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# Further development of mathematical description for combined toxicity: A case study of lead–fluoride combination

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ABSTRACT

In this article, we check and develop further some postulates of the theory and mathematical modeling of combined toxic effect that we proposed earlier [1]. To this end, we have analyzed the results of an experiment on rats exposed during 6 weeks to repeated intraperitoneal injections of lead acetate, sodium fluoride or both. The development of intoxication was estimated quantitatively with 54 functional, biochemical and morphometric indices. For mathematical description of the effect that lead and fluorine doses produced alone or in combination, we used a response surface regression model containing linear and cross terms (hyperbolic paraboloid). It is shown that the combination of lead and fluoride features the same 10 types of combined effect that we found previously for the lead and cadmium combination. Special attention is given to indices on which lead and fluorine produce an opposite effect.



## 1. Introduction

In our previous paper [1] we discussed the state of the art in the complicated and controversial domain of the combined toxicity theory and its mathematical modeling and investigated this problem taking as a case study an experiment on rats subjected to lead–cadmium subchronic intoxication.

We analyzed the findings of that experiment in order to identify the types of combined toxicity using either common sense considerations based on descriptive statistics or two mathematical models based (a) on ANOVA and (b) on Mathematical Theory of Experimental Design, which correspond to the widely recognized paradigms of effect additivity and dose additivity (Loewe additivity), respectively. This analysis has led us to the following conclusions:

(1) these two paradigms are virtually interchangeable and should be regarded as different methods for modeling combined toxicity rather than as concepts reflecting fundamentally differing processes;

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(2) within both models, there exist more than three traditionally recognized types of combined toxicity (additivity, subadditivity and superadditivity), and we have found at least 10 variants of it depending on exactly which effect is considered and what its level is, as well as on dose levels and their ratio.

Later on, these postulates were in principle confirmed based on the same approach in an analysis of experimental data on the combined toxicity of chromium (VI) and nickel [2].

In these papers we touched but tangentially upon the special case of toxic agents acting oppositely on some indices of organism's status where the combined effect equal to the algebraic sum of effects induced by separate exposures in other words, formal additivity can hardly be interpreted otherwise than toxicological antagonism. As well as Tallarida et al. [3] in a similar pharmacological situation, we proposed to model it in the same manner as used for a combination of toxics acting unidirectionally. In this connection, we dwelt upon some terminological issues and proposed to discriminate between "hidden antagonism" (in the case of subadditivity of unidirectional effects) and "explicit antagonism" (in the case of formal additivity of opposite effects).

Earlier Timbrell [4] proposed to distinguish terminologically between "functional antagonism where the effects are opposite and therefore counterbalanced; chemical antagonism in which a complex is produced; dispositional antagonism in which the absorption, distribution, metabolism or excretion of the toxic compound is influenced; and receptor antagonism where two substances interact with the same receptor and thereby reduce the toxic response". Such distinctions (given that no mathematical description has been provided by this author) are, in our opinion, more interesting for understanding or for searching for an understanding of the mechanisms of combined toxicity rather than for developing its working classification. It should be noted in this connection that "functional antagonism" has virtually the same meaning as our term "explicit antagonism" while the other three types of action considered by Timbrell are, in fact, the different mechanisms of what we propose to call "hidden antagonism", and these mechanisms are not alternatives but may be characteristic of one and the same combination of toxics. For instance, the "dispositional antagonism" was found by us not as a unique type but along with other types of combined chromium-nickel toxicity [2]. Moreover, when one toxic influences the metabolism of another, the net result may be not only antagonism but also potentiation of toxicity as it was demonstrated, for instance, for the naphthalene–lead combination [5].

We deemed it worthwhile to check the above-considered fundamental propositions by means of the most efficient mathematical tool, using, however, some other toxic combinations based, like in our previous paper, on certain experimental findings from our laboratory that had already been published without a mathematical analysis of this kind. To this end, we chose lead–fluoride subchronic toxicity [6], for which we had a sufficiently long list of toxicodynamic and toxicokinetic indices some of which suggested the possibility of an opposite effect.

Originally, we had turned just to this toxic combination because it is typically present in a range of urban areas contaminated with both fluorides (due, first of all, to emissions from electrolytic aluminum and superphosphate production facilities) and inorganic lead compounds (due to primary and secondary metallurgy of lead, copper and alloys of these metals and to persistent environmental contamination with lead accumulated over a long period of automotive transport's operation on leaded gasoline). Besides, a combined lead–fluoride pollution of workroom and ambient air is possible in the ceramic industry where sodium silicofluoride is used along with lead glazes. Finally, this issue attracted our attention in connection with the old discussion about the benefits and risks of water treatment with fluoride (as a method of preventing caries), specifically in connection with fact that in a number of cities in the eastern states of the USA there are still sections of water supply piping made of lead. An evaluation of lead content of the blood in more than 280 000 children in the State of Massachusetts revealed that water treatment with fluoride raised this index as well as the related prevalence of neuropsychiatric disorders [7].

Both lead and fluoride are characterized by high toxicity, affecting adversely a lot of systems in the organism, often with similar targets of toxic action [8–12].

Noteworthy, in particular, is the relationship between the toxicodynamics of both elements and the calcium metabolism and the toxic effects of both elements on the thyroid gland and on the bone tissue. However, there was in the scientific literature very little factual data prior to our experiment on the combined toxicity of lead and fluoride. Thus it was shown in an experiment on rats that when lead was added to the drinking water in combination with fluoride the concentration of this metal rose in both blood and teeth, whereas lead and fluoride combination did not influence the accumulation of fluoride in the same tissues [13]. A reduced learning ability was discovered in the offspring of female rats exposed to a combined effect of lead and fluoride, in comparison with the action of lead alone or of fluoride alone [14]. In the same offspring, exposure to this combination produced the greatest reduction in the glutamate content of the brain (hippocampus), glutamate being the principal mediator of excitation in the central nervous system and playing an important role in the learning processes.

To sum, the proposed analysis of the combined lead–fluoride toxicity along the lines considered above is of not only theoretical but also practical interest

## 2. Materials and methods

### 2.1. Animal experiment

This experiment was carried out on outbred white female rats (from our own breeding colony) with an initial age of about 4 months and body weight of 180–190 g, 15 animals in each exposed and control group. All rats were housed in conventional conditions, breathed unfiltered air and were given standard balanced food and clean

bottled water. The study was planned and implemented in accordance with the "International guiding principles for biomedical research involving animals" developed by the Council for International Organizations of Medical Sciences (1985) and approved by the Ethical Committee of the Ekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers.

