METHODS

Evaluation of Biological Activity of Toxic Agents in a Unicellular Model

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Combined toxicity of inorganic substances: Ag(I)—Cu(II), Cr(VI)—Ni(II) is studied in a culture of *Saccharomyces cerevisiae* yeasts. The dynamics of yeast growth in the presence of individual metals an their combinations is examined. A diagram method for the evaluation of combined toxicity is proposed.

Key Words: *silver; copper; chromium; nickel; biological objects; combined toxicity*

Evaluation of biological activity of various toxic agents with the use of protozoa has a number of advantages over the use of laboratory animals. Protozoan cultures provide sufficient information, are less expensive, and can be employed as basic models for evaluation of toxic activity towards the cells of a higher organism. Hormonal, neurophysiological and other systemic factors of higher organisms are considered theoretically, which is regarded as a drawback of cell models [9].

Biotransformation of d-elements in a unicellular organism is quiet simple [4]. The following processes occur upon interaction of a d-element with a cell: dissolution in the extracellular medium, contact with the plasma membrane, transport of its ions or poorly soluble forms through ion channels or by phagocytosis, intracellular dissolution and distribution, binding to macromolecules or other ligands, and modulation of the degree of its oxidation. It can be hypothesized that analogous processes occur in animal cells [4].

Since DNA serves as a genetic template in all biological systems (from bacteria to man), the use of cell models is feasible. Thus, the molecular mechanisms underlying genetic toxicity of various ions are identical for biological systems at different levels of evolution.

It was demonstrated that unicellular organisms can be used for the elucidation of stress mechanisms operating in higher nervous activity [12]. It is noteworthy that unicellular models can be employed for the investigation of combined action of inorganic compounds.

Combined action of chemical compounds has been poorly investigated. The mechanisms underlying binary effects are known only for a small number of systems [6-8,13]. There is a variety of problems associated with quantitative estimation of combined toxicity. Evaluation of combined toxicity requires large experimental series with a wide concentration range of the studied compounds. This is necessary for substantiating the toxicological profile and statistical correctness of the results. In our view, none test system employing multicellular organisms can meet this demand.

Previously, we studied combined effects of inorganic compounds and γ-radiation on animal cells [2]. The kinetics of the release of endogenous inorganic elements caused by combined action of the salts of these elements was studied in mammals [10]. Combined effects of essential and contaminating elements upon their accumulation in plants and animals have been investigated [1,11]. Basic principles

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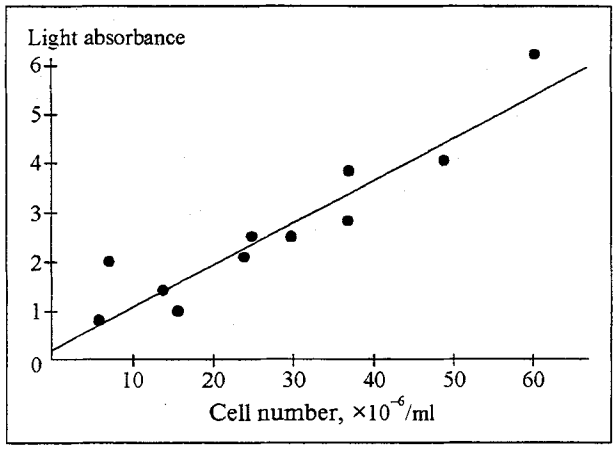

Fig. 1. Light absorbance—cell content relationship.

for evaluation of toxicity at metabolic and physiological levels have been developed [3].

In the present study we examined individual and combined effects of copper, silver, chromium, and nickel salts on *Saccharomyces cerevisiae* yeasts.

MATERIALS AND METHODS

The salts of the studied metals were added to the culture medium before seeding the yeast cells: $AgNO_3$ and $Cu(NO_3)_2$ to a final concentration of 5×10^{-7}-5×10^{-6} and 5×10^{-4}-5×10^{-3} mol/liter, respectively, and solutions $K_2Cr_2O_7$ and $NiSO_4$ to a final concentration 5×10^{-4}-5×10^{-3} mol/liter. These concentration ranges were selected empirically: from the concentration at which the metal produces neither toxic nor biological effect to the concentration inhibiting growth and reproduction of the yeasts.

