SPECIFIC BINDING OF ^{3}H-PHENOZAN, A SYNTHETIC SCREENED PHENOLIC ANTIOXIDANT WITH ISOLATED RAT ENTEROCYTES

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The writers showed previously that the influence of synthetic antioxidants in vivo on the composition and certain structural parameters of cell membrane lipids is "phasic" in character, i.e., relative to control values, changes in the lipid bilayer during the first 3 h and at later stages after intraperitoneal injection of antioxidants into experimental animals were in different directions [2]. On the basis of the results it was postulated that besides their known pathway of action on cell metabolism due to rupture of radical oxidation chains in membrane lipids [1], synthetic antioxidants also act on the cell by another mechanism, namely through receptor interaction with the plasma membrane of the cell. In the first place, such a suggestion can be made relative to a large group of synthetic antioxidants belonging to the screened phenol class, whose structure is very similar to the structure of natural biologically highly active compounds (catecholamines), for which specific receptors exist on the cell surface.

The aim of the present investigation was to study specific binding of ^{3}H-phenozan, a synthetic antioxidant of the screened phenol class, with isolated rat enterocytes.

EXPERIMENTAL METHOD

Isolated enterocytes were obtained from the small intestine of male Wistar rats by the method described in [3].

Binding in ^{3}H-phenozan with the cells (protein concentration 1 mg/ml) was carried out in 50 mM Tris-HCl, pH 7.3, containing 1 mg/ml of bovine serum albumin. The protein concentration in the samples was determined by the method in [6]. Samples measuring 1 ml in volume were incubated for 60 min at 25°C. Specific binding was defined as the fraction of total binding blocked by a thousandfold excess of unlabeled phenozan. The bound and free ligand were separated by vacuum filtration through GF/C filters (Whatman, England) [5].

A compound belonging to the screened phenol class, namely γ-(4-hydroxy-3,5-di-tert-butylphenol)propionic acid (phenozan), and also ^{3}H-phenozan (specific radioactivity 43.6 Ci/mmole) were used as the water-soluble synthetic antioxidant. Phenozan and ^{3}H-phenozan were generously provided by V. V. Eroshov and L. G. Plekhanova, on the staff of the Institute of Chemical Physics, Academy of Sciences of the USSR, and also by N. F. Myasoedov, on the staff of the Institute of Molecular Genetics, Academy of Sciences of the USSR.

EXPERIMENTAL RESULTS

The experiments showed that ^{3}H-phenozan binds reversibly with enterocytes of the rat small intestine (Fig. 1). Analysis of isotherms on Scatchard plots (Fig. 2) showed the presence of two types of specific binding sites for the antioxidant with $K_{D_1} = 0.5 \pm 0.1$ nM and $K_{D_2} = 7.5 \pm 0.5$ nM; the concentration of binding sites was $B_{max_1} = 0.025 \pm 0.005$ pmole/mg protein and $B_{max_2} = 0.2 \pm 0.02$ pmole/mg protein, respectively. Displacement of ^{3}H-phenozan from the specific binding site by an excess of the unlabeled ligand was recorded significantly when concentrations of the radioactive preparation in the incubation medium did not exceed 20 nM, for which ^{3}H-phenozan in concentrations of over 20 nM, determination of the specific character of binding of the antioxidant by enterocytes was made difficult by the

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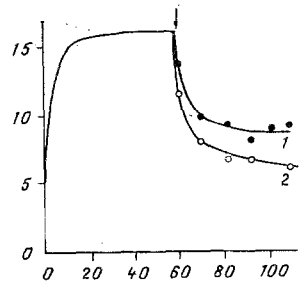

Fig. 1

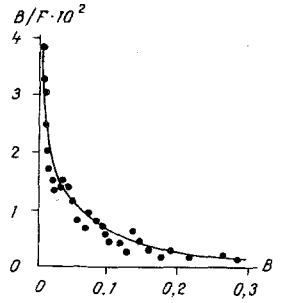

Fig. 2

Fig. 1. Relationship of the bound ^{3}H-phenozan (6 nm) with the isolated rat enterocytes at 25°C. The protein concentration of cell suspension was 1 mg/ml. Along the axis time is in minutes; along the ordinate, binding of ^{3}H-phenozan (in disintegration of 1 mg protein/min·10^{-2}). 1) Addition of thousand-fold excess of unlabeled phenozan. 2) Ten-fold dilution in the incubation medium.

