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ANTIOXIDATIVE ACTIVITY OF SOME PHENOXY AND ORGANOPHOSPHOROUS COMPOUNDS

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Abstract: Experiments were performed in order to check whether biological activity of some organophosphorous compounds widely applied as herbicides: 2,4-dichlorophenoxyacetic acid (**1**) and its sodium salt (**2**), N-phosphonomethylglycine acid (**3**) and its sodium salt (**4**), diethyl 1-butylamino-1-cyclohexanephosphonate (**5**) and diethyl 9-butylamino-9-fluorenephosphonate (**6**) followed from their oxidative activity. The compounds studied differed in their polarity and hydrophobicity. On the contrary, it was found that all herbicides protected erythrocyte membranes against partial peroxidation induced by UV irradiation. The effect was somewhat differentiated and followed the sequence: **5** > **1** > **2** > **6** > **3** > **4**.

The observed differences between the antioxidative activities of the compounds are probably related to differences in their ability to incorporate into the lipid phase of the erythrocyte membrane. Once incorporated, they change fluidity of the membranes. The extent of the changes was determined in fluorescence measurements. Polarization and anisotropy coefficients of erythrocyte membranes modified by micromolar concentrations of herbicides at different temperatures were measured for that purpose. Generally, they followed the sequence found for antioxidative activity of the herbicides studied, which confirms the assumption of close correlation between the depth of incorporation of a herbicide into the erythrocyte membrane and its protective efficiency.

Key Words: Organophosphorous Herbicides, Antioxidative Activity, Erythrocytes

INTRODUCTION

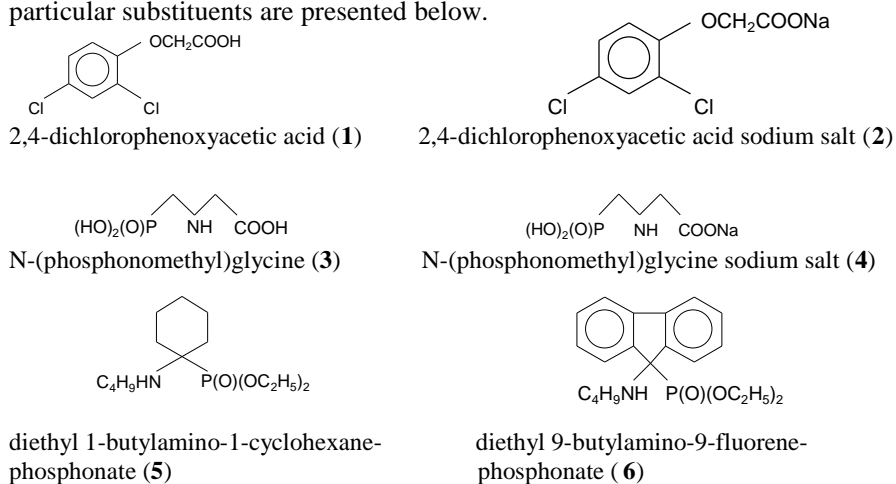
Many organophosphorous and phenoxy compounds are known for their biological activity and are widely used as potent herbicides. Perhaps the best

known of them are 2,4-D (2,4-dichlorophenoxyacetic acid) and its derivatives [1-3], glyphosate (N-phosphonomethylglycine) [4], Trakephon (diethyl 1-butylamino-1-cyclohexanephosphonate) [5], and aminophosphonic acid derivatives of fluorene [6, 7]. It is understood that pesticide activity of organophosphorous compounds, also those studied in this work, is connected with their lipophilicity, which enables them to incorporate into membranes. Therefore, it is reasonable to expect that the primary pesticide effects are the result of perturbations in physicochemical properties of biological membranes [1, 8]. This may lead to the destruction of the membrane when the concentration of the lipophilic compound is high enough. On the other hand, the presence of various compounds in membranes at low concentrations was shown to stabilize the membranes [9, 10]. It is also possible that some compounds applied at low concentrations may exhibit other useful features. Indeed, it has been shown that some compounds, also organophosphorous, when incorporated into erythrocyte membranes, can protect them against oxidation [11-13].