Distilled water was added to control test tubes. The yeasts were grown for 96 h at 28°C on malt wort (dry matter content 6-8%, pH 5.6-6.0). Samples were taken after 0, 24, 28, 32, 48, 52, 56, 60, 72, 76, and 96 h of culturing.

Cell growth was assessed by light absorbance (λ=590 nm, a KFK-2 photoelectrocalorimeter) and cell counting in a Goryaev chamber, after which a calibration curve was constructed (Fig. 1, dilution is taken into consideration). The culturing time—cell number plots (Figs. 2 and 3) were then analyzed, and the effects of silver, copper, chromium, and nickel salts were evaluated.

TABLE 1. Individual and Combined Effects of Silver and Copper Nitrates on *Saccharomyces cerevisiae*

Sample No.	Ag^+, mol/liter	Cu^+, mol/liter	Combined effect
1	5×10^{-7} "—"	5×10^{-4} "—"	"+"
2	8×10^{-7} "—"	8×10^{-4} "+"	"+"
3	1×10^{-6} "+"	1×10^{-3} "+"	"+"
4	2×10^{-6} "+"	2×10^{-3} +	+
5	5×10^{-6} +	5×10^{-3} +	+

Note. "—" = no effect, "+" = cytostatic effect, + = cell death.

RESULTS

Individual effects of silver, copper, nickel, and chromium salts as well as effects of silver—copper and nickel—chromium combinations differed from each other. At 5×10^{-7}-10^{-6} mol/liter, silver ions had no appreciable effect on the yeast growth: the experimental curve coincided with the control (Fig. 2, *a*). At higher concentration (2×10^{-6} mol/liter) a cytotoxic effect was observed, and the light absorbance reached the maximum 5 h later than in the control. A 2.5-fold increase the $AgNO_3$ concentration (5×10^{-6} mol/liter) resulted in cell death.

The concentrations at which copper ions produced cytostatic and cytotoxic effects are about three orders of magnitude higher compared with those of silver ions. However, similar to silver, copper produced cytostatic effect in a relatively narrow concentration range (Table 1).

When added together, silver and copper exhibited cytostatic activity at concentrations at which their individual effects were not observed (Fig. 2 and Table 1). Although silver (8×10^{-7} mol/liter) had no effect in sample 2, in combination with copper (8×10^{-4} mol/liter) it had cytostatic effect. Hence, it can be suggested that copper ions play a more important role in this combination.

Since the curves reflecting cell growth in culture are unnecessary for evaluating combined effects of the studied compounds, we have employed special approaches.

Chromium and nickel acted as synergists (Fig. 3). When added together to culture medium in the concentration range 5×10^{-4}-5×10^{-3} mol/liter, $K_2Cr_2O_7$ and $NiSO_4$ had a more pronounced inhibitory effect on the growth of yeast cells in comparison with their individual effects.

Individual cytostatic effects of $NiSO_4$ were observed at concentrations higher than 2×10^{-3} mol/liter and for $K_2Cr_2O_7$ at concentrations higher than 5×10^{-3} mol/liter.

Two- and three-dimensional dose—effect bolograms can be employed for characterization of combined action [5].

In the present study, the response of the yeasts to silver—copper and chromium—nickel combination was calculated as the ratio of the difference between the experiment and the control to the control. Figures 4 and 5 show experimental and theoretical dose—

