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The Importance of Synergistic Interaction at Low Intensity of Physico-Chemical Agents

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Abstract

Experimental data obtained for the simultaneous action of hyperthermia with different physical or chemical agents on various cellular systems give evidence that the lesser the intensity of physical factor or the concentration of chemical agents, the lower the temperature has to be used to provide the highest or a definite level of synergistic interaction. On this basis, it is inferred that the synergism may take place at small intensities of harmful environmental factors existing in the biosphere. Hence, the assessment of health or environmental risks should take into account the synergistic interaction between harmful agents.

INTRODUCTION

Combined exposures are an essential feature of modern life. It is well known that almost all physical and a wide array of chemical harmful agents, both natural and man made, are capable of interacting with each other in a synergistic manner when the final biological effect exceeds the sum of individual effects produced by interacting agents. Therefore any risk assessment must consider the question whether combined

effects will influence the health outcome. Of all possible situations of combined actions, long term exposure of living objects to low levels of the agents widely presented in the nature is especially important. However, the assessment of potential significance of synergistic interaction between adverse environmental factors acting together at the level of intensity and concentration found in biosphere is still an intriguing and unresolved problem. Real experiments with low intensities found in environmental and occupational settings is prone to large uncertainties. A feasible approach to this problem is to analyze the dependence of the efficiency of synergistic interaction on the intensity of agents used. Hypothetically, some possibilities could be realized. The case when synergism is decreasing with intensities of deleterious agents applied is unimportant. The same is true for the situation when a decrease in the intensity of one factor should be accompanied by an increase in the intensity of another to retain their synergistic interaction at the same level. The only possibility would be of great importance: if the lesser intensity of one of the agents is applied, then the smaller intensity of another agent should be employed for the display of the highest or a definite level of synergy. In such a case, it would be expected that even at low intensity environmental agents may, in principle, interact with each other in a synergistic manner and thereby to enhance their harmful action. In this paper, we present conclusive evidence confirming the last opportunity for various cellular systems and acting noxious agents.

MATERIALS AND METHODS

Zygosaccharomyces bailii haploid and *Saccharomyces cerevisiae* diploid (strain XS800) yeast cells were used in the experiments. Yeast cells were incubated before irradiation for 3-5 days at 30^{o}C on a complete nutrient agar layer to a stationary phase. Aliquots with 10^{6} yeast cells/ml were exposed to various physical agents applied alone or combined with hyperthermia. We used a ^{60}Co γ-ray source Gammacell 220, Atomic Energy of Canada LTD. The γ-ray dose-rate, estimated by a Siemens ionization chamber, was 10 Gy/min. The electron beam from a 25 MeV pulsed linear accelerator was also used in these experiments. The pulse duration at half peak output was 2.7 μs and the pulse repetition rate was at 50 Hz, the average dose rates were 5, 10, 25 and 250 Gy/min as determined by ferrous sulphate dosimetry.

For ultraviolet (UV) irradiation, the cells were exposed with germicidal lamps that emitted predominantly UV light of wavelength 254 nm. Variation of the intensity was achieved by means of calibrated metal wire nets. The fluence rates were measured using a calibrated General Electrical germicidal meter. To avoid photoreactivation, UV exposure, dilution and cell plating were performed under red ambient light, while post-irradiation incubation was carried out under dark conditions.

Ultrasound (20 kHz) was generated by a Fisher sonic dismembrator (Model 300). The ultrasonic dose rates were 0.05 and 0.2 W/cm^2 which was measured by the calorimetric method. To determine the effect induced by ultrasound alone, the absorbed ultrasound heat was completely removed by cooling with water.

Experiments on the simultaneous action of high temperature and other agents were performed as follows. The special dish and tube containing 1.4 (for UV light irradiation) or 9.9 (for ionizing radiation and ultrasound exposures) ml of sterile water was preheated to a specified temperature which was maintained with an accuracy of ±0.2°C. Cell suspension was added immediately before irradiation or sonication (an 0.1-ml aliquot containing $1.5x10^7$ cells for UV-light and 10^8 cells for ionizing radiation and ultrasound exposures). For the simultaneous action of hyperthermia and other physical or chemical agents, the time interval between the introduction of the cells into the preheated water and the beginning of exposure was about 0.1-0.3 min, which was significantly less than the total treatment time. At the end of the treatment, the samples were rapidly cooled to room temperature, and, hence, the exposure to high temperature and another physical agent lasted for the same period of time. Thereafter, cell suspension was diluted to a necessary concentration and plated onto the standard nutrient medium to determine the cell survival by the method of macrocolonies. All experimental series were repeated 3-5 times. The linear regression calculation based on the multi-target single-hit mathematical model was used to fit the survival curves. The details were described elsewhere (Petin and Zhurakovskaya 1995; Petin *et al*. 1997, 1999).

