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Short Communication

THE HEMOLYTIC AND PHYSIOLOGICAL ACTIVITIES OF MIXTURES OF SOME PHENOXY AND ORGANOPHOSPHOROUS HERBICIDES

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Abstract: Experiments were performed investigating the potential to improve the biological activity of some phenoxy and organophosphorous compounds by using them in binary mixtures. The compounds were: 2,4-dichlorophenoxyacetic acid (**1**) and its sodium salt (**2**), dibutyl 1-butylamino-1-cyclohexanephosphonate (**3**) and diethyl 9-butylamino-9-fluorenephosphonate (**4**), all widely used as herbicides. There were two test methods: the inhibition of cucumber (*Cucumis sativus*) growth induced by one single herbicide or by equimolar binary mixtures of herbicides; and, in parallel, the hemolytic efficiency of separate compounds or their mixtures. The hemolytic properties of the compounds were studied as hemolysis is generally a good measure of their toxicity, especially in the case of lipophilic compounds. Pig erythrocytes were used as good models for the determination of toxicity and the kinetics of red blood cell hemolysis. In the plant-based experiments, binary mixtures were found to display additive type toxicity. The compounds' hemolytic activities were of additive or antagonistic types. In some combinations, the addition of a second component did not change the hemolytic efficiency of the first component, and *vice versa*.

Key Words: Herbicide Binary Mixtures, Hemolysis, Physiological Activity

INTRODUCTION

The application of chemicals of specific destination in multi-component mixtures may widen their activity spectrum, especially when they exert their

Abbreviations used: DBBC - dibutyl 1-butylamino-1-cyclohexanephosphonate; DEBF - diethyl 9-butylamino-9-fluorenephosphonate; 2,4-D acid - 2,4-dichlorophenoxyacetic acid; 2,4-D salt - sodium 2,4-dichlorophenoxyacetic

toxicity by different modes of action, and may increase the overall activity in comparison with that of a single compound. The toxicity of a mixture is definable in a range of ways, from synergistic to antagonistic cooperation between the mixed components [1-6].

Evidence exists that the toxicity of many compounds with biological activity, organophosphorous compounds included, is connected with their lipophilicity, which enables them to incorporate into the lipid phase of the cell membrane, and thus to initiate physicochemical changes leading to living organism metabolism perturbation and/or death [7-11].

This paper contains the results of studies on the combined toxicity of phenoxy and organophosphorous compounds that are known for their biological activity and are widely used as potent herbicides. They are: (1) 2,4-D (2,4-dichlorophenoxyacetic acid); (2) its sodium salt; (3) Trakephon (dibutyl 1-butylamino-1-cyclohexanephosphonate – DBBC); and (4) one of the aminophosphonic acid derivatives of fluorene (diethyl 9-butylamino-9-fluorene-phosphonate – DEBF) [7, 9, 12-16]. The toxicity of 2,4-D and Trakephon are also strongly connected with their interaction with cell membranes, e. g., with erythrocyte membranes [7, 9]. The opinion exists that the biological activity of the last of the four studied herbicides may also be at least partially connected with its interaction with the lipid phase of biological membranes [12]. However, its cell membrane-disrupting activity was found to be significantly weaker than the(its) physiological toxicity [9].

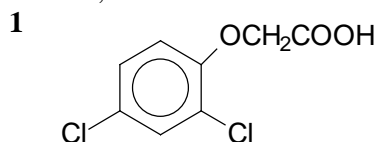
In this study, we checked whether binary mixtures of the compounds studied may exhibit higher physiological and membrane-disrupting activity than that observed for particular compounds on their own. In order to do that, we studied the inhibition of the growth of cucumber (*Cucumis sativus* cv “Wisconsin”) induced by all the compounds applied alone and in equimolar binary mixtures. The compounds’ membrane-disrupting ability was studied with the use of pig erythrocytes.

MATERIALS AND METHODS

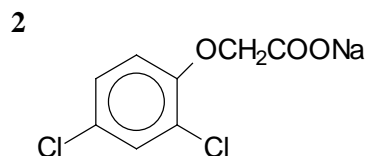
All the herbicides studied were synthesized in the Department of Organic Chemistry, Biochemistry and Biotechnology of the Technical University of Wrocław. The purity was checked by ¹H-NMR and ³¹P-NMR spectra. Their general structure is given below.

