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EXPRESS EXPERIMENTAL EVALUATION OF MUTAGENIC ACTIVITY OF COMPLEX PHOSPHORUS-CONTAINING ORGANIC MIXTURES

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Summary

Researching the mutagenic activity of organic phosphorus-containing mixtures of Esherichia Coli bacteria strain trp A 223 and the continuous cells culture of murine myeloma X63 also detected the presence of weak mutagenic properties in this group of xenobiotics. The concentrations of all compounds 400 and 1000 mg/l have bacteriostatic effect, and their lower concentrations in the range from 10 to 80 mg/l stimulate the mutagenic activity. Phosphorus-containing organic compounds can inhibit the synthesis of proteins, RNA and DNA in cell culture Vero depending on the concentration.

Key words: xenobiotics, carcinogenesis, mutagenesis, atherogenesis, suppression of immune deficiency, reproductive function, Esherichia Coli, cells culture of murine myeloma X63.

ЕКСПРЕС-ЕКСПЕРИМЕНТАЛЬНА ОЦІНКА МУТАГЕННОЇ АКТИВНОСТІ СКЛАДНИХ ФОСФОРОВМІСНИХ ОРГАНІЧНИХ СУМІШЕЙ

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Резюме

Дослідження мутагенної активності фосфорвмісних органічних сумішей на бактеріях Esherichia Coli штам trp A 223 і перевиваємій культурі клітин мишачої мієломи X63, виявили наявність слабких мутагенних властивостей досліджуваних ксенобіотиків. Всі сполуки в концентраціях 400 і 1000 мг/л володіють бактеріостатичним ефектом, а в більш низьких концентраціях в діапазоні від 10 до 80 мг/л вони стимулюють мутагенну активність. В залежності від концентрації, фосфорвмісні органічні сполуки здатні пригнічувати синтез білків, РНК і ДНК в культурі клітин Vero.

Ключові слова: ксенобіотики, канцерогенез, мутагенез, атерогенез, пригнічення імунологічної недостатності, репродуктивна функція, бактерії Esherichia Coli, клітини мишачої мієломи X63.

ЭКСПРЕСС-ЭКСПЕРИМЕНТАЛЬНАЯ ОЦЕНКА МУТАГЕННОЙ АКТИВНОСТИ СЛОЖНЫХ ФОСФОРОСОДЕРЖАЩИХ ОРГАНІЧЕСКИХ СМЕСЕЙ

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Резюме

. Исследования мутагенной активности фосфосодержащих органических смесей на бактериях Esherichia Coli штамм trp A 223 и перевиваемой культуре клеток мышиной миеломы X63 выявили наличие слабых мутагенных качеств исследованных

ксенобиотиков. Все соединения в концентрациях 400 і 1000 мг/л обладают бактериостатическим эффектом, а в больших концентрациях в диапазоне от 10 до 80 мг/л они стимулируют мутагенную активность. В зависимости от концентрации, форсфорсодержащие органические соединения способны угнетать синтез белков, РНК и ДНК в культуре клеток Vero.

Ключевые слова: ксенобиотики, канцерогенез, мутагенез, атерогенез, угнетение иммунологической недостаточности, репродуктивная функция, бактерии Esherichia Coli, клетки мышиной миеломы Х63.

Introduction. Environmental pollution by the chemicals manufacture of chemicals industry is growing every year and creates a global threat to the biosphere, including health. The leading polluters of human habitation are oil refining, oil industry, chemical production chemicals pesticides, herbicides, detergents, polyesters, nitro compounds, alkylating agents, pharmaceuticals and others [1, 2].

During the last 10 years the number of acute poisoning significantly is decreased with the significant increasing of chronic diseases and pathological conditions caused by environmental. In full measure it can be attributed to the production of complex organic phosphorus-containing mixtures which have surface-active properties and belong to ionic surfactants (SAS) [4, 5]. Phosphorus detergents according to the technical regulations, take a leading position in the world due to the volume and range of products made from them. Low concentrations of chemicals can carry great threat to public health which during some years can slowly and insensibly form the development of secondary symptomatic signs of diseases and pathological conditions. The analysis of the scientific literature shows that in the past 20 years the number of occupational diseases decreased, while the overall mixtures morbidity significantly increased [6,7]. Lagerly it can be connected with the pollution by chemical compounds of water, soil, air, food, and significant burden on the biosphere by chemical, petrochemical, pharmaceutical and electrochemical industries. Along with the studies of the most developed world countries including Ukraine over 20-30 years report about growing of overall morbidity and increasing cases of forming long-term effects - carcinogenesis, mutagenesis, atherogenesis, suppression of immune deficiency, reproductive function and so on. [3]. So, it known that reducing of the overall immune resistance may contribute to the deviation and unfavorable course of infectious diseases, inhibition of reparative processes which lead to tumor growth, mutagenesis, generative dysfunction [6]. Numerous studies indicate that a large number of chemicals are circulated in the environmental and they have