RESULTS

It is now generally accepted that the highest synergistic interaction is observed under the simultaneous action of harmful agents. The increasing in the interval

between exposure results in a diminution of synergy. That is why in this paper we analyse only simultaneous application of agents. Cell killing is the main end-point envisaged. Fig. 1 provides an example of the basic experimental data used in this investigation. To estimate quantitatively the sensitization action of hyperthermia, one can apply the thermal enhancement ratio (Stewart and Denekamp 1978) defined as the ratio D_3/D_1 or t_3/t_1 (Fig. 1). This ratio indicates an increase of cell radiosensitivity by high temperature. However, it does not reflect the kind of interaction (whether it was independent or synergistic). To calculate the synergistic effect we used the synergistic enhancement ratio (k), defined as the ratio of the calculated radiation dose (assuming an additive effect of radiation and hyperthermia) to that observed from the experimental survival curve for the simultaneous action of radiation (or other agents employed) and hyperthermia at a fixed level of survival. For example for 1% survival, $k = D_2/D_1 = t_2/t_1$ (Fig. 1). For exponential survival curves, this parameter is independent of the survival level for which it is calculated. For sigmoidal survival curves, the synergistic enhancement ratio was calculated for 10% survival. Both these parameters are plotted in Fig. 2 against the irradiation temperature for XS800 diploid *Saccharomyces cerevisiae* cells simultaneously exposed to ^{60}Co γ-rays (10 Gy/min) and high temperature. The noticeable feature of Fig. 2 is that the thermal enhancement ratio (curve 1) increases indefinitely with increasing exposure temperature, while the synergistic enhancement ratio (curve 2) at first increases, then reaches a maximum, which is followed by a decrease. This implies that the synergistic interaction between hyperthermia and ionizing radiation is observed only within a certain temperature range. Noteworthy is the fact that such a dependence of synergistic effect on temperature under which the exposure was occurred was also obtained upon the simultaneous combination of hyperthermia with UV light (Petin et al. 1997, 2000), ultrasound (Petin et al. 1999; Komarova et al. 2000) and some chemical inactivating agents (Lisovsky et al. 1994; Pantukhina and Petin 1999). Hence, one can conclude that for a given intensity of physical factors or concentration of chemical agents there would be a specific temperature that maximizes the synergistic interaction. Any deviation of the acting temperature from optimal value results in a decrease of synergism.

To evidence the importance of synergistic effects at low intensity of inactivating agents, we analysed the dependence of synergistic interaction on the intensity of physical factors or on the concentration of chemical agents applied in combination with hyperthermia. Using survival curves data published for simultaneous action of hyperthermia and ionizing radiation on bacteriophage (Trujillo and Dugan 1972), bacterial spores (Reynolds and Garst 1970; Reynolds and Brannen 1973), yeast (Petin and Berdnikova 1979; Petin and Zhurakovskaya 1995) and mammalian cultured cells (Ben-Hur et al. 1974; Ben-Hur 1976), we were able to calculate the synergistic enhancement ratio for various cell systems and irradiation condition. It allowed us to establish the correlation between the dose rate and the exposure temperature, which both provide maximum or other arbitrary levels of synergistic interaction (Fig. 3). Open circles denote the results of our calculations based on experimental results published in just now cited papers. The value of synergism was the highest for bacterial spores (k_{max} = 2.2), diploid yeast cells (k_{max} = 1.7) and was intermediate for bacteriophage (k = 1.3) and cultured mammalian cell (k = 2.2). This was due to the fact that for last cell systems the highest synergistic effect was not obtained for all dose rates used in the experiments. One can see that linear relationships are found between these values for various cellular objects. This means the general importance of the dose rate of ionizing radiation in the manifestation of synergistic interaction. It can be inferred that the temperature at which ionizing radiation is delivered should be diminished to obtain the maximum or a definite synergistic effect with dose rate decreasing and *vice versa*.