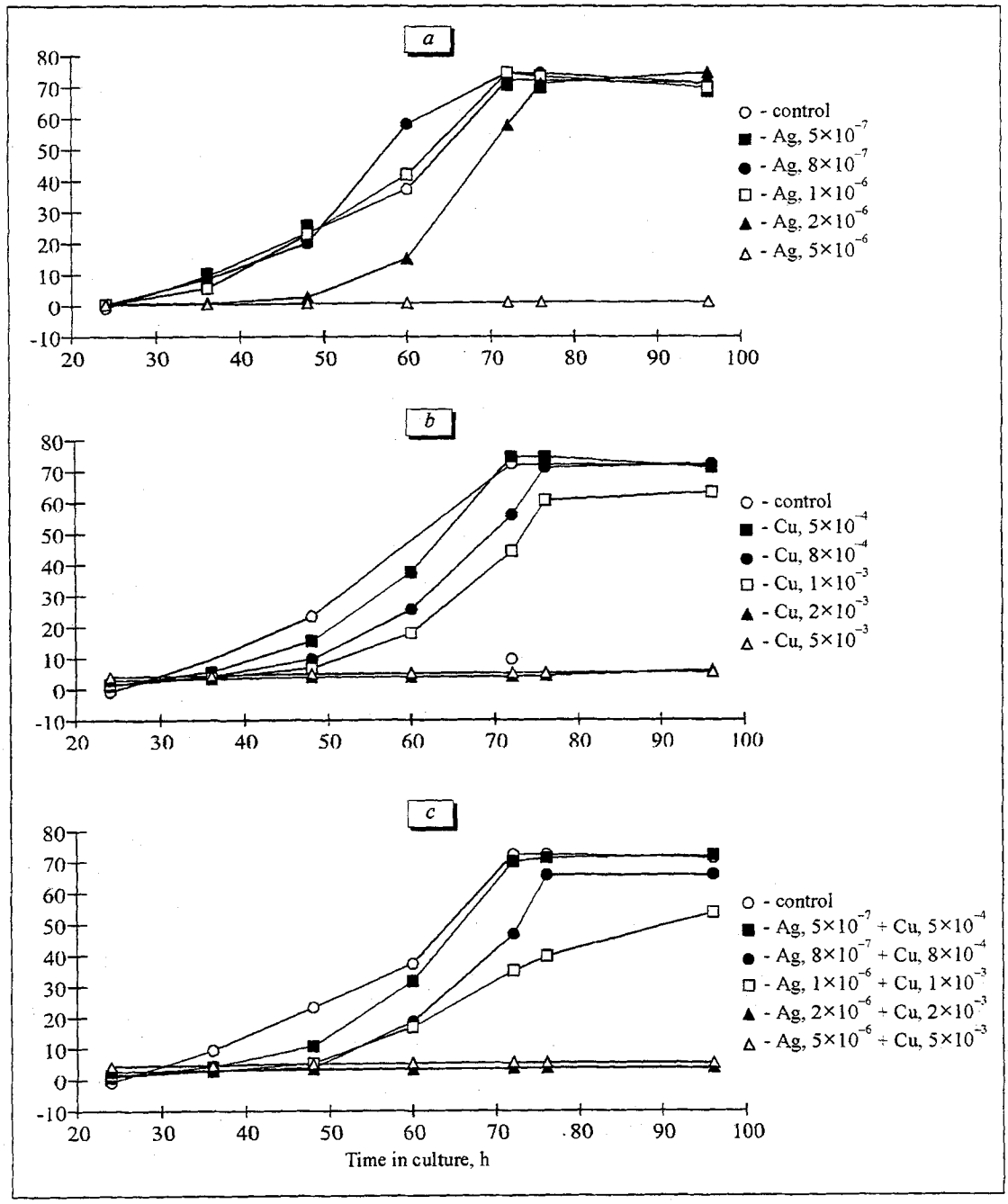

Fig. 2. Effect of silver (*a*), copper (*b*), and silver+copper (*c*) combination on the growth of *S. cerevisiae* 776. Here and in Fig. 3: ordinate: cell number, $\times 10^{-6}$/ml.

response curves. Theoretical curves were constructed by summing individual effects [8] calculated from the following formula:

$$R = \frac{[N^{Ag}{}_{exp} - N^{Ag}{}_{c}] + [N^{Cu}{}_{exp} - N^{Cu}{}_{c}]}{N^{Ag}{}_{c} + N^{Cu}{}_{c}},$$

where, N_{exp} and N_c is the number of cells in the presence of the given ion in experiment and control, respectively.

A comparison of theoretical and experimental curves shows that combined action cannot be interpreted as a sum of individual effects. Specifically, the sum of individual toxic effects of copper and silver ions is equal to their combined effect but only in the high concentration range: 5×10^{-6} mol/liter for Ag^+ and 5×10^{-3} mol/liter for Cu^{2+}. At lower concentrations the differences are statistically significant (Fig. 4). In this case the experimental curve is above the theoretical curve, indicating that copper and silver act as synergists [7].

