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Oxidation of Low-Density Lipoproteins in the Presence of a Fullerene-Containing Silica Gel

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Abstract—The results of a study on the oxidation of low-density lipoproteins (LDLs) in the presence of a fullerene-containing silica gel in the sorbent phase (a solid stationary phase) and in the phase of an LDL solution (a liquid mobile phase) are presented. It was found that the main distinctive feature of the process of LDL oxidation in the presence of a fullerene-containing silica gel consists in its heterogeneity; that is, the oxidation simultaneously occurs in both of the phases. In this case, the effect of the oxidation process in a solid phase on the kinetics in a liquid phase can be described in terms of the nonuniform distribution of reactant concentrations in the phases of the system and the formation of a donor–acceptor complex. It was found that the concentration of oxidation products is an exponential function of LDL concentration; this fact is indicative of a free-radical chain mechanism of the LDL oxidation reaction in the presence of the sorbent. This relationship was described by an empirical equation, which is the same for either of the phases, and the parameters of this equation have precise physical meanings. It was found from calculations that both the formation of donor–acceptor complexes and the nonuniform distribution of LDL in the two-phase system are responsible for a considerable difference between the kinetics of sorbent-initiated LDL oxidation in the sorbent phase and the solution phase.

INTRODUCTION

Low-density lipoproteins (LDLs) undergo oxidation in the presence of a fullerene-containing silica gel [1], as well as in the presence of transition metals [2]. As a result of this reaction, oxidation products are formed, the presence of which in blood plasma accelerates the development of atherosclerosis [3–7]. Sedov *et al.* [8] believed that the capability of a fullerene-containing silica gel for oxidizing LDLs is a barrier to the introduction of this silica gel into the practice of sorption technologies for the correction of lipid exchange. However, a special feature of LDL oxidation in the presence of a fullerene-containing silica gel consists in the fact that the sorbent granules are insoluble in physiological solution and blood plasma. Therefore, they can be easily separated from the solution of unadsorbed LDLs [1]. At the same time, it is believed *a priori* that LDL oxidation occurs and hence lipid oxidation products are formed in both a sorbent phase (a solid stationary phase) and an LDL solution phase (a liquid mobile phase). The results of a study on the kinetics of LDL adsorption on a fullerene-containing silica gel, which was performed by the isotherm method, made it possible to recognize the following two steps in the adsorption of LDLs on a fullerene-containing silica gel: the formation of an activated state of LDLs and the oxidation of LDLs. These steps of the process occur consecutively. The first step always precedes the oxidation of LDLs, and it is favorable for the formation of a transient complex (an activated state). It ends in the formation of a molecular complex (as a result of shifting electron density from LDLs to modified silica gel), which is favorable for a redox reaction with the formation of stable oxidation products. The second step occurs at a much later time, and it is accompanied by electron transfer from a molecular orbital (MO) of LDLs to a molecular orbital of fullerene; in this case, stable oxidation products are formed. Another reason for the two-step adsorption is the heterogeneity of the system: the sorbent phase is a solid stationary phase, and the LDL solution phase is a liquid mobile phase. At equal initial LDL concentrations and times of contact between solutions and the sorbent, the rates of oxidation are expected to be different for aqueous LDL solutions and adsorbed LDLs.

In this work, we present the results of a study on the oxidation of LDLs in the presence of a fullerene-containing silica gel in the sorbent phase and in solution. We attempted to evaluate the significance of these results with the use of statistical thermodynamic approaches to describe two-phase systems [9] in terms of the nonequilibtium distribution of reactants between phases. We also revealed the process parameters responsible for the effect of heterogeneous oxidation on the kinetics of formation and accumulation of lipid oxidation products in blood plasma in the course of plasmosorption.