To check the universality of this regularity the data on simultaneous effect of hyperthermia combined with UV light (Petin et al. 1997, 2000) or ultrasound (Petin et al. 1999; Komarova et al. 2000) on yeast cells, as well as with tris(1-aziridinil)-phosphine sulfide (thio-TEPA) (Johnson and Pavelec 1973) and cis-diamminedichloroplatinum (II) (cis-DDP) (Urano et al. 1990) on cultured mammalian cells were involved. The last two set of data include the relationship between exposure temperature, concentration, and rate of cell inactivation for chemical agents used in clinical chemotherapy. Hence, they have no direct attitude toward environmental harmful agents and they used here to verify the universality of the rule manifested. Using data published in the above cited works, we could obtain the

relationships between the intensity of physical factors or the concentration of chemical agents with the exposure temperature which both provide the greatest synergy (Fig. 4). Here again, open circles denote the results of our calculations based on the survival curve data published earlier. In all cases, at a smaller intensity of the physical factor or concentration of the chemical agents, it was required to reduce the acting temperature to preserve the highest synergistic effect.

DISCUSSION

The experimental data presented in this paper and obtained by authors with yeast cells and published by other authors with cultured mammalian cells after simultaneous action of heat and various physical and chemical agents revealed two remarkable features. First of all, for any constant intensity of physical agent or concentration of chemical compounds there is an optimal temperature at which their synergistic interaction shows the highest effectiveness. In other words, there exists a definite temperature range inside which the synergistic interaction takes place. For temperatures below this range, the synergistic interaction is not observed and cell killing is mainly induced by physical or chemical factor applied. On the contrary, for temperatures above this range, the synergistic interaction is also lacking while cell killing is caused for the most part by heat. Similar rule reveals for other deleterious agents and not only on cellular but also on the whole organism level. Using experimental data on (i) effect of radiation dosage and estrogen in the production of mammary cancer in the rat (Segaloff and Pettigrew 1978), (ii) lung carcinogenesis during in vivo cigarette smoking and radon daughter exposure in rats (Chameaud et al. 1982) (iii) relative risks for esophaged cancer according to daily amounts of tobacco used and alcohol consumed (Saracci 1987), it was demonstrated (Ryabova and Petin 2000) the existence of optimal relationship between applied agents providing the highest synergistic effect. The explanation of this fact may be based on the hypothesis that synergism is expected to result from the additional effective damage arising from interaction of sublesions produced by every agents. These sublesions are considered noneffective after each agent taken alone. According to the mathematical approach developed upon this idea (Petin and Komarov 1997; Petin et al. 1999, 2000), the synergy is determined by the lowest number of sublesions induced by both agents.

The model predicts the highest synergy under condition of equal number of sublesions produced by each agents used in combination. Any deviation from this optimal relationship resulted in the decrease of synergy (Petin and Komarov 1997; Petin et al. 1999, 2000).

The second relevant feature, followed from the data presented in this paper is the evidence of the importance of synergistic effects at low intensity of acting agents. Experimental results obtained for the simultaneous treatment of hyperthermia with ionizing radiation, ultraviolet light, ultrasound and some chemical drugs on various cell systems clearly indicate that the intensity of the physical factor or the concentration of chemical agent predestines the effectiveness of their synergistic interaction with heat. One can conclude therefore that time factor may be considered as a determinant of synergy. It was shown that the lesser intensity of one of the agent applied, the lesser temperature under which the treatment occurred should be used to provide the highest or a specified level of synergism. These results seem to be related to the fact that, if the intensity decreases, then the effective lethal dose is delivered over a long time, so that the duration of heat incubation increases, which could explain the lower temperature that should be applied to the cells.

Petin et al. (1993) extrapolated to the region of low dose rate the data on the dependence of synergistic effect of simultaneous treatment of heat and ionizing radiation on cultured mammalian cells on dose rate. Although the results were widely scattered, it was suggested the existence of a certain interval of dose rates (10^{-5}-10^{-3} Gy/min) inside which the synergistic interaction of ionizing radiation and heat may be observed at physiological body temperatures. It is curious that such dose rates were measured within the 30-km zone around the Chernobyl nuclear reactor.