Fig. 2. Specific binding of ^{3}H-phenozan with the isolated rat enterocytes. The data are given on Scatchard plots. The incubation of cell suspension was at 25°C, the protein concentration in a suspension of 1 mg/ml. B) Total concentration of complexes (in nM); F)-concentration of free ligands (in nM).

considerable nonspecific sorption of the preparation on the cell surface. Moreover, when the concentrations of free ligand in the incubation medium were calculated, during work with such low nanomolar concentrations of ^{3}H-phenozan, the fraction (very considerable) of the labeled preparation adsorbed on the walls of the tubes had to be taken into account, for otherwise correct calculation of the specific binding parameters of ^{3}H-phenozan with the cells would be impossible. Within the concentration range from 20 to 10 μM of total concentration of ^{3}H-phenozan in the incubation medium, total binding of the labeled preparation per milligram protein of cell suspension was of the order of 1.5%. During specific binding of ^{3}H-phenozan with enterocytes, saturation took place in the course of 10 min during incubation at 25°C.

Thus the presence of specific binding sites of a phenol-containing antioxidant on plasma membranes of rat enterocytes was demonstrated. It can be tentatively suggested that the process of specific binding of phenozan is determined by the close agreement between its structure and the chemical structure of several natural phenol-containing ligands, a competitor of which for specific binding sites is most probably the antioxidant specified above. By now indications have appeared in the literature that during analysis of the action of different amphiphilic substances on the cell, both the direct nonspecific effect on membrane permeability, the change in flowability of the bilayer, and so on, and also the corresponding effects of these substances on membrane proteins (in particular, receptor proteins), and also direct interaction of the preparations with these proteins [4], must be taken into account. It can be concluded from all the facts described above that when the biological effects of phenol-containing antioxidants are assessed, attention must be paid not only to nonspecific interaction of these substances with biomembranes, as a result of which the physicochemical parameters of the membrane bilayer are changed and, in particular, those which depend on the level of lipid peroxidation [2], but also to complex-formation of antioxidants with specific binding sites on cell plasma membranes. The latter can explain the biological effects of phenolic antioxidants in below micromolar doses. The results of this investigation indicate that synthetic antioxidants of the screened phenol class may be used as polyfunctional biologically active compounds possessing several different mechanisms of action on cell metabolism.

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EFFECT OF HYDROGEN PEROXIDE ON INACTIVATION OF CYTOCHROME P-450

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In rabbit liver microsomes cytochrome P-450 is inactivated in reactions of hydroxylation of substrates I (dimethylalanine, amidopyrine, p-nitroanisole) and II (aniline) types. Inactivation of the enzyme is potentiated in the presence of the catalase inhibitor NaN_3 [1, 3]. This indicates the possibility of damage to the hemoprotein by hydrogen peroxide (H_2O_2), formed in the NADPH-dependent hydroxylase cycle. Inactivation of cytochrome P-450 is accompanied by its decolorization. Inactivation of other electron-carrier enzymes under these same conditions has not been found [4].

The aim of this investigation was to study inactivation of purified and isolated cytochrome P-450 LM_2 under the influence of H_2O_2.

EXPERIMENTAL METHOD

Reagents. The sodium dihydrogen phosphate, phenobarbital sodium, and NaN_3 were obtained from Merck (West Germany), catalase, glucose, β-mercaptoethanol, and sodium dodecylsulfate were from Serva (West Germany), the 5,5-dithio-bis-(2-nitrobenzoic) acid was from Boehringer (West Germany), and emalgen 13 was from Kao-Atlas (Japan). The remaining reagents were of highly pure grade.

Cytochrome P-450 LM_2 was isolated from liver microsomes of rabbits treated with phenobarbital [2]. To remove the dithiothreitol from the preparation of cytochrome P-450, dialysis was carried out overnight at 4°C against 100 mM sodium-phosphate buffer (pH 7.4).

The concentration of cytochrome P-450 was measured on Hitachi-557 and HP-85 spectrophotometers using a two-wave system, by the method in [7].

The glucose-oxidase system (GOS) generating H_2O_2 contained 100 mM sodium-phosphate buffer (pH 7.4), 110 mM glucose, and 1 μM cytochrome P-450. The reaction was started by addition of glucose oxidase (0.18 U/ml) with activity of 93,200 U/g at 37°C. The concentration of glucose and glucose oxidase were chosen so that the rate of H_2O_2 generation corresponded to the rate of its generation in the NADPH-dependent hydroxylase cycle. The H_2O_2 concentration was determined by the thiocyanate method [10]. The content of SH-groups in the protein molecule was determined with the aid of 5,5-dithio-bis-(2-nitrobenzoic) acid [5].

The aggregate state of the cytochrome P-450 was studied by gel-penetrating high efficiency liquid chromatography on TSK-G 3000 SW columns (KLB, Sweden) and also by gel-filtration on TSK-CELTOYOPEARL HW-60 (Toyo Soda, Japan).

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