EXPERIMENTAL

Low-density lipoproteins were isolated from blood plasma with the use of ultracentrifugation [10, 11]. MSA 200 silica gel with a pore size of 200 nm, a specific surface area of 10 m^2/g, and a pore volume of

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0.7 ml/g was used as a sorbent. The silica gel was modified with a fullerene in accordance with the procedure [8] based on the impregnation of silica gel granules with a solution of C_{60} in *ortho*-dichlorobenzene with a concentration of 30 or 3 mg/ml followed by solvent removal by evaporation in a vacuum chamber at 50°C, long-term washing with water and ethanol, and drying in a thermostat at 150°C. In this procedure, the ability of silica gel to embed molecules of a certain size in its porous structure was used. The fullerene concentration was 2.5 wt %.

All experiments were performed at 20°C. The solutions of LDLs were prepared in 0.15 M NaCl + 0.005 M Tris HCl (pH 7.4). The masses of initial LDLs and LDLs present in the phases were calculated from protein concentrations in the starting solution, in the decanted liquid phase, and in the wash water from the solid phase (Lowry method) or by determining total cholesterol with the use of a commercial kit from Boehringer Mannheim. Adsorption was performed in 10-cm^3 polycarbonate test tubes, in which 2-cm^3 portions of LDL solutions of different concentrations were added to 100-mg portions of the dry sorbent. The contents of test tubes were stirred in the course of experiments. The adsorption capacity of the adsorbent for LDLs was characterized by the elimination factor (*el*), which was calculated from the relation

$$el = (\Phi_0 - \Phi_1)/\Phi_0 = 1 - \Phi_1/\Phi_0. \quad (1)$$

Here, Φ_0, Φ_1, and Φ_2 are the masses (g) of LDLs in the system, the liquid phase, and the solid phase, respectively. Taking into account that $\Phi_0 = C_0V_0$, $\Phi_1 = C_1V_1$, $\Phi_2 = C_2V_2$, and $\Phi_0 = \Phi_1 + \Phi_2$, finally, for the adsorption capacity, we have

$$el = 1 - C_1V_1/C_0V_0 = C_2V_2/C_0V_0. \quad (2)$$

Here, C_0, C_1, and C_2 are the concentrations (g/ml) of LDLs in the system, the liquid phase, and the solid phase, respectively; V_0, V_1, and V_2 are the volumes (ml) of the system, the liquid phase, and the solid phase, respectively.

The relationships for calculating LDL concentrations in the mobile liquid (C_1) and stationary solid (C_2) phases were derived from Eqs. (1) and (2) on condition that LDLs are irreversibly bound to the adsorbent; according to published data [10], this is true of the test system

$$C_1 = C_0(1 - el), \quad (3)$$

$$C_2 = C_0el. \quad (4)$$

The accumulation of lipid oxidation products in the solution and on the sorbent was detected by monitoring an increase in fluorescence intensity (*F*) at different time intervals (the excitation and emission wavelengths were 365 and 430 nm, respectively) [12]. An analyzer designed at the Vavilov State Optical Institute was used. The fluorescence intensity of a fluorene solution in dioxane (0.625 μg/ml) was taken as a unit of *F*. The process of LDL oxidation was additionally examined by determining the concentration of lipid hydroperoxides with the use of an iodometric procedure [13]. Moreover, lipid oxidation products were detected by the accumulation of diene conjugates in plasma passed through a sorbent column. Plasma samples were delipidized with a chloroform–methanol mixture and evaporated to dryness; the residue was dissolved in hexane, and the absorbance was measured at 233 nm. The concentration of diene conjugates was calculated using a molar absorption coefficient of 2.6×10^5 cm^{-1} [14].

RESULTS AND DISCUSSION

Figure 1 demonstrates the fluorescence intensity of LDLs (bound or unbound to the sorbent) as a function of the time of contact between LDLs and the sorbent. According to Lenz *et al.* [2], an increase in *F* is due to the formation of lipid oxidation products as a consequence of the oxidation reaction of unsaturated fatty acids in the presence of a fullerene. It can be seen that, in the presence of the fullerene-containing silica gel, the oxidation of both LDLs unbound to the sorbent and LDLs bound to the sorbent takes place. Because the supernatant and the sorbent form a two-phase system, the data obtained suggest the heterogeneity of the reaction; that is, oxidation occurs simultaneously in either phase.