In this work we tried to find whether the oxidative effect, if present, is connected with the incorporation of the mentioned pesticides into erythrocyte membranes (RBC) irradiated with UV. They were used at sublytic concentrations so as not to break membrane integrity. This was checked by fluorometric measurements that show the membrane fluidity changes induced by the compounds studied. The measured parameters were polarization and anisotropy coefficients.

MATERIALS AND METHODS

The compounds studied were synthesized in the Department of Organic Chemistry, Biochemistry and Biotechnology of the Technical University of Wrocław. The purity was checked by $^1\text{H-NMR}$ and $^{31}\text{P-NMR}$ spectra. The general structure of the organophosphorous and phenoxy compounds studied and particular substituents are presented below.



The oxidation studies were performed on erythrocyte membranes from fresh heparinized pig blood, prepared according to Dodge *et al.* [14]. Erythrocyte ghosts were suspended in phosphate solution (131 mM NaCl, 1.80 mM KCl, 0.85 mM MgCl₂, 11.80 mM Na₂HPO₄·2H₂O, 1.80 mM Na₂H₂PO₄·H₂O) of pH 7.4 at protein concentration *ca.* 1mg/ml. Lipid peroxidation in the RBC membrane was induced by UV irradiation (a low-pressure bactericidal lamp, BakMed, Łódź, Poland, 95% energy emitted at wavelengths 253.7 nm, with an intensity of 3.5 μW/cm²) for 1 hour. The degree of lipid peroxidation was determined spectrophotometrically by measuring the concentration of malonic dialdehyde (MDA) released in the samples. MDA is one of the end products of lipid peroxidation and gives colour reaction with thiobarbituric acid. Further details were given elsewhere [11]. The fluidity experiments were performed on erythrocyte ghosts, which were subjected to the action of the compounds studied. Two fluorescent probes were used at 1 μM concentration. They were DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate)], purchased from Molecular Probes Inc. (Eugene, USA). The measurements were performed with an SFM 25 spectrofluorometer (KONTRON, Zurich, Switzerland) at 25°C and 37°C. The excitation and emission wavelengths were 354 nm and 429 nm, respectively. The polarization (P) and anisotropy coefficients (A) were calculated according to the following formulas [15-17]:

$$P = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + GI_{\perp}); \quad A = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + 2GI_{\perp}) \quad (1)$$

where I_{\parallel} is the intensity of fluorescence emitted in the direction parallel to the polarization plane of the exciting light, I_{\perp} is the intensity of fluorescence emitted in the perpendicular direction, and G is the diffraction constant used to correct for the inability of the instrument to transmit differently polarized light equally.

RESULTS AND DISCUSSION

The results of antioxidation studies are shown in Table 1. It can be seen that all the compounds protected lipids of erythrocyte membranes against oxidation by UV irradiation and that efficiency of this protection increased with the compounds' concentration. Generally, the sequence of the protective ability of the compounds studied was as follows: **5** > **1** > **2** > **6** > **3** > **4**. However, it must be noted that for the concentration of 1 mM compound **4** protected membrane lipids better than compounds **3** and **6**.

The results of membrane fluidity measurements are shown in Figure 1 as the dependencies of polarization (P) and anisotropy (A) coefficients on concentrations of compounds **5** and **6**, respectively. Both compounds influenced membrane fluidity in a similar way, although compound **6** seems to exert a slightly stronger effect.

Tab. 1. Percentage of oxidation inhibition of erythrocyte membrane lipids vs. concentration of compounds.

Compound	Concentration [mM]	Oxidation inhibition [%]
1 2,4-dichlorophe-phenoxyacetic acid	0.5	21.5
	1.0	42.2
	3.0	70.1
2 2,4-dichlorophe-phenoxyacetic acid sodium salt	0.5	19.0
	1.0	40.3
	3.0	66.4
3 N-(phosphono-methyl)glycine	0.5	16.2
	1.0	24.3
	3.0	60.0
4 N-(phosphono-methyl)glycine sodium salt	0.5	15.5
	1.0	30.1
	3.0	56.2
5 Diethyl 1-butyl-amino-1-cyclohexa-nephosphonate	0.5	28.4
	1.0	53.5
	3.0	72.1
6 Diethyl 9-butyl-amino-9-fluorene-phosphonate	0.5	18.0
	1.0	28.5
	3.0	65.2

Standard deviation did not exceed 4%.