Fresh heparinized pig blood was used in the hemolytic experiments. The blood was centrifuged for 3 minutes at 1000 g, the plasma removed and the cells washed twice with an isotonic phosphate buffer solution (131 mM NaCl, 1.79 mM KCl, 0.86 mM MgCl₂, 11.80 mM Na₂HPO₄·2H₂O, 1.80 mM NaH₂PO₄·H₂O) of pH 7.4. The erythrocytes were then incubated for 1 h at 37°C in the same solution containing 0.4 mM concentrations of the pesticides studied or equimolar mixtures of two of the pesticides, each mixture at a 0.4 mM overall concentration. Other mixtures were made up from a 0.4 mM solution of one

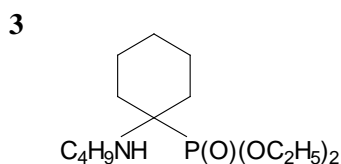
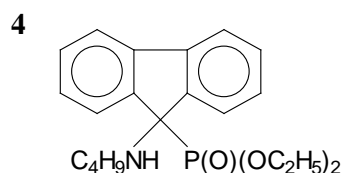
component and a 0.1 mM solution of the second one. After modification, samples were taken at 10 min intervals and centrifuged, and the supernatant was assayed for hemoglobin content at 540 nm. During all the stages of the procedure, we insured that the suspension was well mixed.



2,4-dichlorophenoxyacetic acid



sodium - 2,4-dichlorophenoxyacetate

dibutyl 1-butylamino-1-cyclohexane-
Phosphonatediethyl 9-butylamino-9-fluorene-
phosphonate

The toxicity of the herbicides studied was determined using a toxicity unit model (TU) [1], where a value of 1 TU was assigned to the effective concentration of a compound causing 50% inhibition (EC_{50}) of growth of cucumber (*Cucumis sativus* cv “Wisconsin”). The toxicity of the mixtures was calculated as a sum; $TU_{mix} = C_1/EC_{50} + C_2/EC_{50}$, where C_i is the compound concentration in the mixture. TU_{mix} was compared to the measured toxicity. The experiments were run in a SANYO® growth chamber for 96 h with a 9:15 light:dark cycle at 25°C. The effects of five concentrations of each compound were determined in triplicate. Seeds were germinated at 25°C for two days in darkness. Tests with binary mixtures were performed in the same way. Concentrations of each herbicide were added at proportions of their respective EC_{50} 's, so that the sum of the concentrations was equivalent to the five tested concentrations (0.5 to 2.0 units). For example, to achieve 1.5 unit toxicity for a mixture of compounds **1** and **3**, they were used at concentrations of 3.6 μ M and 202.5 μ M, respectively (see Table 1).

RESULTS AND DISCUSSION

The values of EC_{50} found for 2,4-D, DBBC and DEBF and the calculated toxicities for their binary mixtures are collected in Table 1.

Qualitatively different results were obtained in the study of the hemolysis of erythrocytes treated with particular compounds individually and in binary mixtures. Compounds **1** and **2** caused no hemolysis when used at a 0.1 mM concentration, while the same concentration of compound **3** brought about 40%

Tab. 1. The partial toxicity (TU) and the effective concentrations of cucumber growth inhibition (EC_{50}) for the binary mixtures.

Pesticide	EC_{50} (μ M) ($\pm 95\%$ confidence limits)	Binary mixtures				
		$\Sigma 0.5$ TU	$\Sigma 0.75$ TU	$\Sigma 1.0$ TU	$\Sigma 1.5$ TU	$\Sigma 2.0$ TU
1	4.8 (4.3–5.4)	1.2	1.8	2.4	3.6	4.8
3	270 (244–296)	67.5	101.25	135	202.5	270
4	550 (506–600)	137.5	206.25	275	421.5	550

hemolysis. The level of red blood cell hemolysis observed for the **1 + 3** mixture (not shown here) was the same as that for compound **3** used alone, and that for the mixture of **2 + 3** was significantly lower than caused by **3** (Fig. 1). It means that in the first of mentioned mixtures, there was no interaction between the compounds, while in the second mixture, the compounds acted as antagonists. In turn, compound **1** completely inhibited the hemolytic efficiency of compound **4**, which, when used alone at a 0.4 mM concentration, caused 60% hemolysis of erythrocytes (Fig. 2), while compound **2** had no influence on this efficiency (not shown here).

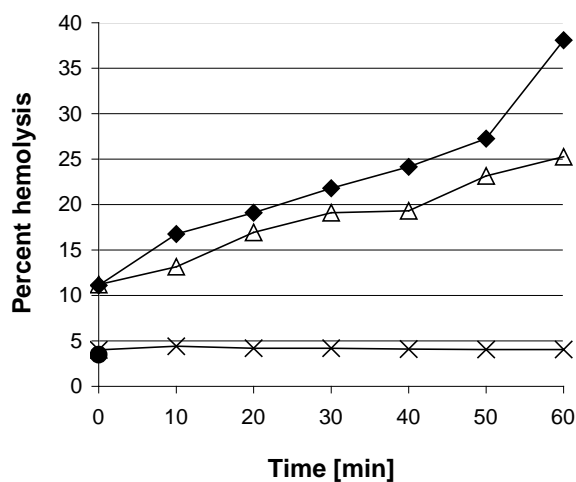


Fig. 1. The hemolysis of erythrocytes induced by 2,4-D salt (compound **2**) and DBBC (compound **3**), used individually and in binary mixture. ■ - DBBC 0,1 mM (**3**), ▲ - DBBC 0,1 mM and 2,4-D salt 0,1 mM (**3+2**), × - 2,4-D salt 0,1 mM (**2**), ● - control.