Figure 2a illustrates the results of a study of LDL oxidation in the presence of the fullerene-containing sorbent with the use of another method for identifying lipid oxidation products (by measuring the ratio between the concentrations of lipid hydroperoxides (LHPs) and cholesterol (Ch) [LHP]/[Ch]). This method gives information only on the concentration of lipid oxidation products in the lipid phase of a particle. As can be seen in Fig. 2a, oxidation on the sorbent occurs at a higher rate and leads to a twofold increase in the concentration of lipid hydroperoxides. These independent experimental data provide support for the heterogeneity of the process, that is, the simultaneous occurrence of the oxidation process in two phases, and for a higher rate of the process in the solid phase. Quantitative differences between functions shown in Figs. 1 and 2a can result from both different sensitivities of the methods used for determining lipid oxidation products and different oxidizabilities of the test LDLs.

Analogous studies were performed with the use of blood plasma. According to Klimov and Nikul'cheva [14], the presence of molecules with two conjugated double bonds, which are determined spectrophotometrically by the appearance of a maximum at 233 nm in the absorption spectrum, is a sensitive test for hydroperoxides in blood plasma. Figure 2b illustrates the results of a study of the biochemical composition of blood plasma before and after its contact with the fullerene-containing sorbent (for different times). It can be seen that at long times of contact between blood plasma and the fullerene-containing sorbent, the formation of diene conjugates was also observed both in the

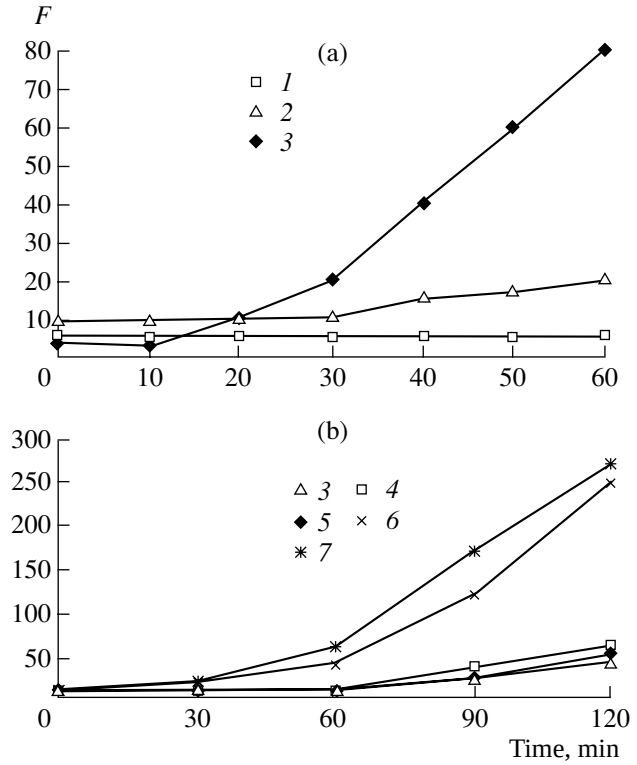

Fig. 1. Fluorescence intensity (F) of aqueous LDL solutions in (*1–4*) liquid and (*5–7*) solid phases as a function of the time of contact with a fullerene-containing silica gel. Initial LDL concentrations (C_0) are (*1–3, 5)* 0.5, (*6*) 1.0, and (*4, 7*) 2.5 mg/ml; (*2, 3*) vitamins A and E are added.