The toxics used were sodium fluoride and lead acetate. The model of subchronic intoxication was created by repeated intraperitoneal injections of the salts under study to rats 3 times a week during 6 weeks (totally, 18 injections). The dosage of the salts corresponded to 0.025 $LD_{50}$ and amounted to 1.45 mg/kg for sodium fluoride, and 5.5 mg/kg for lead acetate. Animals in the control group were administered normal saline in the same volume (0.5 ml per rat).

After the exposure period, the following procedures were performed for all rats: weighing; estimation of CNS ability to perform the temporal summation of subthreshold impulses – a variant of withdrawal reflex and its facilitation by repeated electrical stimulations in an intact, conscious rat [15]; recording of the number of head-dips into holes and number of crossed squares on a hole-board, which is frequently used for studying behavioral effects of toxicants and drugs (e.g. [16]); collection of daily urine for analysis of its density, urine output as well as lead, fluoride, coproporphyrin, delta-aminolevulinic acid (δ-ALA), and creatinine contents; sampling of capillary blood from a notch on the tail for examining the standard hemogram, reticulocytes count, hemoglobin content, and for cytochemical determination of succinate dehydrogenase (SDH) activity in lymphocytes (by the reduction of nitrotetrazolium violet to formazane, the number of granules of which in a cell is counted under immersion microscopy).

Then rats were killed by decapitation and blood was collected by exsanguination. Biochemical indices determined from the serum included calcium, total protein, albumin, globulin, bilirubin, cholesterol, glucose, ceruloplasmin, malondialdehyde (MDA), alkaline phosphatase, alanine- and asparate-transaminases (ALT, AST), gamma glutamine transferase, amylase, and cholinesterase. The fluoride content of the urine and of the bone tissue (after pyrohydrolysis) was determined potentiometrically with the help of a Shimadzu atomic absorption spectrophotometer. The thyroid hormones and the thyrotropic hormone contents were determined in the blood serum by the ELISA method on a Multiskan EX Microplatge Photometer with on-board software. The bone marrow smears from the femur were fixed with methanol and stained by the Pappenheim method for counting the number of micronuclei per 1000 polychromatophilic erythrocytes.

The femurs released from the muscle sheath were fixed in 10% neutral formalin and then were decalcified in Trilon B. For making tissue specimens, bone fragments were passed through a set of alcohols of increasing concentration and then were embedded in wax. Microsections were prepared with longitudinal orientation of preparations and were stained with hematoxylin–eosin and with picrofuchsine by the van Gieson method. We measured the thickness of the bone plate of the diaphysis using an ocular micrometer, whilst the number of osteocytes/osteoblasts in the bone diaphysis and the proportion of bone trabeculae in the metaphysis preparation were estimated with the help of Avtandilov grid [17].

The statistical significance of differences between the mean values of indices was estimated by means of multiple comparisons with Bonferroni correction.

### 2.2. Mathematical analysis of combined toxicity

As was mentioned in Section 1, earlier we used two different mathematical models for mathematical description of the combined effect produced by lead and cadmium [1], one of which was based on ANOVA and the other on Mathematical Theory of Experimental Design, corresponding to the paradigms of effect additivity and dose additivity, respectively. To analyze the data of a similarly designed experiment involving lead and fluoride with aim of verifying the model-insensitivity of main postulates based on our previous work, we used the Response Surface Methodology (RSM), which is a generalization of the ANOVA and MTOED methods. In this approach, the conditions of effect additivity and dose additivity are brought together in the notion of "zero interaction": if the effect $Y$ produced by toxicants acting in combination is outside the zero interaction response surface (ZIRS), this situation is equivalent to the conditions of effect additivity and dose additivity being satisfied; if $Y$ falls below/above ZIRS, synergism/antagonism takes place [18–22].

The regression equation describing the response surface $Y = Y(x_1, x_2)$ in RSM may be constructed by fitting the coefficients of the regression equation to experimental data (Table 1), where $Y$ is an index of organism's status, $x_1$ and $x_2$ are the doses of toxicants participating in the combination. In the case of two two-level toxicants even if one of the levels is equal to zero (as in our case), the response surface may have one possible shape (hyperbolic paraboloid)

$$Y(x_1, x_2) = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2, \quad (1)$$

where $b_0, b_1, b_2$ and $b_{12}$ are the coefficients of the regression equation. Myers et al. [22] noted the special importance of two-level experiments in response surface works, although, in principle, the Response Surface Methodology is applicable to any experiment design. According to the response surface approach, even in the case of two-level toxic agents the model (1) enables one to predict the magnitude of response $Y$ for any combination of toxicant doses within the experimental range for each of them (rather than at two points only).

It is believed that two toxicants produce a unidirectional effect on response $Y$ if the one-way response functions $Y(x_1, 0)$ and $Y(0, x_2)$ act in the same direction (both functions either increase or decrease with an increase in $x_1$ or $x_2$); similarly, two toxicants produce an opposite effect on response $Y$ if the one-way response functions $Y(x_1,0)$ and $Y(0, x_2)$ act in opposite directions (one function increases while the other decreases).

We can consider in the same manner the behavior of the response to a change in the dose of one toxicant (for example, $x_1$) the dose of the other toxicant being different from zero (for example, $x_2$), *i.e.* the behavior of the function $Y(x_1, x_2)$. For instance, if we vary the dose of the first toxicant $x_1$,

**Table 1**
Some functional and morphometric indices to the condition of rat organism after the subchronic exposure fluoride and lead separately or in combination ($x \pm$ s.e).