Dineva et al. (1993) studied the genetic consequences of the combined action of chronic irradiation with ionizing radiation and lead nitrate on *Arabidopsis thaliana* seeds. Seed were collected from natural populations growing for five years in the 30-km zone around the Chernobyl nuclear reactor at areas with different levels of radioactive contaminant (1, 5, 25, and 300 μGy per hour), then treated with lead nitrate (3.39 g/l) and tested for the frequencies of mutant embryos and embryos with lethal genotypes. The authors (Dineva et al., 1993) showed the dependence of synergism on the dose rate of chronic irradiation of populations studied. The

synergistic effect reached the greatest value (about of 2.5) at an optimal dose rate (5 μGy/h), being much lower at other rates.

Taking all these data as a whole, one can conclude that for a long duration of interaction, which are important for problems of health physics, small intensities of deleterious environmental factors may synergistically interact with each other either with environmental heat or physiological temperatures of homoiothermal animals and man. Hence, the assessment of health or environmental risks from numerous natural and man-made agents at the level of intensities or concentrations found in environmental and occupational settings should take into account the synergistic interaction between harmful agents.

CONCLUSIONS

It can be therefore concluded that (i) the exposure rate of environmental agents is an important relevant determinant of synergy; and (ii) there perhaps may be exist an universal rule independent of object tested and agents employed: the lesser intensity of one of the agent applied, the smaller intensity of another agent has to be used to provide the highest or a definite level of synergistic interaction. It can be inferred that, in principle, the synergistic effect may take place between small intensities of harmful environmental factors existing in the biosphere. This inference can have important bearing on the possible outcome of combined exposure and the risk assessment of numerous deleterious agents existing in contemporary life.

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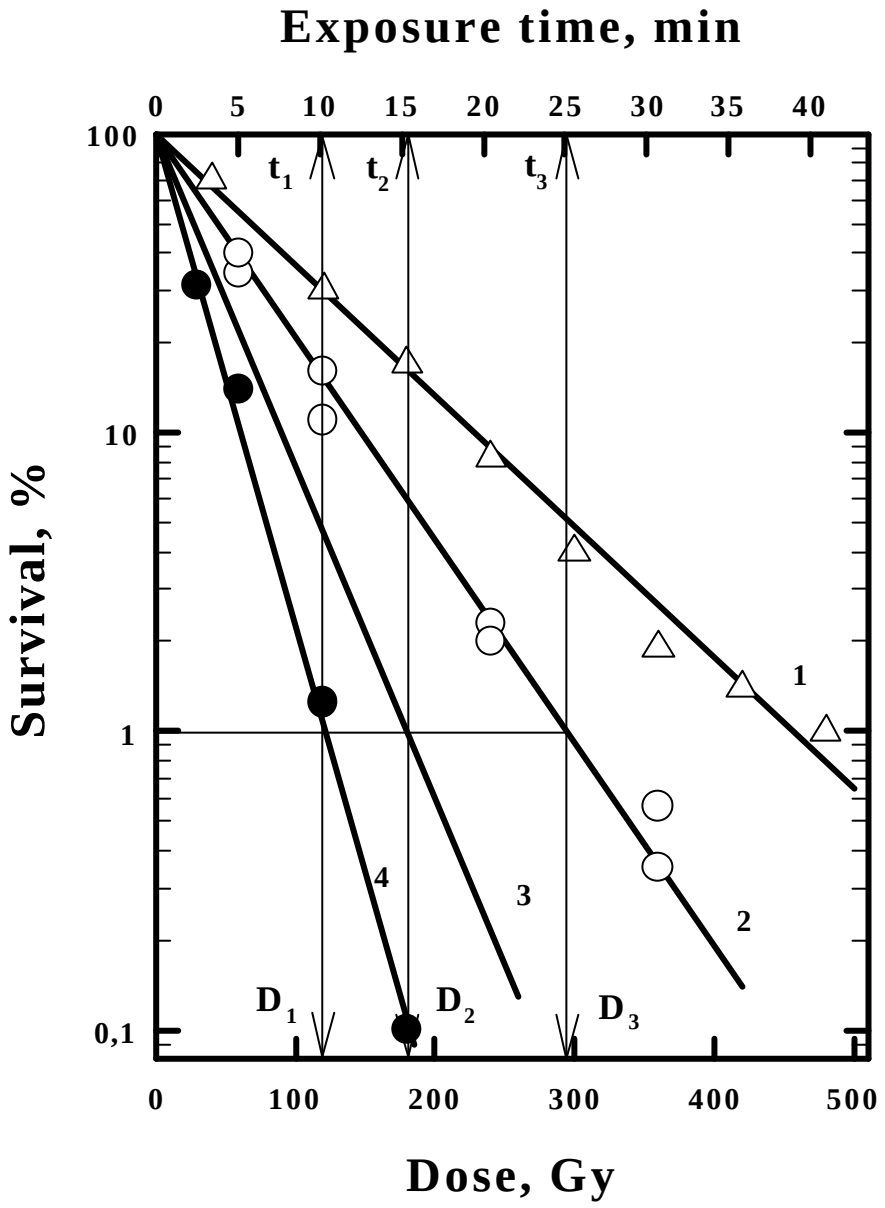

