The Inhibition of Glutathione Reductase by Quinones
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Abbreviations: GSSG, oxidized glutathione; GSH, reduced glutathione; DTT, dithiothreitol; TN, turnover number; TN/Kmax, bimolecular rate constant.

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Introduction
Glutathione reductase (EC 1.6.4.2) contains FAD and redox active disulfide in the active center (1–3) and catalyzes the reversible oxidation of NADPH by GSSG. This enzyme is responsible for the maintenance of sufficiently high concentrations of GSH and is one of the key enzymes in the protection against the “oxidative stress” – an increased concentration of radicals and other activated forms of oxygen (4). Therefore its inhibitors and inactivators are often used as anti-tumour, antiparasitic or antimalarial agents (5–7). It is supposed that heteroaromatic inhibitors of glutathione reductase bind to the interface domain at the site which is distinct from NADP(H) and GSSG binding sites (7, 8).

The data of present communication show that certain quinones, including the naturally occurring ones may act as potent inhibitors of glutathione reductase. This may be an additional factor of their toxicity apart from their redox cycling and reaction with cellular nucleophiles (9).

Materials and Methods
Yeast glutathione reductase (Sigma) was additionally purified up to A340/A460 = 7.7–7.9 [1]. The enzyme concentration was determined using ε460 = 11 mM−1 cm−1. NADPH, NADP+ and DTT (Serva), GSSG and GSH (Chemapol, Czechoslovakia), rhein (4,5-dihydroxy-9,10-anthraquinone-2-carboxylic acid, Aldrich), duroquinone (Sigma) were used as received. Alizarin (2,3-dihydroxy-9,10-anthraquinone), 9,10-anthraquinone-2-sulfonate, 1,5-dioxy-9,10-anthraquinone and 9,10-phenanthrenequinone (Reakhim, USSR) were recrystallized from ethanol or benzene. Christophanol (2-methyl-4,5-dihydroxy-9,10-anthraquinone) was extracted from the cultural fluid of Verticillium dahliae, javanicin (2-methoxymethylene-3-methyl-5,8-dioxy-7-methoxy-1,4-naphtoquinone) – from Fusarium decemcellulare, and pigment G-2N (2-methyl-4,7,9,12-tetraoxy-5,6-dihydropyrene[al]-naphtacene-8,13-dione) – from Frankia sp. ANP 190107 as described earlier [10, 11]. The structural formulae of some uncommonly used quinones are presented in Fig. 1. The reaction rate was determined using a Hitachi-557 spectrophotometer according to the NADPH absorption change (Δε340 = 6.2 mM−1 cm−1) at 25 °C in 0.1 M K-phosphate-citrate, pH 7.0, containing 1 mM EDTA. The enzymatic GSH-linked reduction of NADP+ was done in the presence of 10 mM DTT [12]. TN of the enzyme is a number of NADP(H) consumed by FAD per 1 s.

Results and Discussion
By analogy to previous data [1, 2], the GSSG-linked oxidation of NADPH by glutathione reductase follows a “ping-pong” mechanism. TNmax of the enzyme (at saturating concentration of both substrates) at pH 7.0 is 240 s−1.

Since the partially substituted quinones may react with GSH formed during the reaction of glutathione reductase [9], only the fully substituted compounds were tested. It was found that rhein was potent inhibitor of glutathione reductase, acting as the mixed-type inhibitor for NADPH (Fig. 2). The inhibition is not linear according to the in-

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Fig. 1. Structural formulae of quinones used: chrisophanol (1a), rhein (1b), javanicin (II), G-2N (III).

The double reciprocal plot of inhibition degree vs. inhibitor concentration [6] gives \( K_i = 1.1 \mu M \) and the maximal degree of inhibition, 93%. The inhibition of rhein is linear according to the slopes (reciprocal TN/\( K_m \) of NADPH) giving \( K_i = 8 \mu M \). Javanicin was another mixed-type inhibitor possessing \( K_i \) of 70 \( \mu M \) and 100 \( \mu M \) (according to intercepts and slopes). Other quinones were either linear competitive inhibitors (alizarin, \( K_i = 20 \mu M \); chrisophanol, \( K_i = 34 \mu M \); G-2N, \( K_i = 40 \mu M \); 1,5-dioxyanthraquinone, \( K_i = 70 \mu M \)), either uncompetitive inhibitors for NADPH (duroquinone, \( K_i = 200 \mu M \); anthraquinone sulfonate, \( K_i = 50 \mu M \); phenanthrene quinone, \( K_i = 1.8 \mu M \), maximal inhibition degree, 73%). Since phenanthrene quinone was the oxidant of glutathione reductase (TN\(_{max}\) = 5 s\(^{-1}\), TN/\( K_m \) = \( 10^4 \) M\(^{-1}\) s\(^{-1}\)) the inhibition patterns were obtained after the substraction of rates of this reaction. All quinones tested were the uncompetitive inhibitors for GSSG.

The reverse reaction of glutathione reductase - GSH-linked reduction of NADP\(^+\) is characterized by a transition from “ping-pong” to “sequential” mechanism at high concentrations of NADP\(^+\) [12]. This is indicated by the transition of a series of parallel lines to the converging ones in the Lineweaver-Burk plots (Fig. 3). In this case rhein de-
increases TN/K_m of NADP^+ and does not affect TN_max, while anthraquinone sulfonate almost does not influence the reaction kinetics.

The data presented indicate that certain quinones (rhein, phenanthrene quinone) are potent inhibitors of glutathione reductase, similar to certain flavin derivatives, used as antimalarial agents [7]. Thus the additional factor of influence of quinones on the system of glutathione metabolism is demonstrated, which may attribute to their toxicity to some extent. This is especially interesting for naturally occurring oxyquinones which activity is associated with the inhibition of mitochondrial electron transport chain (rhein, G-2N) [11, 13] or redox cycling (javanicin) [10]. It seems that quinones being uncompetitive inhibitors for NADPH bind to the interface domain of enzyme which is distinct from NADP(H) and GSSG-binding sites [8]. Such a character of binding is proposed for flavins and nitrofurans [6, 7], which inhibit glutathione reductase in a similar way. Evidently, oxyquinones being competitive inhibitors for NADPH bind at its binding site in oxidized glutathione reductase. It is known that certain oxyquinones bind to NAD(P)H-binding centers of flavoproteins [13, 14]. The mixed character of inhibition of rhein (Fig. 2) and javanicin may be explained by their binding both at the NADP(H)-binding site and at the interface domain. The binding of rhein to the NADP(H)-binding site is evident from data of Fig. 3. The reasons for different specificity of rhein comparing to other oxyquinones remain unclear. It is possible that 2-carboxyl group of rhein takes a definite part in the complexation, since an analogous 2-methyl-substituted compound, chrisophanol (Fig. 1) inhibits glutathione reductase in a different way.