| Index | Control | Lead | Fluoride | Pb + F |
|---|---|---|---|---|
| Final body mass, g | 236.3 ± 4.3 | 219.2 ± 6.2 | 230.8 ± 4.37 | 221.7 ± 3.2 |
| Temporal summation of sub-threshold impulses, s | 12.42 ± 0.79 | 14.50 ± 1.17 | 11.94 ± 1.26 | 18.34 ± 0.56*,• |
| Number of head-dips into holes during 3 min | 6.80 ± 0.51 | 4.60 ± 0.81 | 6.20 ± 1.21 | 3.70 ± 0.60 |
| Number of squares crossed over within 3 min | 13.9 ± 1.62 | 11.80 ± 2.09 | 17.20 ± 4.45 | 5.70 ± 1.27• |
| Hemoglobin, g/l | 96.0 ± 2.6 | 78.7 ± 2.1*,• | 105.7 ± 4.6 | 78.4 ± 2.8*,• |
| Erythrocytes, $10^{12}$ g/l | 6.48 ± 0.22 | 5.29 ± 0.14*,• | 6.59 ± 0.15 | 5.20 ± 0.11*,• |
| Reticulocytes, ‰ | 16.7 ± 2.6 | 63.7 ± 8.9*,• | 23.1 ± 5.9 | 86.7 ± 12.3*,• |
| Lymphocytes, % | 73.4 ± 5.9 | 61.1 ± 2.9* | 66.9 ± 2.2 | 57.2 ± 3.9* |
| Segmented neutrophils, % | 15.5 ± 1.6 | 29.7 ± 2.2* | 21.5 ± 2.1 | 34.7 ± 3.1*,• |
| Monocytes, % | 4.5 ± 1.0 | 6.2 ± 1.2 | 6.6 ± 1.3 | 5.2 ± 1.2 |
| Eosinophils, % | 4.5 ± 0.75 | 2.5 ± 0.50 | 4.2 ± 0.68 | 2.67 ± 0.56 |
| Banded neutrophils | 1.29 ± 0.18 | 1.22 ± 0.15 | 1.21 ± 0.21 | 1.53 ± 0.50 |
| Diuresis | 30.6 ± 4.0 | 31.0 ± 5.4 | 29.4 ± 3.1 | 39.9 ± 3.9 |
| Urine specific density | 1005.6 ± 0.8 | 1005.8 ± 0.7 | 1005.5 ± 0.7 | 1005.7 ± 0.6 |
| Succinate dehydrogenase (SDH) activity, number of formazane granules per 50 lymphocytes | 705.5 ± 4.1 | 599.9 ± 7.3* | 590.3 ± 7.6* | 579.8 ± 9.9* |
| ALT activity in blood serum, mM/h l | 0.53 ± 0.08 | 0.67 ± 0.06• | 0.47 ± 0.07 | 0.55 ± 0.05 |
| AST activity in blood serum, mM/h l | 0.62 ± 0.10 | 0.66 ± 0.09 | 0.51 ± 0.12 | 0.57 ± 0.10 |
| De Ritis coefficient | 1.22 ± 0.15 | 1.10 ± 0.19 | 1.12 ± 0.24 | 1.11 ± 0.19 |
| MDA in blood serum, nmol/l | 4.17 ± 0.28 | 5.31 ± 0.32* | 4.67 ± 0.26 | 5.00 ± 0.35 |
| Ceruloplasmin in blood serum, mg/l | 25.5 ± 1.3 | 34.8 ± 2.1*,• | 24.6 ± 2.3 | 32.9 ± 2.4• |
| Coproporphyrin in urine, nM/l | 83.2 ± 37.7 | 453.1 ± 72.4*,• | 105.0 ± 18.6 | 479.8 ± 86.5*,• |
| Coproporphyrin, in urine, nM/day | 1.88 ± 0.70 | 13.71 ± 3.00*,• | 3.06 ± 0.59 | 17.54 ± 2.02*,• |
| δ-ALA in urine, μmol/l | 13.66 ± 2.79 | 152.0 ± 14.0*,• | 14.38 ± 1.64 | 164.8 ± 14.25*,• |
| δ-ALA in urine μmol/day | 0.38 ± 0.07 | 4.33 ± 0.58*,• | 0.42 ± 0.05 | 6.47 ± 0.68*,•,■ |
| Total protein content of blood serum, g/l | 79.7 ± 3.6 | 71.3 ± 2.4• | 83.8 ± 1.9 | 69.8 ± 2.5• |
| Alkaline phosphatase in blood serum, nmol/(s l) | 102.4 ± 8.6 | 127.2 ± 6.9• | 99.9 ± 12.3 | 170.9 ± 11.8•,■ |
| Activity of γ-glutamine transferase in blood serum, nmol/(s*l) | 3.34 ± 0.27 | 4.27 ± 0.44 | 3.33 ± 0.37 | 3.87 ± 0.27 |
| Calcium in blood, mmol/l | 2.91 ± 0.04 | 2.39 ± 0.15* | 2.36 ± 0.08* | 2.17 ± 0.08* |
| Creatinine in blood serum, μmol/l | 72.3 ± 2.4 | 95.2 ± 16.4 | 93.8 ± 4.9 | 80.9 ± 7.2 |
| Cholinesterase in blood serum, units/l | 970.4 ± 111.2 | 298.8 ± 22.4*,• | 990.0 ± 84.4 | 255.2 ± 23.9*,• |
| Albumins content of blood serum, g/l | 39.50 ± 0.78 | 32.18 ± 0.37*,• | 39.61 ± 0.70 | 32.31 ± 0.93*,• |
| Globulins | 42.9 ± 5.2 | 42.9 ± 2.3 | 47.4 ± 2.2 | 40.1 ± 4.5 |
| A\|G index | 0.997 ± 0.137 | 0.759 ± 0.038* | 0.842 ± 0.035 | 0.740 ± 0.057* |
| Cholesterol | 1.84 ± 0.084 | 1.72 ± 0.07 | 1.92 ± 0.13 | 1.67 ± 0.14 |
| Glutathione | 19.6 ± 0.5 | 19.3 ± 0.6 | 19.2 ± 0.8 | 18.5 ± 1.3 |
| Creatinine in urine/day | 0.048 ± 0.011 | 0.046 ± 0.006 | 0.049 ± 0.010 | 0.055 ± 0.009 |
| Creatinine in urine/L | 2.66 ± 0.91 | 2.08 ± 0.43 | 1.78 ± 0.41 | 1.49 ± 0.30 |
| Amylase | 2956 ± 147 | 3009 ± 298 | 3066 ± 255 | 2899 ± 190 |
| Catalase | 0.84 ± 0.55 | 0.51 ± 0.27 | 0.62 ± 0.16 | 0.30 ± 0.04 |
| Glucose | 6.66 ± 0.15 | 6.38 ± 0.14 | 6.09 ± 0.14* | 6.26 ± 0.20 |
| Micronuclei per 1000 polychromatophilic erythrocytes | 0.63 ± 0.26 | 2.63 ± 0.65* | 1.88 ± 0.52 | 1.29 ± 0.42 |
| Thyrotropic hormone of hypophysis in blood serum, mmol/l | 0.20 ± 0.04 | 0.18 ± 0.01 | 0.16 ± 0.02 | 0.12 ± 0.02 |
| Thyroxin in blood serum, pmol/l | 31.99 ± 1.59 | 39.51 ± 2.59• | 30.15 ± 1.71 | 40.21 ± 3.02• |
| Triiodothyronine in blood serum, pmol/l | 2.88 ± 0.27 | 2.97 ± 0.38 | 3.05 ± 0.28 | 2.15 ± 0.39 |
| Fluoride in urine, μg/day | 22.1 ± 2.5 | 26.3 ± 2.7 | 36.5 ± 2.2* | 52.0 ± 4.4*,•,■ |
| Fluoride in urine/L | 761.6 ± 61.2 | 933.0 ± 77.9• | 1300.8 ± 89.0* | 1321.3 ± 61.0*,■ |
| Fluoride in bone, mg/kg | 12.57 ± 0.53 | 10.25 ± 0.94• | 45.00 ± 4.94* | 33.50 ± 3.48*,■ |
| Lead in blood, μg/dL | 0.015 ± 0.002 | 22.66 ± 5.66*,• | 0.022 ± 0.002 | 22.39 ± 5.12*,• |
| Lead in urine, μg/day | 0.20 ± 0.06 | 5.03 ± 1.33*,• | 0.24 ± 0.02 | 5.90 ± 1.26*,• |
| Lead in urine mg/L | 6.81 ± 0.49 | 146.3 ± 19.7*,• | 8.41 ± 0.50■ | 148.0 ± 23.7*,• |
| Lead in bone, mg/kg | 1.98 ± 0.29 | 305.5 ± 9.5*,• | 4.64 ± 0.73 | 279.1 ± 17.5*,• |
| Thickness (mm) of diaphysis bone wall | 0.57 ± 0.015 | 0.66 ± 0.025• | 0.82 ± 0.025* | 0.85 ± 0.042* |
| Number of osteoblasts/osteocytes in a square of Avtandilov grid | 16.28 ± 0.57 | 23.82 ± 0.64• | 32.70 ± 0.83* | 24.70 ± 0.61 |
| Specific proportion of bone trabeculae in metaphysis (as %% of area) | 45.06 ± 1.26 | 31.74 ± 0.76*,• | 55.88 ± 1.70* | 48.18 ± 1.62•,■ |