Fig. 1. Survival curves of Zygos*accharomyces bailii* haploid yeast cell: curve 1 - heat treatment (45 ^{o}C) alone; curve 2 - ionizing radiation (^{60}Co) at about 10 Gy/min and room temperature; curve 3 - calculated curve for independent action of ionizing radiation and heat; curve 4 - experimental curve after simultaneous thermoradiation action.

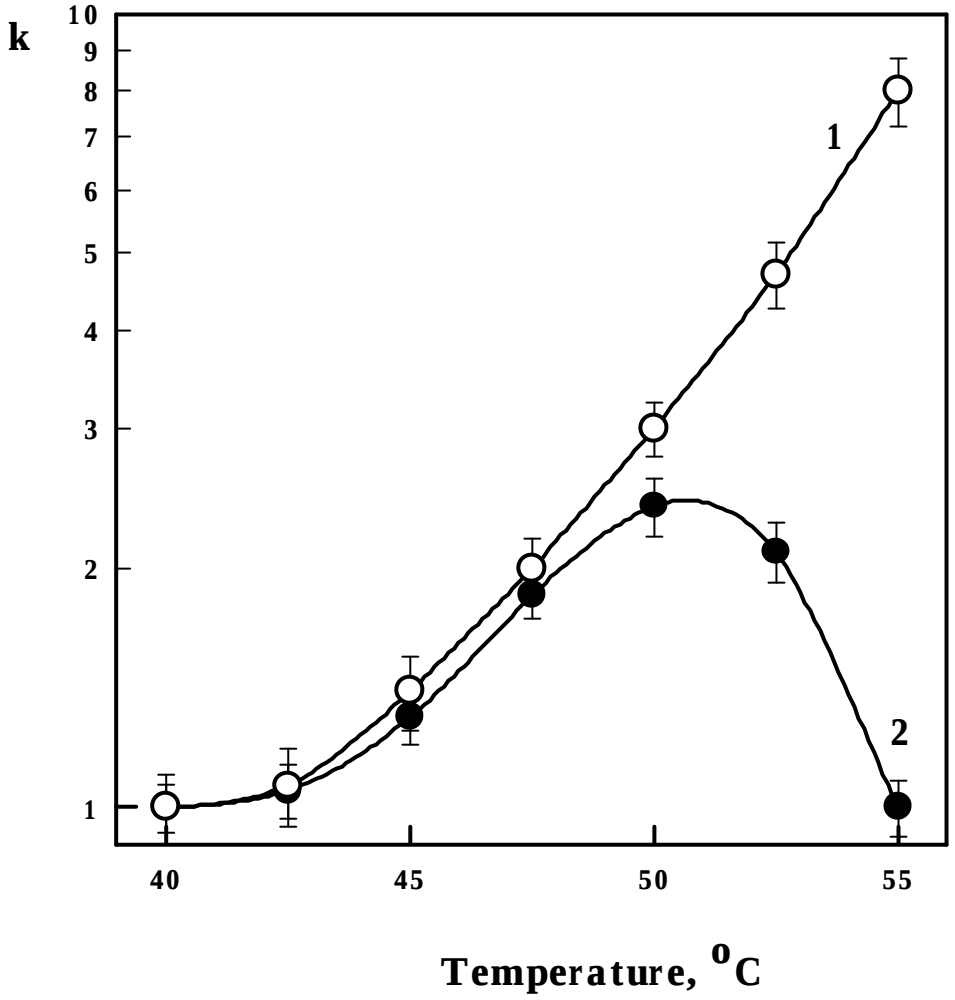

Fig. 2. Thermal enhancement ratio (curve 1) and synergistic enhancement ratio (curve 2) of *Saccharomyces cerevisiae* diploid yeast cell (strain XS800) as a function of temperature exposed (^{60}Co) at 10 Gy/min.

