25] that such complex formation results in shortening of the nifedipine relaxation time by $\sim$30–50%. In the present study, it was found that the addition of 1 mM nifedipine to the methanol solution of cholesterol with GA results in the appearance of a very short component in the decay kinetics of the echo signal with a characteristic relaxation time of about 40 ms (Fig. 6).

We suppose that this effect is due to incorporation of nifedipine into the cholesterol–GA aggregates. The observation of bi-exponential kinetics means the slow exchange rate between free nifedipine and the complex. This result might be also considered for practical application: cholesterol associated with GA due to its biocompatibility and physiological clearance can be used as a drug delivery system with slow drug release.

3.4 Protection Role of GA for the Oxidation of Unsaturated Compounds by Ozone

Taking into account the abovementioned role of ozone and the products of the cholesterol ozonolysis for the development of atherosclerosis [31], we have attempted to investigate the protection role of GA toward oxidation of cholesterol and other unsaturated organic compounds by ozone molecules. At first, we present the example of the effect of GA micelles formed in water–ethanol solution on the model reaction of carotenoid canthaxanthin with ozone molecules. Carotenoids are known as a class of natural dyes which intensive color is determined by the length of unsaturated polyene chain. This is why in this experiment the reaction was

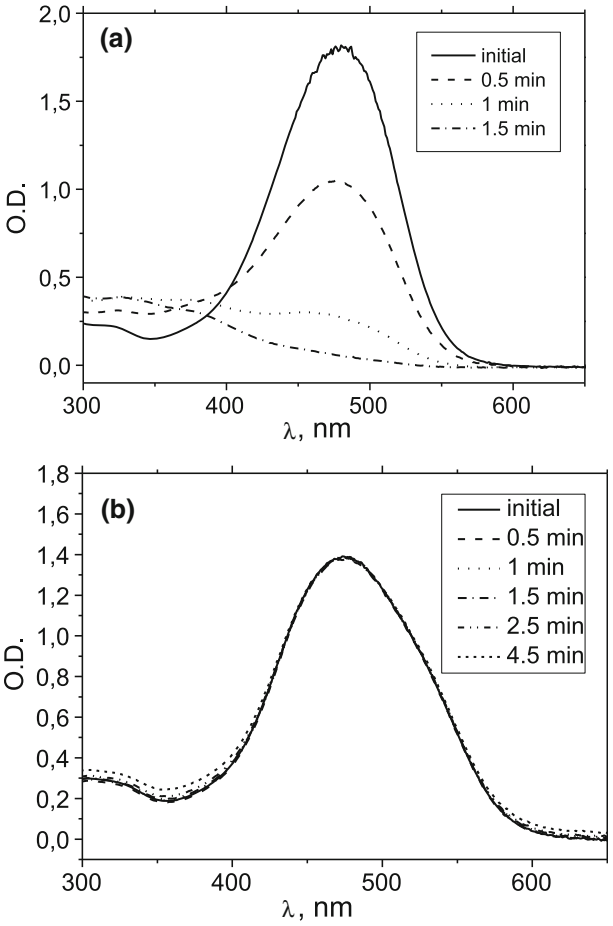


Fig. 7 Optical absorption spectra of ~0.02 mM canthaxanthin solution in 10% ethanol after different time of ozone treatment in the absence (**a**) and in the presence (**b**) of 1 mM GA

monitored by measurement of the carotenoid absorption spectra. Figure 7 shows the changes in the canthaxanthin absorption spectra during ozonolysis of 0.02 mM canthaxanthin in 10% ethanol solution in the absence (Fig. 7a) and in the presence (Fig. 7b) of 1 mM GA. One can see that the incorporation of a carotenoid molecule into the GA micelle results in significant decrease of the oxidation rate.

Since the cholesterol–GA complexes are insoluble in aqueous solutions, in this study the effect of GA on the oxidation rate of cholesterol molecules by ozone was investigated in the solid state. For this purpose, the intensities of the NMR signals of ozonolysis products of dry films of pure cholesterol and 1:2 cholesterol complex with GA have been compared after their dissolution in deuterated acetone. The decrease of the intensity of the signals corresponding to ozonide by ~4.5 times was observed in the presence of GA. The ability of GA to reduce the oxidation rate of

unsaturated organic molecules might be important for practical application in pharmacology, cosmetics and food processing.

4 Conclusion

The formation of stable aggregates of cholesterol and some of its oxidation products with GA was observed for the first time. The measurement of T_2 relaxation time of the protons of cholesterol and GA shows the existence of complexes with stoichiometry of 1:2. The analysis of thermodynamic parameters for GA interaction with cholesterol points that their binding is comparable with that detected for the self-associates of cholesterol and its associates with components of cage membranes. This, as we believe, makes GA a perspective reagent for practical application. Taking into account also the ability of GA to gain the permeability of cage membranes, where near 90% of cholesterol is located, this study might be the beginning of development of a new approach to the regulation of the cholesterol level. The special attention should be paid to the ability of GA to reduce the oxidation rate of cholesterol and other hydrophobic molecules, and to bind its oxidation products even in nonaqueous media.

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