* Statistically significant difference from the "control" group.
• The same from the "fluoride" group.
■ The same from the "lead" group by multiple comparison test with Bonferroni correction, $p < 0.05$.

the value of the second toxicant being fixed $x_2 = x_2^*$ the pattern of monotonicity of the function $Y(x_1, x_2^*)$ determines the direction of the effect (increase or decrease) produced by the first toxicant with the second toxicant being present at a dose of $x_2 = x_2^*$. Similar reasonings hold for the function $Y(x_1^*, x_2)$ if we fix the dose of the first toxicant $x_1 = x_1^*$.

Thus, we can identify the pattern of combined action featured by the toxicants for a given combination of doses

$(x_1^*, x_2^*)$ considering the change in the dose of one toxicant with the dose of the second one being fixed. If the corresponding one-way functions $Y(x_1, x_2^*)$ and $Y(x_1^*, x_2)$ feature the same direction, a unidirectional effect takes place (near a given combination of doses $(x_1^*, x_2^*)$); if the direction is opposite, the toxicants act in opposite directions by analogy with the one-way functions $Y(x_1, 0)$ and $Y(0, x_2)$.

Consider some important features of model (1). If coefficient $b_{12}$ of Eq. (1) is not statistically significantly different form zero, we deal with a zero interaction case, *i.e.* additivity. If the combined effect of the toxicants departs from additivity, its estimation, as was noted in the Introduction, is essentially different for unidirectional and opposite actions of the agents in the combination on an index under consideration. Moreover, inside these two classes (unidirectional and opposite actions) one should pay attention to the position of the saddle point on the hyperbolic paraboloid (1), the coordinates of which are given by the equalities

$$x_1^0 = -\frac{b_2}{b_{12}}, \quad x_2^0 = -\frac{b_1}{b_{12}}. \tag{2}$$

If the coordinates $x_1^0$ and $x_2^0$ lie outside the experimentally studied range of doses, we have the classical isoboles of additivity, synergism and antagonism (allowing for the directionality of toxicant effects). If one or both coordinates $x_1^0$ and $x_2^0$ lie within the range of doses under study, one can observe various types of combined effect on index $Y$ for different dose ranges and various response levels of $Y$ [1].

In this paper, the dependent variable $Y$ (effect) in regression equation (1) represents one of the functional, biochemical or morphometrical indices describing the status of the organism (Table 1). The predictor variables are the doses of the toxicants *Lead* (variable $X_1$) and *Fluoride* (variable $X_2$). In our experiments, both predictor variables have two gradations: absence of the toxicant and presence of the toxicant at a dose of 0.025 $LD_{50}$.

## 3. Results and discussion

### 3.1. Descriptive statistics and estimation of combined toxicity based on common sense considerations

As can be seen from Table 1, both fluoride and lead as well as their combination caused changes (in comparison with the control group) in a large number of indices. Many of the observed changes may be categorized as nonspecific ("integral") features of intoxication characterizing the disturbance of homeostasis on organism level which is observed virtually in any chronic intoxication. In our case, such features are, for example, weight loss; disbalance between excitation and inhibition processes in the central nervous system (judging by the temporal summation of sub-threshold impulses), motor activity (measured by the number of squares crossed), and exploratory behavior (judging by the number of head dips into holes); general suppression of the energy metabolism showing itself as a decrease in the blood lymphocyte SDH activity, while there was some enhancement of lipid peroxidation judging by the MDA content of the blood serum. Judging by some of these indices, lead is more toxic subchronically in relation to them as compared with fluoride even if given in doses isoeffective in relation to their $LD_{50}$.

Other changes may be classified as relatively specific for the effects of lead and/or fluoride. First of all, this applies to the typical indices for the effect of lead on red blood (reduction in the hemoglobin content and the number of erythrocytes with an increased percentage of reticulocytes in them) and to the indices that reflect disturbances in porphyrin metabolism caused by this metal (a sharp increase in the δ-ALA and coproporphyrin in urine). Characteristic of both lead and fluoride toxicities are disturbances of calcium metabolism which manifest themselves in a reduction in the calcium content of the blood. However, the activity of alkaline phosphatase, one of the key enzymes that control this metabolism, was increased in our experiment only under exposure to lead or its combination with fluoride. According to Shanthakumari and Subramanian [14], an increase in the serum level of both alkaline and acidic phosphatase was shown also to be present in rats administered fluoride with drinking water for 8 and 16 weeks.

Both fluoride and especially lead display some mutagenic property judging by the increased number of micronuclei in the polychromatophilic erythrocytes of the bone marrow.

Comparison of the values obtained for the groups of separate and combined exposure shows that, for the majority of the toxicodynamic indices, the combined effect is more marked than the effect of fluoride alone or lead alone, and in some cases the difference of the combined effect from the effect of separate exposure is statistically significant. In cases where the effect under consideration due to a separate exposure is observed for one of the toxicants only but is significantly enhanced in the presence of the second one, this combination may be deemed to act synergistically. Examples of such synergism are shifts in all indices of nervous activity, reticulocyte count, excretion of δ-ALA and alkaline phosphatase content of the blood serum. In other cases, where this or that shift is provoked by both toxicants acting separately but is more marked in the case of their combination, a combined toxicity is evident but it would be difficult to determine whether there is a deviation from additivity and what the sign of this deviation is.