the specific term consequences. They can carry out specific impact on the human organism without noticeable general toxic effects manifested not during their action, or immediately after it, but in the long term life of individuals, often from chemical exposure postponed for years and even decades [8,9]. For society is chemically very dangerous due to distant effects in subsequent generations, which may be accompanied by chromosomal aberrations and gene mutations that can change gene pool population. Knowledge of the mechanisms of mutations and their consequences help to grasp the danger of mutagenic load on the environment. In addition to those mechanisms formation of mutagenic action remain largely unstudied. This also applies to new groups phosphorus organic compounds commonly used as flotation reagents, emulsifiers, surfactants, and others. [10, 11]. The lack of prognostic potential safety characteristics for the warm-blooded animals, large volume production and their spread contact with the population require necessary justification of pathochemical mechanisms of formation of long-term effects during prolonged effects on the body by the small dose of subtoxic xenobiotics and development of pathogenetic correction of the structural and metabolic disorders. It means that the availability priority is operational evaluation of the presence of possible long-term outcomes, including mutagenic effect. Have given above, **the purpose of this work** express is an experimental researching of presence mutagenic effects under the influence of complex phosphorus-containing organic mixtures using in vitro the microorganisms and tissue cultures.

Materials and methods. Choice of group of complex phosphorus-containing organic mixtures was largely determined by the necessity to study the pathochemical mechanisms of structural and metabolic disorders with prolonged subtoxic action of xenobiotics, the absence of prognostic characteristics of potential safety and widespread of new detergents group in the environment, manufacture and life. Three brands of phosphorus-containing detergents with regulated physico-chemical properties were studied. They have such tradenames: Efasol – mix of alkylphosphates and fraction of secondary alcohols C_{10}-C_{20}; Syntafon 10-18 - a mixture of mono- and diesters of alkylphosphoric acids which are made from fraction of primary fatty acids C_{10}-C_{18}; Polifos - 72 - a mixture of fraction of synthetic primary alcohols C_7-C_{12} and phosphoric anhydride. Phosphorus-containing detergents are used in chemical, oil, mining, metallurgical, construction, pharmaceutical, pulp and paper industry like detergents, emulsifiers, flotation reagents, stabilizers, anti-corrosion agents, antistatic, lubrikants and others. The aggregation state of all substances is viscous and readily soluble in water and liquid organic solvents. The research program included the study of possible gene mutations in short-term experiments on microorganisms and continuous cell cultures [7, 8]. In this

aspect, mutagenic effect has been studied on bacteria Esherichia Coli (strain K-12 F037), grown in special culture medium with addition of ampicillin (20 mg / ml) and followed using of SOS-chromotest which provides for the definition of increasing the synthesis of β-galactosidase and reducing the levels of alkaline phosphatase under influence the xenobiotics (9.10). The activity of alkaline phosphatase and β-galactosidase was determined by Miertus: $R = A_{420}^{b} * t^{p} / A_{420}^{p} * t^{b}$, where A - light absorption at 420 nm; t - reaction time; b - the activity of β-galactosidase; p - the activity of alkaline phosphatase. Induction factor is used to compare the results.

Into the cells the protein synthesis is activated by the influence of factors inducing single-strain breaks of DNA, which refers to SOS-regulon. Esherichia Coli MRE 600 was incubated for 10 min at 37 ° C with 35S-methionine to study the induction of protein fractions. Bacterial cells lysed by boiling in 1% solution dodecyl sulfa (DS), proteins are separated by electrophoresis in polyacrylamide gel. Gels were stained by Kumasi G-250, dried and exposed on X-ray film (RM-B) during two weeks. Avtoradiograms are densitymetried by Ultroscan XL for quantifing the radioactive protein fractions.