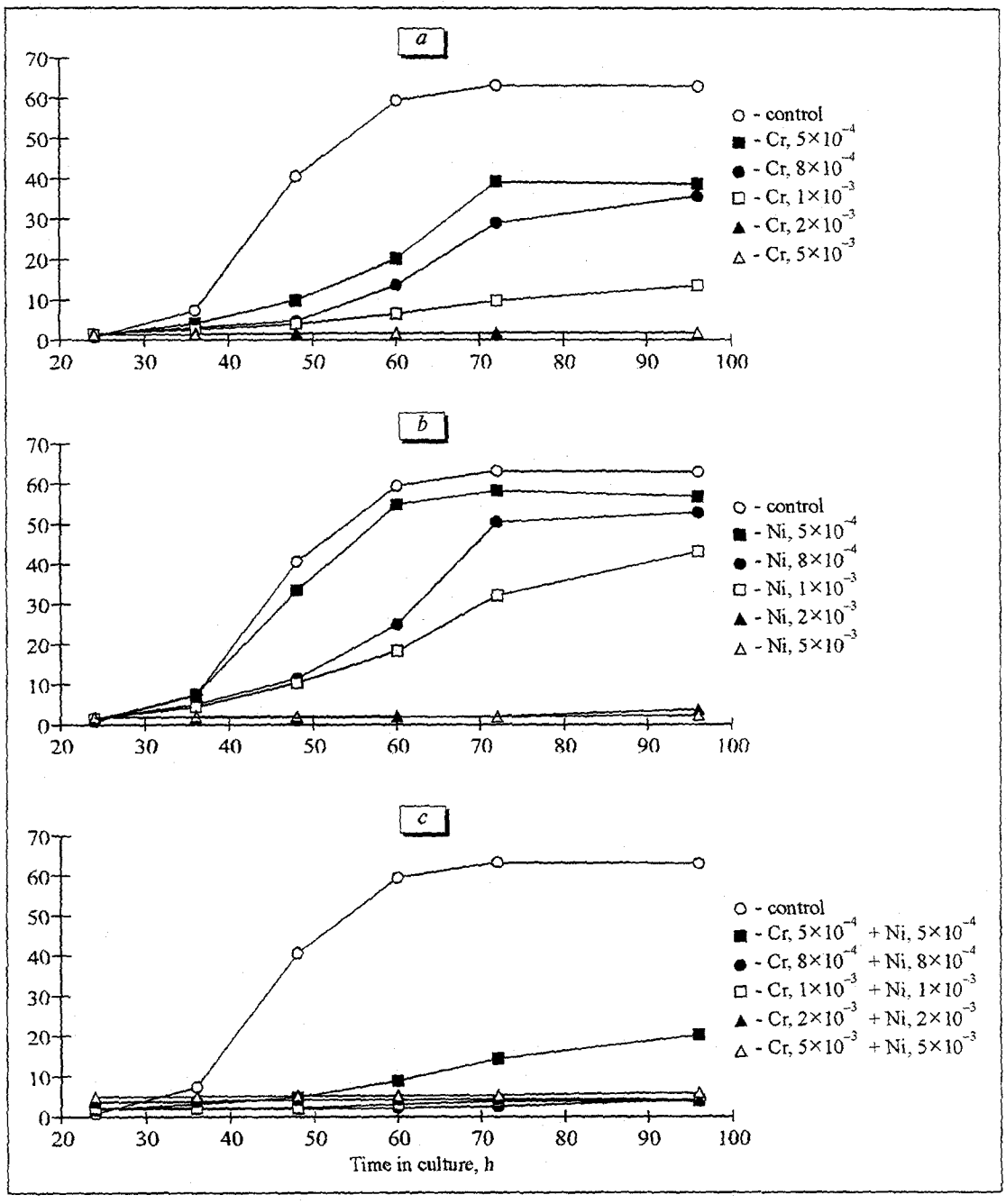

Fig. 3. Effect of chromium (*a*), nickel (*b*), and chromium+nickel (*c*) combination on the growth of *S. cerevisiae* 776.

A similar situation was observed in experiments with Cr(VI) and Ni(II): their effect was additive in the concentration range $>2\times10^{-3}$ mol/liter, at lower concentration it was synergistic. Since the dose—effect isoboles for nickel and chromium lie close to each other, we managed to employ the method for evaluation of combined action [7]. The "sail line" obtained at a constant total concentration of Ni and Cr equal to 1.5×10^{-3} mol/liter is convex towards the abscissa (dose). As expected, the corresponding experimental point is above the "sail." The effect of nickel in this combination is prevailing: the "sail" is raised in the region of nickel higher concentration (Fig. 6).