The combination of compounds (**3 + 4**), no matter which was added to the incubation solution first, caused hemolysis that may be defined as the additive effect type. It seems they do not influence each other's action (Fig. 3). Indeed, the same is true for the other mixtures.

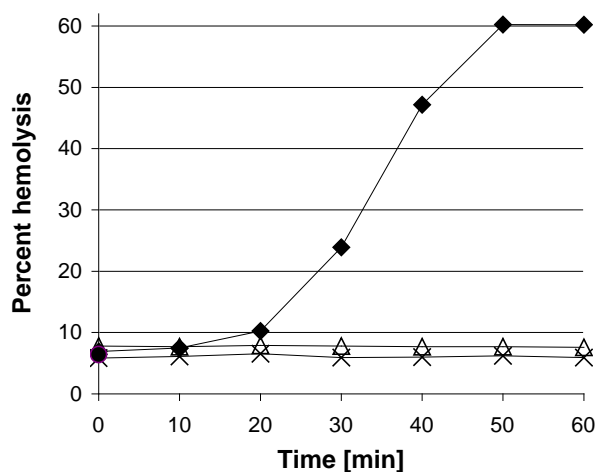


Fig. 2. The hemolysis of erythrocytes induced by 2,4-D acid (compound 1) and DEBF (compound 4) used individually and in binary mixture. ■ - DEBF 0,4 mM (4), △ - DEBF 0,4 mM and 2,4-D acid 0,1 mM (4+1), * - 2,4-D acid 0,1 mM (1), ● - control.

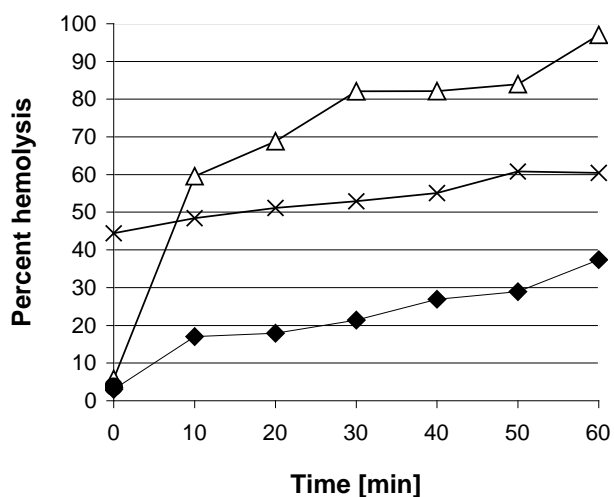


Fig. 3. The hemolysis of erythrocytes induced by DBBC (compound 3) and DEBF (compound 4) used individually and in binary mixture. ■ - DBBC 0,1 mM (3), △ - DBBC 0,1 mM and DEBF 0,4 mM (3+4), * - DEBF 0,4 mM (4), ● - control.

The results of physiological experiments concerning the inhibition of the growth of cucumber by binary mixtures of the compounds studied are summarized in Table 2. 2,4-D amine salt (compound 2) was used in these experiments. Its combinations with DBBC (compound 3) and DEBF (compound 4) were found to have an additive effect. The toxicity of binary mixtures of these latter

compounds was diminished in comparison with the effects of both components used in equivalent quantities, which indicates an antagonistic interaction between them. For comparison, see the values summarized in Tab. 1.

Tab. 2. The combined actions of pesticides studied

Mixture	Toxic units (TU)	Combined action
2 + 3	1.03 (0.92 – 1.12)	Additive
2 + 4	1.17 (1.02 – 1.32)	Additive
3 + 4	1.55 (1.40 – 1.70)	Antagonistic

The results of the physiological experiments are somewhat contradictory to those obtained during the hemolytic experiments, and it is hard to explain them. It seems that the toxic effects observed are a combination of membrane destabilization and the direct interaction of the pesticides with metabolism-driving cell components. In the case of more membrane-active compounds, a concurrence for the same interaction sites, indicating similar mechanisms of toxicity, seems to exist. No simple explanation can be offered for all the cases. For instance, it was shown earlier [9] that Trakephon's (compound **3**) physiological toxicity was much weaker than that of DEBF, and its hemolytic efficiency much better. This should indicate significantly different mechanisms of toxicity of these compounds, or the domination of one of the components of toxicity. The results of the hemolytic experiments do not permit such a conclusion to be drawn, and further investigation is required.

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