There is a wide range of chemicals that can disrupt the protein synthesis, RNA and DNA. The study of such processes was performed on the continuous cell culture of green monkey epithelium (Vero), which in vitro was exposed to the toxic effects by different concentrations of xenobiotics at once [6]. The studing of possible mutations in the locus of X-linked and gene synthesis of hypoxanthine-huanine-phosphoribozyl-transferring enzyme (HHPRT) was conducted on the continuous cell cultures Vero [9, 10, 11]. The authors concluded that the detection of mutant clones growing with the presence of 6-tiahuanine or 8-azahuanine are performed by hypoxanthine-huanine-phosphoribozyl-transferring enzyme wich catalyzes the conversion these non-toxic substrates to ribophosphorylated mutagens. The frequency of mutation HHPRT gene was assessed by the number of cells revertants $HHPRT^{+}$-$HHPRT^{-}$. Bacteria Esherichia Coli (strain trp A 223) and murine myeloma cell culture (HEZ) with BALb / also were used to determine the mutagenic activity of phosphate detergents. The number of cells revertants demonstrated the mutagenic effect in comparison with the controls.

Bacteria Esherichia Coli trp A strain 223were grown on meat peptone broth to stationary phase of growth. Aliquots of culture were treated by different concentrations of substances for 30 minutes. After which it was made series of 10-fold dilutions and seeding in Petri dishes with agar medium Mg. The plates were incubated during 18 hours at 37 ° C, then the colonies number was counted. The ratio of the number of colonies (K) in Petri dishes with agar medium Mg, containing tryptophan (1 mg / ml), describes the tryptophan synthetase

gene mutation frequency (K tryptophan$^-$ / K tryptophan$^+$). Evaluation of mutagenic activity in cell culture X63 was carried out as follows: phosphorus-containing mixture was added to the culture medium and incubated during1 hour at 37 ° C. Then the cells were dispersed into a 96-wells plate with the macrophages like feeder. The cells are not capable to produce HHPRT$^-$ remained alive during the line cultivation on the growth medium with the 8-azaguanine (50 mg / ml). The cells are capable to produce HHPRT$^+$ remained alive during the line cultivation on the growth medium with hypoxanthine-aminopterin-thymidine (HAT). Clones growing in culture plate wells were counted in two weeks using inversion microscope. The frequency of gene mutations HHPRT is estimated by the number of HHPRT $^+$ - HHPRT$^-$clones revertants .

The study of biosynthetic processes also carried out in cell culture of murine myeloma X63 in the presence of studied xenobiotics. The intensity of protein synthesis of RNA and DNA were determined by the incorporation level of radioactive precursors in TCA-insoluble precipitate. ^{3}H-timidyn (2,0 mCi / ml) and ^{3}H-uridyn (5,0 mCi / ml) were used to evaluate the synthesis of DNA and RNA, and ^{14}C-leucine (2,5 mCi / ml) - for the protein. Samples were treated on nitrocellulose filters according to the accepted methods, and radioactivity is measured by toluene scintillation β-counter "Beckman 7800". Processing of the results was carried out using Student's t test, Fisher.

Results and discussion. It is known that factors which induce single-strain breaks of DNA are combined with activation of SOS-regulon protein synthesis. To study the induction of individual protein fractions, cells Esherichia Coli strain MRE-600 were incubated for 10 minutes at 37^0C with 35S-methionine as it described above. The results showed that concentrations of phosphorus-containing organic mixtures up to 5.0 mg/l (0.005 mg/ml) did not effect on the spectral composition of proteins Esherichia Coli strain MRE-600. SOS-chromotest researching of bacteria Esherichia Coli strain K-12F037 found that concentrations of phosphorus-containing organic mixtures untill 0.4 mg/ml and 1.0 mg/ml, respectively, 400 and 1000 mg/l, significantly inhibited the activity of β-galactosidase and alkaline phosphatase (Table 1).

Table 1

Effect of complex organic phosphorus-containing mixtures on SOS- chromotest of bacteria Esherichia Coli strain K-12F037 (M±m).

Substances, (mg/ml)	β-galacto-sidase	Alkaline phosphatase	Induction factor of β-galacto-sidase	Induction factor of alkaline phosphatase
Control	1,68±0,24	1,47±0,18		
Efasol - 0,4 mg/ml	0,86±0,07 *	0,94±0,08 *	0,511	0,639
1,0 mg/ml	0,72±0,06 *	0,76±0,05 *	0,428	0,517
Syntaf 10-18 - 0,4 mg/ml	0,93±0,05 *	0,82±0,06 *	0,553	0,557
1,0 mg/ml	0,68±0,07 *	0,64±0,05 *	0,404	0,435
Polifos-72 - 0,4 mg/ml	0,75±0,06 *	0,73±0,04 *	0,446	0,496
1,0 mg/ml	0,63±0,05 *	0,55±0,06 *	0,375	0,374
Mitomycin - 0,4 mg/ml	2,93±0,27 *	1,68±0,18 *	1,744	1,142
1,0 mg/ml	4,35±0,38 *	1,52±0,14 *	2,589	1,034

Note:* - the significance level $p \leq 0,05$ is valid for the control.