Thus, the effects of silver—copper and nickel—chromium combinations are examined for the first time in *S. cerevisiae* yeasts (strain 776). New methodological approaches have been employed in the experiments: construction of the growth curves, moment of the toxic agent addition, and choice of the concentration range. A modified method for the evaluation of combined effects of inorganic sub-

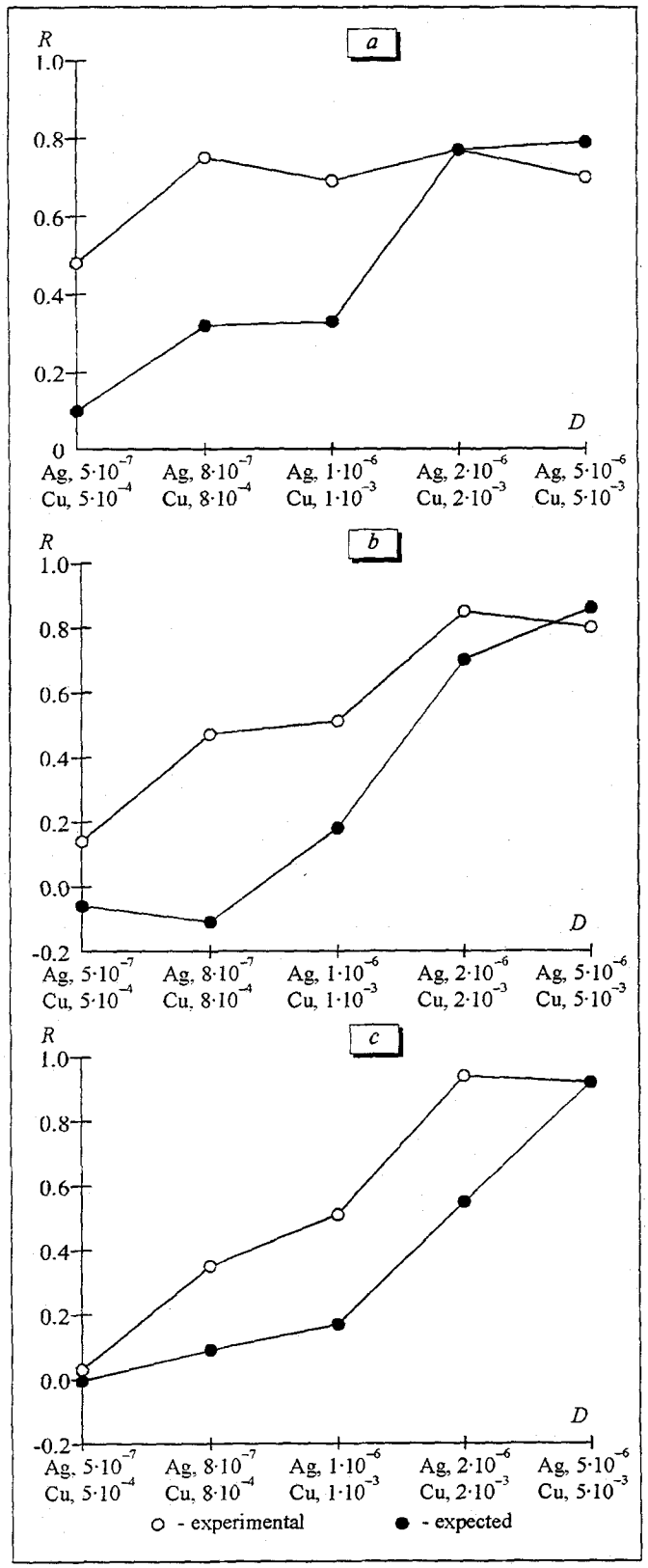

Fig. 4. Dose (*D*)—response (*R*) curves for combined action of silver and copper during 48 h (*a*), 60 h (*b*) and 72 h (*c*).