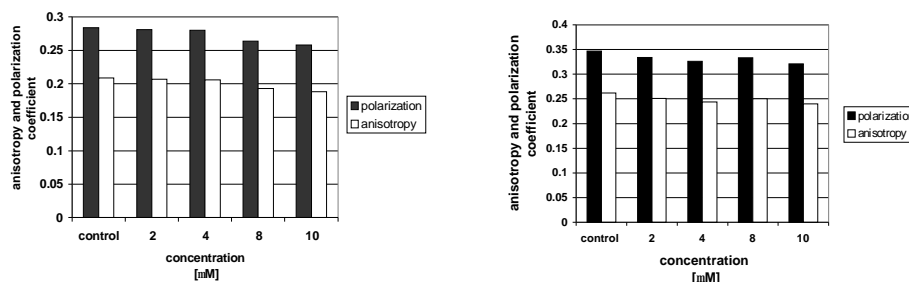


Fig. 1. Polarization and anisotropy coefficients versus concentration of compound **5** (left) and **6** (right) with fluorescent probe DPH at 37°C.

Table 2 contains the values of P obtained for two probes (DPH and TMA-DPH) used at 10 μ M concentrations and at different temperatures (25°C and 37°C). As expected, fluidity was found to be higher at elevated temperatures. Different values of polarization coefficients indicate a different localization of both the probes used in the lipid bilayer of the RBC membrane. No significant difference in sensitivity of both probes to fluidity changes inside the membrane was detected.

Tab. 2. Polarization coefficients for compounds studied used at concentration 10 mM at 25°C and at 37°C with fluorescent probes DPH and TMA-DPH.

Compounds	Polarization coefficient P			
	DPH probe		TMA-DPH probe	
	25°C	37°C	25°C	37°C
1	0.304	0.267	0.349	0.263
2	0.320	0.284	0.357	0.323
3	0.278	0.240	0.353	0.327
4	0.298	0.266	0.361	0.320
5	0.333	0.258	0.365	0.304
6	0.366	0.274	0.373	0.321
Control	0.330	0.280	0.345	0.302

Figure 2 presents the concentration of compounds that inhibit UV caused oxidation by 50% of membrane lipids. In order to do that, all the compounds, with the exclusion of **6**, were used in concentrations higher than those given in Table 1. The sequence of efficiency of antioxidative protection was the same as that mentioned above for the highest concentration used in oxidation experiments.

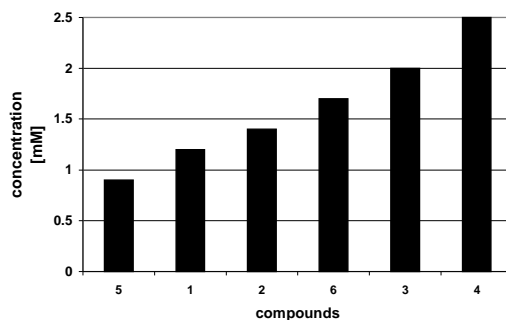


Fig. 2. Compound concentrations causing 50% oxidation inhibition.

The general conclusion emerging from the results obtained is that the organophosphorous and phenoxy pesticides studied may act as moderate antioxidative agents. Perhaps this is the reason why no increased activity of some antioxidant enzymes was observed during studies on oxidative stress in tilapia liver exposed to compound **1** [18]. The antioxidative efficiency of the compounds studied followed their efficiency to modify fluidity of erythrocyte membranes. Since that effect depends on the localization of the compounds in the membrane, the obvious conclusion is that also the antioxidative action is governed by the possibility of incorporation of the compounds into the membranes and the concurrent change in their physicochemical properties. The

same conclusion was already formulated in the case of studies on the antioxidant activity of various bifunctional surfactants [19, 20] or toxicity of some organophosphorous insecticides [8].