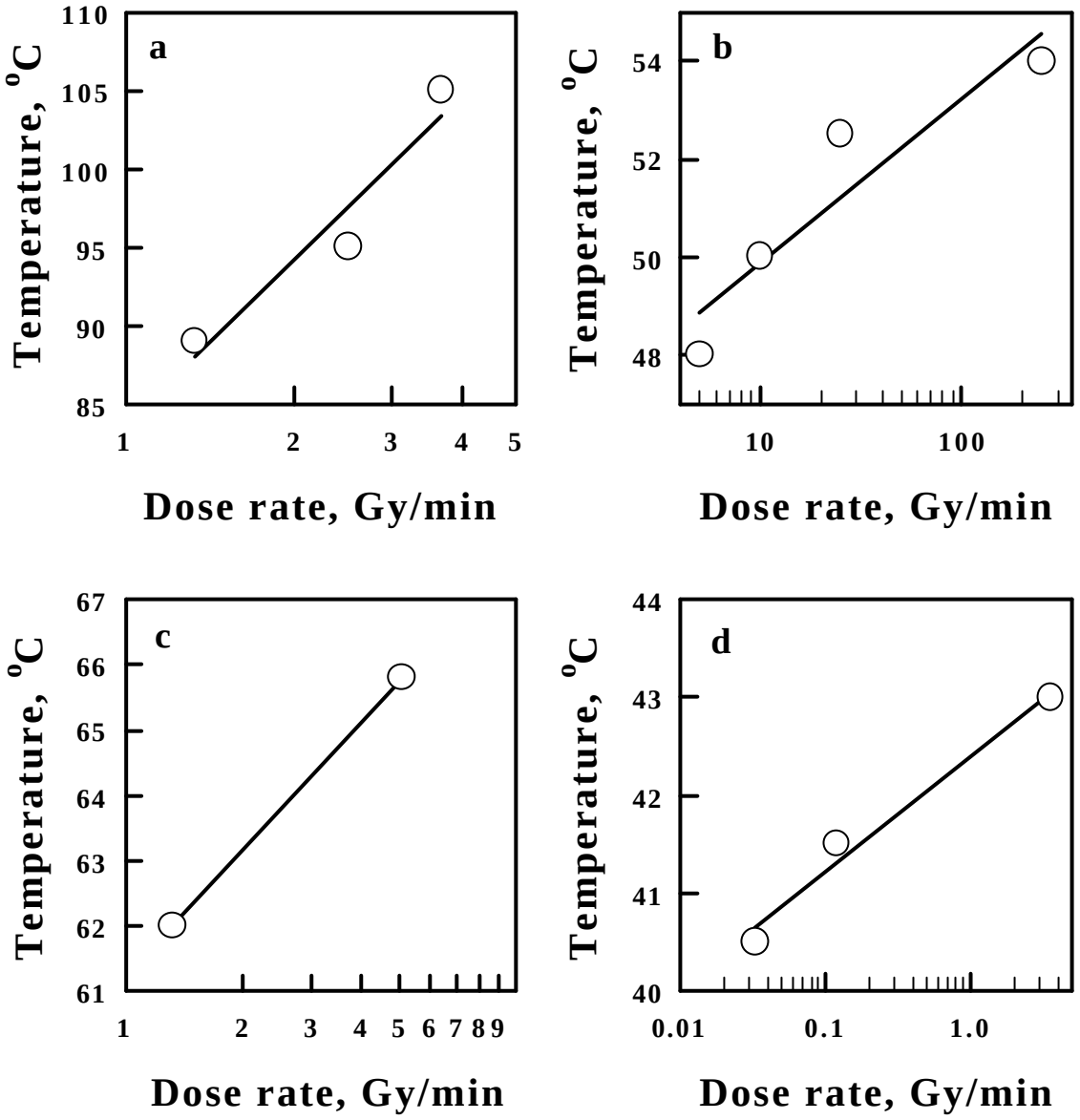

Fig. 3. Correlation of dose rate and exposure temperature providing the same synergistic interaction under simultaneous thermoradiation action: **a** - bacterial spores (*Bacillus subtilis*); **b** - diploid yeast cells (*Saccharomyces cerevisiae*, XS800); **c** - bacteriophage (T4); **d** - cultured mammalian cell (Chinese hamster cells).