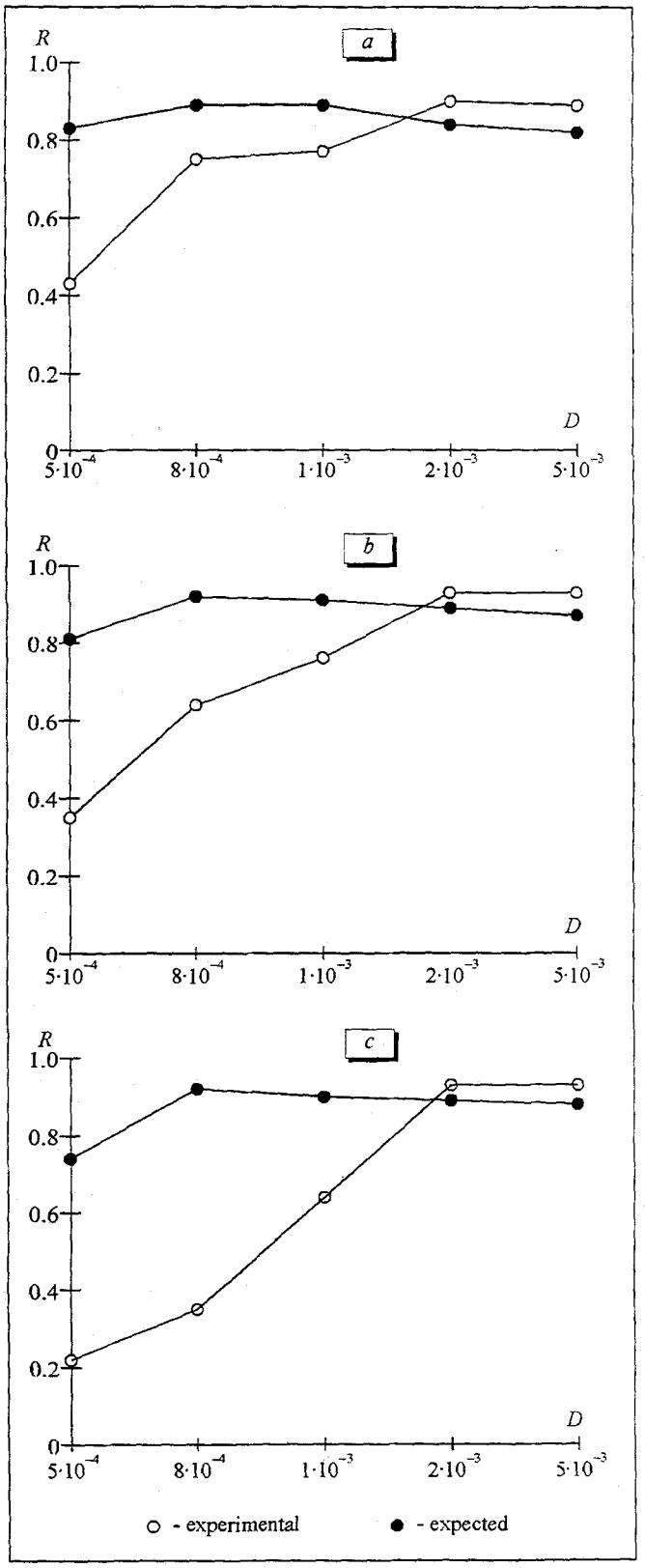

Fig. 5. Dose (*D*)—response (*R*) curves for combined action of chromium and nickel during 48 h (*a*), 60 h (*b*) and 72 h (*c*).

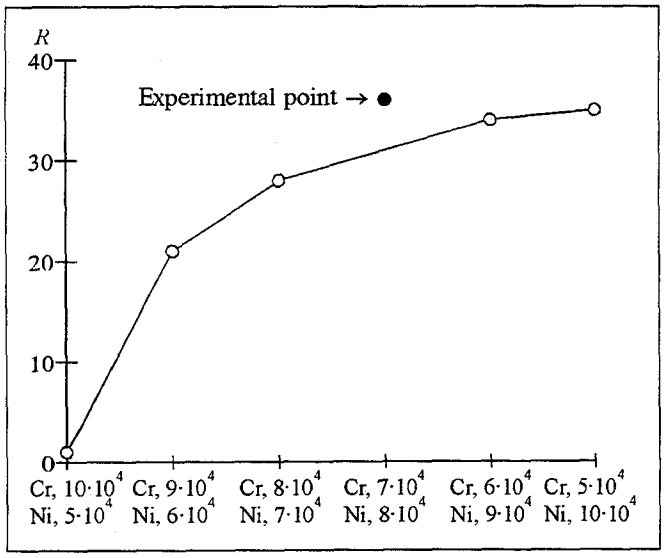

Fig. 6. "Sail line" for combined action of $K_2Cr_2O_3$ on yeast cells [7].

stances has been proposed. It is shown that combined effects of Cu, Ag, Ni, and Cr are additive or synergistic depending on their concentrations in a combination.

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