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REFERENCES

1. Suwalsky, M., Benites, M., Villena, F., Aguilar, F. and Sotomayor, C.P. Interaction of 2,4-dichlorophenoxyacetic acid (2,4-D) with cell and model membranes. **Biochim. Biophys. Acta** 1285 (1996) 267-276.
2. Medyantseva, E.P., Vertlib, M.G., Kutyreva, M.P., Khaldeeva, E.I., Budnikov, G.K. and Eremin, S.A. The specific immunochemical detection of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid pesticides by amperometric cholinesterase biosensors. **Anal. Chim. Acta** 347 (1997) 71-78.
3. Oruç, E.Ö. and Üner, N. Effects of 2,4-Diamin on some parameters of protein and carbohydrate metabolisms in the serum, muscle and liver of *Cyprinus carpio*. **Environ. Poll.** 105 (1999) 267-272.
4. Cole, D.J. Mode of action of glyphosate – a literature analysis. In: **The herbicide glyphosate**. (Grossbard, E. and Atkinson, D., Eds.), Butterworths, London (1985) pp. 48-74.
5. Perkow, W. **Wirsubstanzen der Pflanzenschutz und Schadlingsbekämpfungsmittel**. Part II. (Parey, P. Ed.), Verlag, Berlin und Hamburg (1983/1988) p. 24.
6. Lejczak, B., Kafarski, P. and Gancarz, R. Plant growth regulating properties of 1-amino-1-methyl phosphonic acid and its derivatives. **Pest. Sci.** 22 (1988) 263-275.
7. Gancarz, R. and Dudek, M. Structure-activity studies of aminophosphonic derivatives of fluorene. **Phosphorus, Sulfur and Silicon** 114 (1996)135-142.
8. Videira, R.A., Antunes-Madeira, M.C., Lopes, V.I.C.F. and Madeira, V.M. C. Changes induced by malathion, methylparathion on membrane lipid physicochemical properties correlate with their toxicity. **Biochim. Biophys. Acta** 1522 (2001) 360-368.
9. Hagerstrand, H. and Isomaa, B. Amphiphile-induced antihemolysis is not causally related to shape changes and vesiculation. **Chem. Biol. Interactions** 79 (1991) 335-347.
10. Isomaa, B., Hagerstrand, H., Paatero, G. and Engblom, A.C. Permeability alterations and antihemolysis induced by amphiphiles in human erythrocytes. **Biophys. Biochim. Acta** 860 (1986) 510-524.
11. Kleszczyńska, H., Oświęcimska, M., Witek, S. and Przesalski, S. Inhibition of lipid peroxidation in the erythrocyte membranes by quaternary

- morpholinium salts with antioxidant function. **Z. Naturforsch.** 53c (1998) 425-430.
12. Kleszczyńska, H., Sarapuk, J., Oświęcimska, M. and Witek, S. Antioxidative activity of some quaternary ammonium salts incorporated into erythrocyte membranes. **Z. Naturforsch.** 55c (2000) 976-980.
 13. Kleszczyńska, H. and Sarapuk, J. New aminophosphonates with antioxidative activity. **Cell. Mol. Biol. Lett.** 6 (2001) 83-91.
 14. Dodge, J.T., Mitchell, C. and Hanahan, D.J. The preparation and chemical characteristics of hemoglobin-free ghosts of erythrocytes. **Arch. Biochem.** 100 (1963) 119-130.
 15. Lakowicz, J.R. Fluorescence polarization. In: **Principles of Fluorescence Spectroscopy**. Plenum Press. New York and London, (1983) pp. 112-151.
 16. Campbell, L.D. and Dwek, R.A. Fluorescence. In: **Fluorescence in Biological Spectroscopy**. The Benjamin Cunnings Publishing Company Inc. Menlo Park, London (1984) pp. 91-120.
 17. Lentz, B.R. Membrane „Fluidity” from fluorescence anisotropy measurements. In: **Spectroscopic Membrane Probes** (Loew, L.M. Ed.), CRC Press Inc. Boca Raton, Florida (1988) vol. 1, pp. 13-41.
 18. Oruç, E.Ö. and Üner, N. Combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes and lipid peroxidation in liver of *Oreochromis niloticus*. **Comp. Biochem. Physiol.** 127 (2000) 291-296.
 19. Kleszczyńska, H. and Sarapuk, J. The role of counterions in the protective action of some antioxidants in the process of red cell oxidation. **Biochem. Mol. Biol. Int.** 2 (1998) 385-390.
 20. Kleszczyńska, H., Oświęcimska, M., Sarapuk, J., Witek, S. and Przystański, S. Protective effect of quaternary piperidinium salts on lipid oxidation in the erythrocyte membrane. **Z. Naturforsch.** 54c (1999) 424-428.