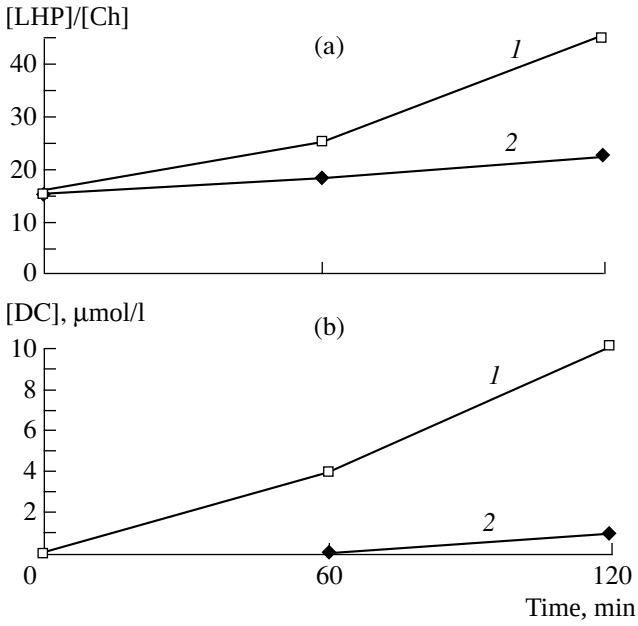

Fig. 2. (a) Ratio between the concentrations of lipid hydroperoxides and cholesterol and (b) the concentration of diene conjugates (DC) in blood plasma in the course of contact with a fullerene-containing silica gel: (*1*) sorbent and (*2*) supernatant phases of the system.

blood plasma and on the sorbent surface. The ratio between diene conjugate concentrations in the solid and liquid phases after long-term contact of blood plasma with the fullerene-containing sorbent was 10 : 1. At the same time, at short contact times, diene conjugates were detected only on the sorbent surface. Thus, lipid oxidation products are formed in both the liquid phase (the mobile phase is blood plasma or an aqueous LDL solution) and the solid phase (the stationary phase is the sorbent). Consequently, the process of LDL oxidation in the presence of the fullerene-containing silica gel should be considered as a heterogeneous reaction.

A special feature of the kinetics of oxidation in either phase consists in the occurrence of two characteristic portions in the curves of fluorescence intensity (F) plotted against the time (t) of contact between an LDL solution and the sorbent. These portions differ in the character and rate of change in F with t. In the first portion, $(t \in (0–t_1)) – F$ does not depend on t and equals the value of F for the initial LDL solution; in the second portion of the curve, $(t > t_1) – F$ increases with t. The time t_1 was different for LDLs separated from the blood plasma of different patients. According to Chin *et al.* [15], blood plasma may contain LDLs different in oxidizability. Then, the constancy of F with time at $t < t_1$ is due to the presence of LDLs with low reactivity. The presence of vitamins A and E in an LDL solution exerts a similar effect on the kinetics of oxidation. According to Voskresenskii [5], the above vitamins are antioxidants and inhibit the oxidation reaction. In this context, the difference between the values of t_1 in curves *2* and *3* (Fig. 1) can be explained by a change in the concentration of antioxidants in the experiments under comparison.

A decrease in the value of t_1 by a factor of 2 to 3 was observed in the solid-phase oxidation of LDL. An increase in F in the region of $t > t_1$ reflects the process of LDL oxidation, which results in the formation and accumulation of lipid oxidation products in the LDL solution. In this case, as can be seen in Fig. 1, the kinetics of oxidation processes in different phases is dramatically different. The differences are much greater than experimental errors, and this fact is indicative of a fundamental difference between processes occurring in different phases. The solid-phase oxidation process is characterized by a higher rate of increase in F. At equal LDL concentrations in the initial solutions, this rate is higher than the rate observed in the F–t plot for a liquid phase by a factor of 4 to 5. In turn, the initial concentration of an aqueous LDL solution also affects the absolute values of F in different phases to a variable degree. Thus, it can be seen in Fig. 1b that, as the concentration of an LDL solution increases by a factor of 5 at the same contact time, the values of F in the liquid and solid phases increased by factors of 1.5 and 5, respectively.

Figure 3 demonstrates the distribution of LDLs between the phases over a wide range of initial concen-

trations (C_0) of aqueous LDL solutions, as calculated using Eqs. (1)–(4). It can be seen that over the entire range of C_0 the concentrations of LDLs in the solid phase (C_2) are higher than those in the liquid phase (C_1). However, maximum differences between C_1 and C_2 were observed at high initial LDL concentrations. Thus, at C_0 = 2.5 mg/ml, this difference is greater than one order of magnitude. These regularities result from the small pore volume of the sorbent (the total pore volume is smaller than 1 ml/g [16]) and the high adsorption capacity of the sorbent at a high LDL content [17]. Thus, it is obvious that the adsorption capacity of the sorbent toward LDLs (*el*) and the pore volume are parameters that affect the rate of oxidation because they are responsible for the distribution of LDLs in a two-phase system, that is, for the concentrations of LDLs in mobile and stationary phases.