It is well known that fluoride, being a metabolic antagonist of iodine, also suppresses the hormonal function of the thyroid gland. In our experiment, both fluoride and lead caused a statistically insufficiently significant reduction in the thyrotropic hormone level, but under a combined exposure this effect grew stronger and reached statistical significance. Neither fluoride nor lead produced a reduction in triiodothyronine level, but it was reduced under the combined effect (*i.e.* overt synergism took place). On the contrary, at exposure to lead alone or in combination with fluoride the level of thyroxine was raised.

The tendency toward a combined action of obviously subadditive type unexpectedly manifests itself when estimating mutagenicity: whereas fluoride and lead acting separately provoked a statistically significant increase in the number of micronuclei (3 and 4 times more than in the controls, respectively), under the combined exposure this increase in comparison with the control index was only 2-fold and statistically not significant.

The excretion of fluoride with urine at the end of the combined exposure period was significantly higher than for exposure to fluoride alone. The fluoride content of the bone tissue for the combined exposure was, on the contrary, somewhat reduced in comparison with the separate action of fluoride. Though this reduction is insufficiently significant statistically, one should not ignore the fact that a similar tendency to a reduced background fluoride content of the bone under the effect of lead is observed for the separate exposure to the latter as well. Unfortunately, we do not have any data on the fluoride content of the blood, but the logical inference would be that lead (through its influence on calcium metabolism or by any other mechanism) interferes with the uptake of fluoride in the bone, thereby promoting an increase in its content of the blood as the central toxicokinetic pool, and hence not only in urine (as it was really discovered) but also in the target organs that are sensitive to the toxic effect of fluoride. Circumstantial evidence for this hypothetical mechanism is the fact that, whereas the toxic effect of fluoride on these organs under the combined exposure proved to be enhanced according to the indices considered above, its toxic effect on the bone, as is discussed below, was noticeably reduced. Similarly, at combined fluoride–lead exposure there was less lead in the bone than at exposure to lead alone, although this difference (as well as a small enhancement of lead excretion with urine) was not statistically significant. The same pattern was also observed with the attenuation of histopathological changes in the bone in comparison with the "lead only" group (see below).

If we assume that the action of toxic elements on the bone marrow is associated with their transition into the cellular microenvironment not only from the blood but also directly from the bone trabeculae, a reduction in their content in the bone tissue could explain the above-mentioned paradoxical subadditivity (antagonism) of the effects of lead and fluoride on the formation of micronuclei in polychromatophilic erythrocytes.

Lead and, even to a greater extent, fluoride (alone or combined with lead) caused an increase in the thickness of the bone diaphysis wall. Given the same direction of action of both toxicants on this index, the combined effect is actually determined by one of them and is not enhanced by the action of the second one, which again may be interpreted as subadditivity of effects. The cellularity of the diaphysis is statistically significantly higher than in the control group for the separate action of both lead or, to a greater extent, fluoride and their combination. However, in the combined exposure group, this index is somewhat lower than for exposure to fluoride alone, which, again, may be considered as a manifestation of lead–fluoride antagonism. The effect of lead manifested itself in a decrease in the number of bone trabeculae in the metaphysis, while the effect of fluoride in an increase in them. With such opposing direction of action, the combined exposure yielded a quasi-normalization of this index – what we proposed to tag as "the explicit. antagonism" [1].

As it was stressed by as earlier [1,24], the type of combined toxicity (especially of chronic or sub chronic one) can be different depending on which indices of intoxication (and, thus, what organ, system or organism's function) this type is assessed for, and just this dependence was demonstrated for combined action of lead and cadmium [1]. This important aspect of the combined toxicity problem is not often paid due attention to, mainly because the majority of experimental work in this field has involved acute *in vivo* intoxications or *in vitro* models, and only one definite effect has been registered rather than many different effects. However, when the polytropism of toxicity characteristic of many chemical elements is taken into consideration, the variability of combined action becomes evident. For instance, the following summarizing statement given in the ATSDR [23] overview document is of interest: *The predicted direction of interaction for the effects of these mixtures (Pb–As and Pb–Cd) is not consistent across endpoints. This observation is most striking for the effects of cadmium on the toxicity of lead. The predicted direction is greater than additive for the neurological effects (the critical effect) and testicular effects (a less sensitive effect), less than additive for renal and hematological effects, and additive for cardiovascular effects".

As can be seen from the above discussion of our results, a similar variability is demonstrated in the case of the lead and fluoride toxicity too, and in the next sub-Section it will be confirmed by mathematical analysis.

### 3.2. Mathematical analysis of combined toxicity

The type of combined toxicity featured by fluoride and lead was defined by the coefficients of model (1). As well as in our previous paper [1], we use a coding consisting of three symbols to show the statistical significance and the signs of three coefficients, $b_1$, $b_2$, $b_{12}$. The symbol "0" means that a corresponding coefficient is equal to zero or is different from zero statistically insignificantly, while the symbols (+) and (−) mean the presence of statistical significance and the sign of the corresponding coefficient. Given this coding, in our previous study we identified 10 possible classes (with subclasses), which correspond to various types of combined effect produced by two toxicants. In the present study, we confirm the existence of the same 10 types (Table 2).

Class 0 includes indices on which the toxicants in the doses used did not have any statistically significant effect ($p > 0.05$). Class 1 includes indices on which only one toxicant produced a significant impact (either lead or fluoride). The indices of class 1 are divided into two parts: in the first part, the effect of lead is observed to prevail (these indices correspond to codes (+0 0) or (−0 0)); in the second one (fluoride in urine/L and thyrotropic hormone of hypophysis in blood serum), fluoride is dominant. Note that for one of the indices from this group (*Number of head-dips into holes*) for which, as follows from Table 2, a statistically significant effect is observed for lead only, analysis based on descriptive statistics (see Section 3.1) led to the conclusion of synergism being present. Such differences in the identification of the type of combined effect are also observed for the indices *Reticulocyte count* and *Number of squares crossed over within 3 min*. These differences are due to differences in the approach to estimating combined toxicity.