But it should be noted that the classical mutagen mitomycin increased the activity in these concentrations of β-galactosidase and virtually not impacted the alkaline phosphatase. Analysis of the results showed that under the influence of phosphorus-containing detergents reducing the activity of β-galactosidase and alkaline phosphatase it was observed the factor inhibition of their induction. In this case we can assume that too high concentrations of xenobiotics are capable of bactericidal or bacteriostatic effects which were obtained due to the concentrations of 0.4 and 1.0 mg/ml or 400 and 1000 mg/l.

Assessment effect the concentration 10 mg/l (0.01 mg/ml) of organic phosphorus-containing mixtures detected the increasing of β-galactosidase and alkaline phosphatase activities during SOS-chromotest on the bacteria Esherichia Coli strain K-12F037. So, when it was compared with the classic mutagens mitomycin there was an increasing the enzyme activity due to suppression factor induction of β-galactosidase and increasing alkaline phosphatase induction factor (Table. 2). Comparative analysis of the results gives reason to believe that phosphorus- containing organic mixtures have weak mutagenic properties. The

concentration 1 mg/l (0.001 mg/ml) of xenobiotics did not change the activity of β-galactosidase and alkaline phosphatase enzymes.

Table 2

Effect of the organic phosphorus-containing mixtures concentration 10 mg/l (0,01mg/ml) on SOS- chromotest of bacteria Esherichia Coli strain K-12F037.

Substances, (10 mg/ml)	β-galacto-sidase	Alkaline phosphatase	Induction factor of β-galacto-sidase	Induction factor of alkaline phosphatase
Control	1,68±0,24	1,47±0,18		
Efasol	2,34±0,27 *	2,23±0,21 *	1,392	1,517
Syntaf 10-18	2,46±0,31 *	2,35±0,26 *	1,464	1,598
Polifos-72	2,75±0,33 *	2,44±0,19 *	1,636	1,142
Mitomycin - 0,4 mg/ml 1,0 mg/ml	2,93±0,27 * 4,35±0,38 *	1,68±0,18 * 1,52±0,14 *	1,744 2,589	1,142 1,034

Note:* - the significance level $p \leq 0,05$ is valid for the control.

Researching the mutagenic activity of organic phosphorus-containing mixtures of Esherichia Coli bacteria strain trp A 223 and the continuous cells culture of murine myeloma X63 also detected the presence of weak mutagenic properties in this group of xenobiotics. It was found the mutagenic activity increasing almost in 3-4 times in the gene locus tryptofansyntetase of bacteria Esherichia Coli strain trp A 223 under the influence of xenobiotics concentrations of 10.0 mg/l and 20.0 mg/l (Table. 3). Wherein this activity increasing was in 2 times less than under the influence of UV radiation, which is a standard mutagenic factors. Results of the mutagenic activity studying the concentrations of phosphorus-containing detergents 40.0 mg/l and 80.0 mg/l on cells culture X63 detected the increasing in 2-3 times of mutagenic activity in the gene locus $HHPRT^+$ - $HHPRT^-$ compared to control levels. However, it must be said that the number of revertanted clones by the action of phosphorus-containing detergents was lower almost in 2-3 times than by the influence of classical mutagen ethylmethanesulfonate (Table. 3).

Table 3

Effect of the organic phosphorus-containing mixtures on the mutagenic activity of bacteria Esherichia Coli strain trp A 223 and the clones of $HHPRT^+$ - $HHPRT^-$.of cells culture of murine myeloma X63

Xenobiotic	Esherichia Coli strain trp A 223. K trypt-/K trypt+, (mg/l)		Clones of $HHPRT^+$ - $HHPRT^-$.of cells culture of murine myeloma X63			
			$HHPRT^+$ (8-azaguanine)		$HHPRT^-$ (HAT), ml/l	
	10,0	20,0	40,0	80,0	40,0	80,0
Control	10,7±1,4 *		8		3	
Efasol	34,5±2,7 *	46,7±3,2 *	24 *	32 *	8 *	7 *
Syntaf 10-18	29,3±1,8 *	45,8±2,6 *	21 *	29 *	6 *	8 *
Polifos -72	32,6±2,3 *	47,4±2,7 *	19 *	8 *	7 *	6 *
UV radiation	78,4±5,6 *		-		-	
Ethylmethane-sulfonate	-	-	53		23	

Note:* - difference of significance level $p \leq 0,05$ is valid for the control and UV radiation and ethylmethanesulfonate (mutagens).