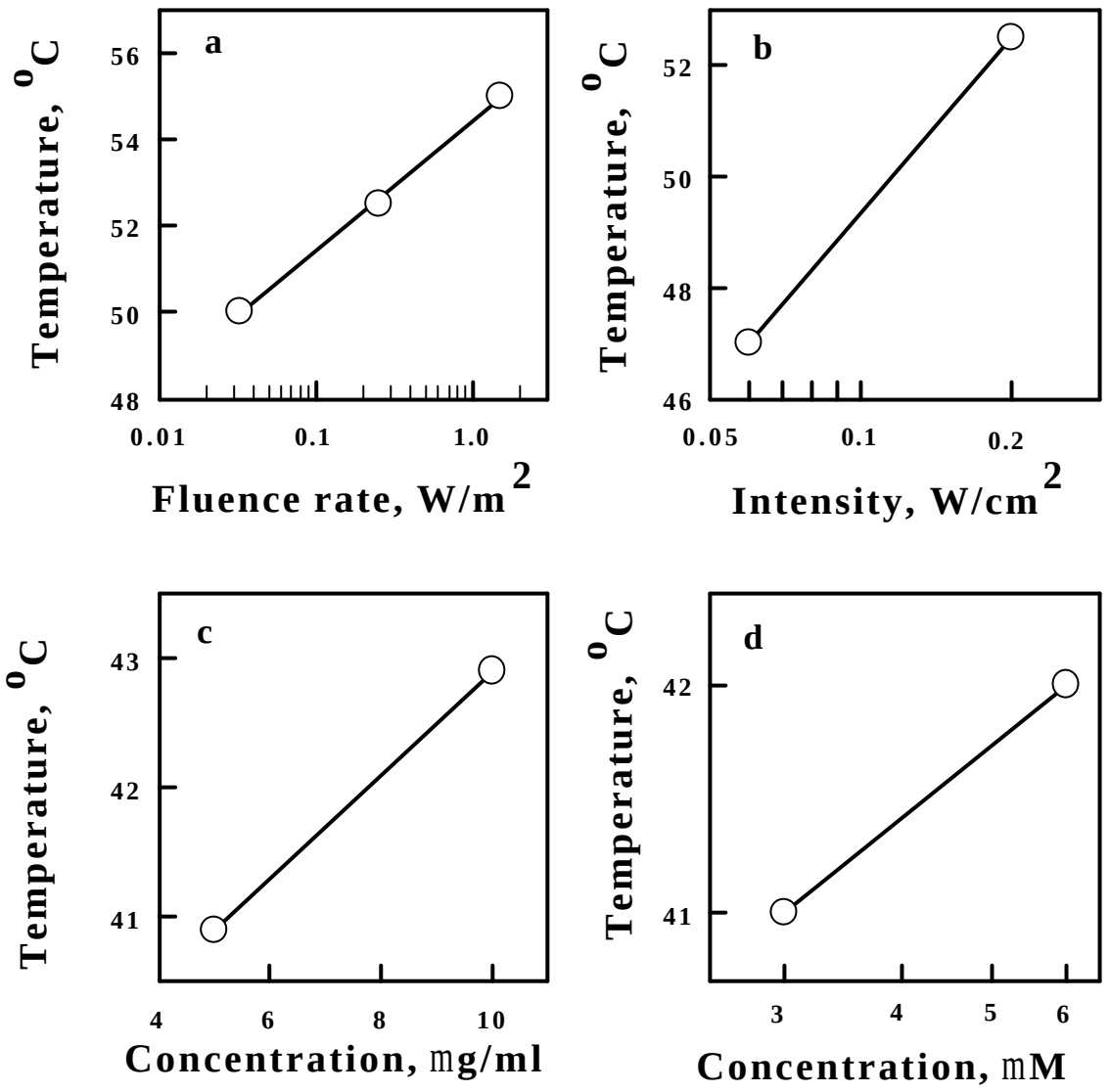

Fig. 4. Correlation of exposure temperature with UV light fluence rate (**a**), ultrasound intensity (**b**), and concentration of tris(1-aziridinil)-phosphine sulfide (thio-TEPA) (**c**) and cis-diamminedichloroplatinum (II) (cis-DDP) (**d**) providing the highest synergistic interaction under their simultaneous action on yeast cells (**a, b**) and cultured mammalian cells (**c, d**).