Subclass 2.1 includes indices which demonstrate dose additivity with both toxicants acting in the same direction

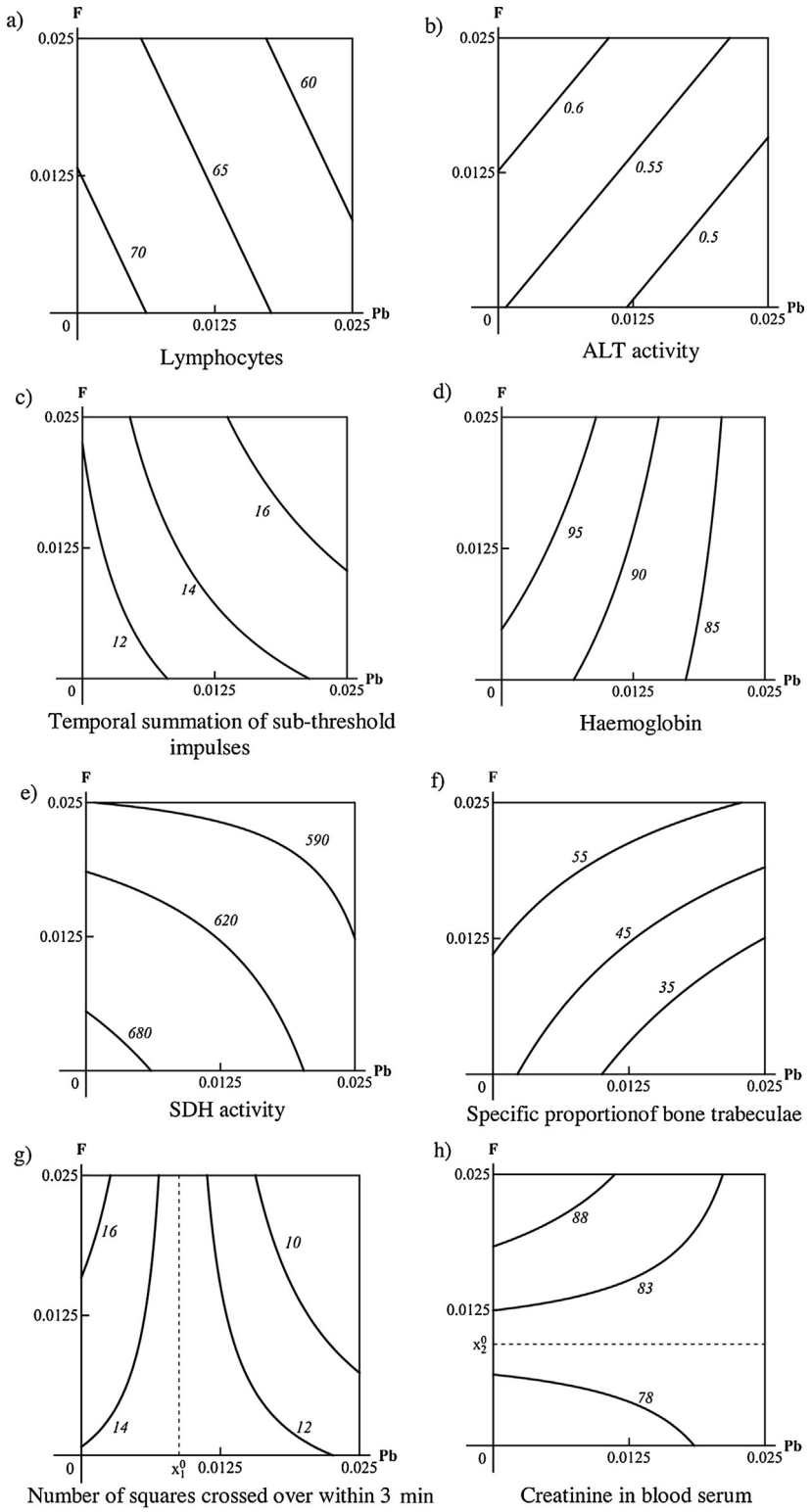


**Fig. 1.** Isoboles: a and b = additivity for unidirectional and opposite effect of *Pb* and *F*; c and d = synergism and antagonism for the unidirectional action of the toxicants; e and f = opposite non-additive actions of *Pb* and *F*, g and h = the ranges of toxicant doses are divided by an asymptote passing through the saddle point $x_1^0$ or $x_2^0$ (the asymptotes are shown as dotted lines). The doses of lead and fluoride are given in $LD_{50}$ units. The number of lines of the isoboles shows the value of the effect *Y* (for example, values 60, 65 and 70 in (a) show the number of lymphocytes for which the isobole has been constructed).

**Table 2**
Grouping of indices describing the status of rat's organism according classes of $b_1$, $b_2$, $b_{12}$ coding as 0, (−) and (+).

| Class | Sub-class | Indices | Coding |
|---|---|---|---|
| 0 | | Banded neutrophils | (0 0 0) |
| | | AST activity | |
| | | De Ritis coefficient | |
| | | Urine specific density | |
| | | Globulins | |
| | | Glutathione | |
| | | Creatinine in urine/day | |
| | | Creatinine in urine/L | |
| | | Amylase | |
| | | Catalase | |
| | | Triiodothyronine in blood serum | |
| | | Monocytes | |
| | | Diuresis | |
| 1 | | MDA | (+ 0 0) |
| | | Ceruloplasmin | (+ 0 0) |
| | | Coproporphyrin/day | (+ 0 0) |
| | | Coproporphyrin/L | (+ 0 0) |
| | | δ-ALA in urine/L | (+ 0 0) |
| | | Activity of γ-glutamine transferase | (+ 0 0) |
| | | Lead in urine/L | (+ 0 0) |
| | | Lead in urine/day | (+ 0 0) |
| | | Lead in blood | (+ 0 0) |
| | | Lead in bones | (+ 0 0) |
| | | Thyroxin in blood serum | (+ 0 0) |
| | | Final body mass | (− 0 0) |
| | | Erythrocytes | (− 0 0) |
| | | Eosinophils | (− 0 0) |
| | | Number of head-dips into holes | (− 0 0) |
| | | Total protein | (− 0 0) |
| | | Albumins | (− 0 0) |
| | | A\|G index | (− 0 0) |
| | | Cholinesterase in blood serum | (− 0 0) |
| | | Cholesterol | (− 0 0) |
| | | Fluoride in urine/L | (0 + 0) |
| | | Thyrotropic hormone of hypophysis in blood serum | (0 − 0) |
| 2 | 2.1 | Lymphocytes | (− − 0) |
| | | Reticulocytes | (+ + 0) |
| | | Segmented neutrophils | (+ + 0) |
| | | Thickness of diaphysis bone wall | (+ + 0) |
| | 2.2 | ALT activity | (+ − 0) |
| | | Fluoride in bones | (− + 0) |
| 3 | | Micronuclei per 1000 polychromatophilic erythrocytes | (0 0 −) |
| 4 | 4.1 | Number of squares crossed over within 3 min | (− 0 −) |
| | 4.2 | Creatinine in blood serum | (0 + −) |
| | | Glucose | (0 − +) |
| 5 | 5.1 | Fluoride in urine/day | (+ + +) |
| | | Temporal summation of sub-threshold impulses | (+ + +) |
| | | δ-ALA in urine/day | (+ + +) |
| | | Alkaline phosphatase | (+ + +) |
| | 5.2 | Succinate dehydrogenase activity (SDH) | (− − +) |
| | | Calcium in blood | (− − +) |
| | | Number of osteoblasts/osteocytes | (+ + −) |
| | 5.3 | Specific proportion of bone trabeculae in metaphysis | (− + +) |
| | | Hemoglobin | (− + −) |

on index $Y$ (*i.e.* both toxicants either increase or decrease the value of the index). The isobole presents a straight line corresponding to dose additivity, the zero interaction surface is a plane corresponding to effect additivity (the equation of zero interaction plane is Eq. (1) for $b_{12} = 0$). An example (lymphocytes) is shown in Fig. 1a.