Figure 4 demonstrates the concentration dependence of F in semilogarithmic coordinates for liquid and solid phases. The concentrations were calculated with the use of Eqs. (1)–(4). By choosing the calculated concentration of LDLs rather than the concentration C_0 of the initial LDL solution, we simultaneously took into account both the concentration of the initial LDL solution and the phase volumes and thereby revealed the character of F as a function of LDL concentration. It can be seen in Fig. 4 that, to a first approximation, the F–C plot in the above coordinates is adequately approximated by a straight line. In this case, the concentration dependence of F fits well both with data for the liquid phase and with data calculated by Eqs. (1)–(4) for the solid phase with consideration for the nonuniform distribution of LDLs in the system. The linear dependence in the above coordinates suggests an exponential increase in F with concentration regardless of the phase in which LDLs are oxidized. That is, the concentration of LDLs is one of the parameters responsible for the reaction rate of LDL oxidation in the presence of a fullerene-containing sorbent regardless of the phase of the system. The dependence of ln$F$ on C can be described by the uniform equation

$$\ln F = A + BC_i = A + D(t - t_0)C_i, \qquad (5)$$

where i = 1 or 2 for the liquid or solid phase, respectively; t_0 is the adsorption equilibration time, which is equal to 20 min [1]; A and D are empirical parameters of the ln$F$–C relationship, which are numerically equal to 3.4 and 0.002 ml mg^{-1} min^{-1}, respectively. The numerical value of parameter A corresponds to the fluorescence intensity of the initial physiological solution at C_0 = 0. Parameter D in Eq. (5) characterizes the dependence of ln$F$ on the time of contact between the LDL solution and the sorbent. Its value was calculated by an analysis of parameter B (the slope of the ln$F$–C_i plot) as a function of the exposure time of the LDL solution. According to Fig. 5, the time dependence of parameter B is adequately approximated by the straight-line equation

$$B = Dt. \qquad (5a)$$

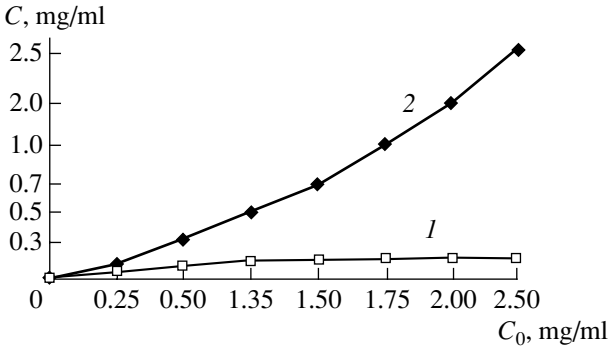

Fig. 3. Distribution of LDLs between (*1*) liquid and (*2*) solid phases as a function of the initial concentration (C_0) of an aqueous LDL solution. The concentrations in the phases were calculated with the use of Eqs. (1)–(4) at V_0 = 2 ml, V_1 = 2 ml, and V_2 = 0.07 ml.

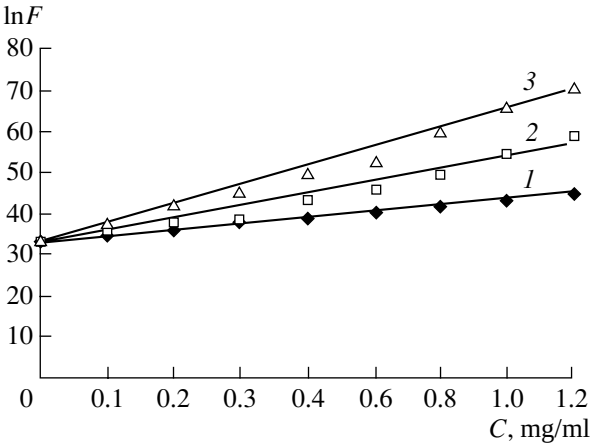

Fig. 4. Fluorescence intensities as functions of concentration of aqueous LDL solutions in liquid and solid phases at the following times of contact with a fullerene-containing silica gel: (*1*) 60, (*2*) 120, and (*3*) 180 min. The concentrations were calculated by Eqs. (1)–(4) at V_0 = 2 ml, V_1 = 2 ml, V_2 = 0.07 ml, *el* = 0.3, and C_0 = 0.5, 1.0, or 2.5 mg/ml.