All of this confirms the presence of weak mutagenic properties this group of chemical compounds. Similar results were obtained when assessing mutagenic activity of phosphorus-containing detergents on cell culture Vero. Both concentration of xenobiotics 40.0 mg/l and 80.0 mg/l increased the number of clones revertants compared with control, but their level was lower than ethylmethanesulfonate exposure condition. All of this makes it possible to affirm that phosphorus-containing detergents have weak mutagenic properties.

The studying of biosynthetic processes carried out in cell culture Vero. In the cell culture the intensity synthesis of protein, RNA and DNA detected the inhibition of incorporation of ^{14}C - protein hydrolyzate, the inclusion of ^3H-uridine and ^3H-thymidine under the influence of xenobiotics concentrations 20 and 40 mg/l (Table. 4).

Table 4

Effect of the organic phosphorus-containing mixtures on synthesis of protein, RNA and DNA in cell culture Vero (M+m).

Xenobiotic	Con-cent-ration, mg/l	Radioactivity of TVY- insoluble fraction (imp/10s×10^6 cells)		
		inclusion of ^{14}C - protein hydrolyzate	inclusion of ^{3}H-uridine RNA of the cell culture	inclusion of ^{3}H- thymidine DNA of the cell culture
Efasol	10,0	1483,4±125,6	1934,5±176,4	4157,2±305,7
	20,0	582,3±16,77	867,6±21,8	2263,8±196,4
	40,0	276,5±12,8	453,2±9,3	1235,4±82,3
Syntaf 10-18	10,0	1524,5±85,9	1888,7±172,5	4266,7±283,5
	20,0	622,7±18,4	537,4±18,3	2193,8±204,2
	40,0	235,6±14,3	310,8±18,5	1342,5±85,5
Polifos-72	10,0	1451,9±107,6	1956,2±153,4	4342,9±274,8
	20,0	537,6±14,5	697,5±24,7	2156,5±178,3
	40,0	195,3±10,2	420,6±18,5	1268,3±85,5
Control	-	1568,4±140,6	1987,4±185,3	4236,2±273,4

The concentrations of phosphorus-containing organic compounds 10 mg/l did not affect the metabolism of proteins and nucleic acids RNA and DNA. Along with this the concentrations of Efasol 20,0mg/l reduced the inclusion of ^{14}C - protein hydrolyzate, ^{3}H-uridine and ^{3}H-thymidine in cell culture in 2.69 times, 2.29 times and 1.87 times respectively. More significant inhibition of incorporation was observed under the influence of Efasol concentration 40,0mg/l: the inclusion of ^{14}C - protein hydrolyzate is decreased in 5.67 times, ^{3}H-uridine in 4.38 times, ^{3}H-thymidine in 5.67 times. Under the influence Syntaf 10-18 and Polifos-72 it was established similar dynamics violation of the synthesis of proteins, RNA and DNA. Studies have shown that xenobiotics increasingly inhibit the synthesis of proteins and nucleic acids.

Conclusions. Therefore, the results show that relatively high concentrations of such phosphorus-containing organic compounds as Efasol, Syntaf 10-18 and Polifos -72 400 and 1000 mg/l (0.4 and 10.0 mg/l) inhibit the metabolic activity of the cell culture Vero. Their

lower concentrations 10.0 mg/l (0.01 mg/ml), activate the increasing synthesis of β-galactosidase enzymes and alkaline phosphatase during of SOS-chromotest on the bacteria Esherichia Coli strain K-12F037. The concentrations 10 and 20 mg/l of phosphorus-containing detergents can increase the mutagenic activity in the locus tryptophansynthetase gene of bacteria Esherichia Coli strain trp A 223, and their concentration 40 and 80 mg/l can increase the mutagenic activity in the locus gene hypoxanthine-guanine-phosphoribozyltransferring enzyme of cell culture murine myeloma X63. In comparison with classical mutagens (ultraviolet radiation and ethylmethane-sulfonate) much less induction of enzyme activity under the influence of phosphorus-containing detergent allows to include these compounds to the weak mutagens, which have not specific mutagenic effect. The concentrations of all compounds 400 and 1000 mg/l have bacteriostatic effect, and their lower concentrations in the range from 10 to 80 mg/l stimulate the mutagenic activity. Phosphorus-containing organic compounds can inhibit the synthesis of proteins, RNA and DNA in cell culture Vero depending on the concentration. Concentration of xenobiotics 1 mg/l (0.001 mg/ml) did not affect the metabolic and mutagenic activities of Esherichia Coli strain K-12F037 and this suggest that this concept is not affecting the genetic apparatus under conditions of express-experimental testing of organic phosphorus- containing mixtures.

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