Subclass 2.2 includes indices responding in opposite directions to lead and fluoride; for example, lead increases ALT activity, while fluoride reduces it (the isobole is a straight line running from left to right upwards; an example for ALT activity is shown in Fig. 1b).

The next group of indices $Y$ demonstrates synergism (*Fluoride in urine per day, Temporal summation of sub-threshold impulses* and *δ-ALA in urine*) or antagonism (*SDH activity* and *calcium in blood*) for unidirectional action. These are some indices from subclasses 5.1 and 5.2. Examples of the isoboles are shown in Fig. 1c and e. The indices $Y$ of subclass 5.3 demonstrate a similar monotonous behavior (as in Fig. 1c and e); however, for *opposite* effects of lead and fluoride (lead reduces $Y$ while fluoride increases it): Hemoglobin (the effect is above ZIRS) and *Specific proportion of bone trabeculae in metaphysis* (the combined effect of Pb and F is below ZIRS) – see Fig. 1d and f.

If for a certain index $Y$ one of the saddle point coordinates ($x_1^0$ or $x_2^0$) falls within the range of *Pb* or *F* doses (doses from zero to 0.025 $LD_{50}$), one can observe various types of combined effect in various dose ranges and for various levels of response $Y^*$ upon which the isobole is constructed. This condition is met by the indices included in class 4 (*Number of squares*, *Creatinine in blood serum* and *Glucose*), as well as the indices *Alkaline phosphatase* and *Number of osteoblasts* from class 5. Examples of the isoboles are shown in Fig. 1g and h. In Fig. 1g (the index *Number of squares crossed over within 3 min*), the range of toxicant doses (0–0.025 $LD_{50}$) has the saddle point coordinate $x_1^0 = 0.0088\,LD_{50}$ for lead falling within it (95% confidence interval for the coordinate $x_1^0$ is (0.0037; 0.0139)). This point divides the range of lead doses into two parts: the right-hand part features the synergism of lead and fluoride acting in the same direction, while in the left-hand one the effect is above ZIRS with the toxicants acting in opposite directions. Isoboles like the ones shown in Fig. 1g, result for the index *Alkaline phosphatase* as well. For this index, synergism is observed on the right of $x_1^0$ (as well as for *Number of squares*), while on the right of it the effect is below ZIRS for Pb and F acting in opposite directions.

In Fig. 1h, the range of toxicant doses (0–0.025 $LD_{50}$) has the saddle point coordinate $x_2^0 = 0.0094\,LD_{50}$ for fluoride falling within it (95% confidence interval for the coordinate $x_2^0$ is (0.0041; 0.0147)). The saddle point $x_2^0$ divides the fluoride dose range into two parts: below $x_2^0$, we observe the antagonism of lead and fluoride acting in the same direction, while above $x_2^0$ these toxicants act in opposite directions. Under action in opposite directions, the indices *Creatinine in blood serum* and *Number of osteoblasts* display an effect above ZIRS, while the effect for *Glucose* is below ZIRS. In both cases corresponding coordinate of the saddle point lies within the experimental dose range together with the 95% confidence interval.

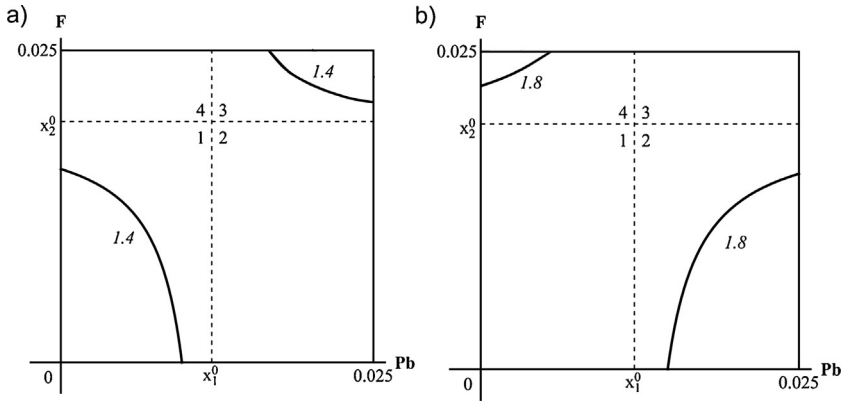


**Fig. 2.** Isoboles for micronuclei per 1000 polychromatophilic erythrocytes: (a) lower effect values; (b) greater effect values (designations as in Fig. 1).

The indices of class 3 in Table 2 are characterized by the fact that *both* saddle point coordinates $x_1^0$ and $x_2^0$ *always* occur within the dose range under study together with their 95% confidence intervals. In this case, the range of toxicant doses splits into 4 quadrants (as in Fig. 2). In the first and third quadrants, we always observe a unidirectional action of the toxicants, while in the second and forth quadrants it is always opposite. According to Table 2, in our case only one index falls into class 3, which is *Micronuclei per 1000 polychromatophilic erythrocytes*. The isoboles for this index are shown in Fig. 2. In the first and third quadrants, we observe antagonism and synergism, respectively (for unidirectional action); in quadrants 2 and 4, the effects are above ZIRS for Pb and F acting in opposite directions. A characteristic feature of the isobole for the indices of class 3 is the simultaneous presence of two branches for one level of effect $Y = Y^*$; in the particular case of $Y$ = micronuclei (Fig. 2), the branches of the isobole for the unidirectional action of Pb and F are observed for lower values of $Y^*$, while the opposite action of Pb and F, for greater values of $Y^*$.

Special consideration should be given to the identified cases of opposite effects produced by the toxic agents. This issue has not yet been given due attention in combined toxicity studies and in the corresponding area of pharmacology. As noted by Tallarida et al. [3]: "Interestingly, little or no attention has been given to active drugs that individually produce effects in the opposing direction."