Thus, the use of statistical thermodynamic approaches for interpreting experimental data allowed us to describe the concentration dependence of F in both of the phases by one Eq. (5) in semilogarithmic coordinates. We also explained higher fluorescence intensities of the solutions of LDL desorbed from the sorbent surface by the nonuniform distribution of LDL in the two-phase system. At the same time, according to the theory of oxidation [18], an exponential character of the F–C relationship is indicative of a free-radical chain process of LDL oxidation in aqueous solution in the presence of the fullerene-containing sorbent. Correspondingly, this process can be explained by a kinetic scheme including chain initiation, propagating reactions, and chain-branching reactions. The experimental data suggest that free radicals can be formed not only in the presence of transition metals (such as Cu^{2+} [2])

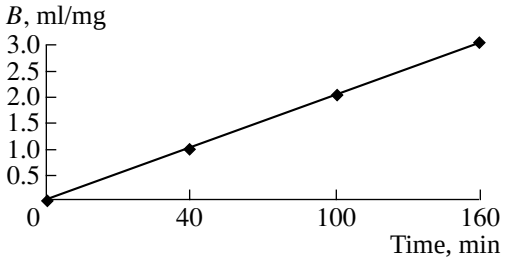

Fig. 5. Effect of the time of contact between aqueous LDL solutions and a fullerene-containing silica gel on the slope B of the $\ln F–C$ plot.

because of one-electron redox reactions but also in the presence of fullerenes, whose specifically degenerated molecular orbitals can take up to six electrons. A conclusion on a free-radical mechanism of the peroxide oxidation of polyunsaturated fatty acids was also supported by the detection of diene conjugates in blood plasma after contact with the fullerene-containing sorbent, because diene conjugation takes place at the step of free-radical formation.

A direct consequence of the heterogeneous oxidation of LDLs in the presence of a fullerene-containing silica gel is the accumulation of lipid oxidation products in blood plasma; these products may deteriorate the status of patients after plasmophoresis. Because of this, the tolerance range of plasmosorption times is an important characteristic of the process of plasmosorption; the knowledge of this range makes it possible to avoid complications. Because the experimental results shown in Fig. 1 demonstrate that lipid oxidation products are formed in the solid phase well before forming in the liquid, the oxidation of LDLs can be subdivided into the following two stages: (1) oxidation that takes place in the solid phase and (2) oxidation that takes place in the liquid phase. However, as found previously [1], the oxidation of LDLs takes place at $t > t_0$ (where t_0 is the adsorption equilibration time). These experimental time parameters seem to be of importance for choosing plasmosorption conditions that prevent the formation of lipid oxidation products in blood plasma, which is recycled back to the body after a correction for the LDL content. They also substantiate the existence of a tolerance range of plasmosorption times, which provides a maximum capacity of the sorbent and minimum damage to blood plasma caused by the presence of lipid oxidation products. For this purpose, it is sufficient to restrict the time of plasmosorption by the induction period t_1 of liquid-phase oxidation and by the equilibration time t_0 of LDL adsorption on silica gel. Finally, the tolerance range of plasmosorption times can be described by the following inequality:

$$t_0 < t < t_1. \quad (6)$$

Thus, we experimentally supported a heterogeneous nature of the oxidation of blood plasma LDLs in the presence of a fullerene-containing silica gel and found considerable differences in the kinetics of LDL oxidation on the sorbent surface and in solution. On this basis, we proposed an inequality for determining the tolerance range of the times of contact between blood plasma and a sorbent at which oxidation products are not formed in a liquid phase.

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