As noted above, the direction in which two toxicants act is typically determined by the one-way "dose–effect" functions $Y(x_1, 0)$ and $Y(0, x_2)$: if both functions increase or decrease with growth in the corresponding argument $X_1$ or $X_2$, the toxicants $X_1$ and $X_2$ are recognized as acting in the same direction, if one of them increases while the other decreases, they act in opposite directions. However, this definition of directionality, being sufficient for understanding the direction in which the toxicants act in isolation, is too simplified for understanding the directionality of complicated and unambiguous combined toxicity. On the contrary, in the response surface theory the direction of action produced by toxicants is determined by the "slope" of the response surface $Y(x_1, x_2)$ toward the axes of the coordinates $X_1$ or $X_2$ at this point $(X_1, X_2)$ and, thus, reflects the direction in which each toxicant acts when in *combination*. For example, model (1) shows that the direction of action of the toxicants combined is not necessarily unambiguously set over the entire range of toxicant doses under study; it can vary in going over from one point $(x_1, x_2)$ to the other.

The 10 indices for which lead and fluoride demonstrate an opposite action can be divided into 3 groups. The first group of indices is characterized by the fact that the direction in which *Pb* and *F* act is manifest over the *entire* range of toxicant doses, since the saddle point (2) for them lies outside the range of the studied doses from zero to 0.025 $LD_{50}$. This includes *all* indices of subclass 2.2 (*ALT activity* and *Fluoride in bones*), as well as some indices from class 5 (in our case, these are *Specific proportion of bone trabeculae* and *Hemoglobin*). Out of these four indices, the first two demonstrate an opposite *additive* action. An example of the isobole for such indices is shown above in Fig. 1b. Fig. 1d shows isoboles for the index *Hemoglobin* (opposite action of Pb and F with the combined effect *above* ZIRS); Fig. 1f shows isoboles for the index *Specific proportion of bone trabeculae*, on which Pb and F produce an opposite action with the combined effect lying *below* ZIRS.

The second group of indices is characterized by the fact that the opposite action of *Pb* and *F* is observable only for certain doses of lead (the indices from subclass 4.1) or fluoride (the indices from subclass 4.2), since for the indices of these classes *one* of the saddle point coordinates (2) falls within the range of doses studied. In addition to the class 4 indices, the same group *may* include some indices from class 5. Thus, lead and fluoride act oppositely on the indices *Number of squares crossed over within 3 min* (subclass 4.1) and *Alkaline phosphatase* (subclass 5.1) for low doses of lead only (an example is shown in Fig. 1g). At the same time, the toxicants act on the indices *Creatinine in blood serum* and *Glucose* (subclass 4.2) and *Number of osteoblasts* (subclass 5.2) in opposite directions only for high fluoride doses (an example is shown in Fig. 1h).

The third group of indices is characterized by the fact that the opposite action of Pb and F may manifest itself in two areas of lead and fluoride doses since *both* coordinates of saddle point (2) fall within the dose range studied. Falling into this group are necessarily the indices of class 3 (in our case, this is *Micronuclei per 1000 polychromatophilic*

*erythrocytes*, Fig. 2), and it *may* include *some* indices from class 5; in this experiment, no such indices are to be found in class 5.

Thus, out of 18 indices for which we observe statistically significant effects of both toxicants, lead and fluoride, or a significant cross term in model (1) (these are indices from classes two to five), 10 indices demonstrate the possibility of an opposite action (either over the entire range of doses or in some sections of it). This points to the importance of studying opposite effects for practical toxicology and conducting further research into this area.

The last but not the least important inference from the discussed above, as well as from earlier published results is the ambiguity of the combined toxicity characterization which can differ depending not only on assessed indices of toxicity (as was stressed in Section 3.1) but also on the dose levels and their ratio. This ambiguity is important from the theoretical point of view, but what is expected from a toxicologist in everyday practice is a clear and unequivocal recommendation concerning a rule for monitoring safe concentrations of mixtures in the environment or for assessing health risks posed by the impact of a mixture. It is therefore that a proposal was put forward to establish an auxiliary concept of "main" or "determinant" type of combined effect for resolving such practical issues [24]. It was recommended that the choice of the main type of combined toxicity should be based on:

- the predominant significance of the type of combined effect that is revealed for doses (concentrations) causing chronic intoxication, and for doses which are close to the lowest observed adverse effect level (LOAEL);
- in cases where the combination under consideration occurs in real conditions mainly in a narrow range of ratios between its components – the priority given to the type of combined effect that is characteristic of this range;
- in cases where the organs and systems the response of which is the most involved in toxicodynamics and toxicokinetics of combined intoxication are known – the priority given to the type of combined effect that dominates with regard to changes in these organs (systems);
- in cases where the substances making up the combination, or at least one of them, are especially harmful to the organism or population (in particular, when carcinogenicity, mutagenicity, influence on the reproductive function are involved) – the priority given to the type of combined effect that is observed for these harmful effects, particularly in cases of synergy.

However, outside the scope of hygienic standard setting and risk assessment it is not only the "main" type but also the entire range of possible types of combined toxicity are of both theoretical and practical interest. For instance, even if additivity is accepted as the main type of combined toxicity based on the above criteria, the possible attenuation of certain manifestations of intoxication as a result of toxicological antagonism inherent to them can blur the clinical picture and modify the doctor's attitude to the diagnostic value of corresponding symptoms and, possibly, therapeutic tactics.

## 4. Conclusions

We compared a descriptive analysis of the combined toxicity of lead and fluoride based *on common sense considerations* with mathematical description of such combined action based on the Response Surface Methodology which generalizes the traditional paradigms of effect additivity and dose additivity. The first approach has revealed the three traditional variants of combined toxicity, additivity, synergism, and antagonism, in relation to various combined toxicity effects. The second approach (like other mathematical methods we had used earlier for describing the combined toxicity of lead and cadmium or chromium and nickel – specifically, those based on the principles of ANOVA or of Mathematical Theory of Experimental Design) has again enabled us to identify 10 subclasses of combined toxicity.

In this combination, as well in those studied by us earlier, we have revealed both unidirectional and opposite toxic action on the organism in relation to various effects and various levels of exposure. We believe that it is in a case of unidirectional action of toxic agents only that it would be reasonable to use the traditional terms "additivity", "more than additive (synergism)" and "less than additive (antagonism)". However, when two toxics act in opposite directions featuring formal (algebraic) additivity, their effects do not add up arithmetically; rather, they get subtracted. In such cases, the use of the above terms provokes terminological uncertainty, sometimes, even a misunderstanding between a toxicologist and a mathematician in their assessment of combined toxicity. Comparison of an observed effect produced by two toxics acting in combination with the zero interaction response surface makes it possible to describe the type of combined toxicity irrespective of the direction of their action.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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