Physiological Activity of Some Organophosphorous Compounds and Their Influence on Mechanical Properties of Erythrocytes

Halina Kleszczyńska[a,*], Dorota Bonarska[a], Janusz Sarapuk[a] and Krzysztof Bielecki[b]

[a] Department of Physics and Biophysics, Agricultural University, Norwida 25, 50–375 Wrocław, Poland. Fax: (+48)-071–32–05–172. E-mail: halina@ozi.ar.wroc.pl
[b] Department of Botany and Plant Physiology, Agricultural University, Cybulskiego 32, 50–205 Wrocław, Poland

*Author for correspondence and reprint requests

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Hemolysis and fluidization of erythrocytes (RBC) membranes by some newly synthesized aminophosphonates as well as their potency to induce electrolyte efflux from cucumber (*Cucumis sativus* cv "Wisconsin") cotyledons were studied. Also, the chlorophyll content in aminophosphonate-treated cotyledons was affected. The compounds studied differed mainly in hydrophobicity of their substituents at the carbon, nitrogen and phosphorus atoms. It was found that aminophosphonate potency to fluidize RBC membranes depended on the combination of its overall lipophilicity and/or the kind of substituent at the P atom. Especially, iso-propyl groups enhanced that potency. The sequence of aminophosphonates that exhibited the strongest fluidization activity was paralelled by their physiological and hemolytic activities; in the latter case for these compounds that hemolyzed RBC under used concentrations.

A general conclusion is that both the stereochemistry and lipophilicity determine the efficiency of the aminophosphonates studied. This efficiency is most probably related to the interaction of aminophosphonates with the lipid phase of biological objects.

Introduction

Aminophosphonates are nowadays a subject of intensive studies with start of their agrochemical application. Perhaps the best known compounds are N-phosphonomethylglycine and dibutyl 1-butylamino-1-cyclohexanephosphonate. It was suggested that biological activity of some aminophosphonates is the result of their interaction with membranes of biological objects and that optimal lipophilicity is necessary to make this activity high (Gancarz and Dudek, 1996).

In this work we studied some newly synthesized aminophosphonates to determine whether they may find an application as pesticides and to check whether the mechanism of their activity is related to lipophilicity only. We have already shown that a convenient model to study the interaction of exogenous agents with the lipid phase of biological membranes are erythrocytes (Kleszczyńska *et al.,* 2000a; 2000b; 2000c; Sarapuk *et al.,* 2000; 2001). The parameter studied was hemolysis, which is commonly regarded as the result of interaction of the compound studied with the lipid phase of the erythrocyte (RBC) membrane. Since not every compound hemolyzes RBC at reasonable concentration we have also measured RBC ghosts membrane fluidization by means of a fluorescent probe.

The results of model experiments were compared with physiological toxicity of the compounds studied. These were performed with the use of cucumber (*Cucumis sativus* cv "Wisconsin") cotyledons. Efflux of electrolyte from cotyledon discs was measured and chlorophyll content. The obvious assumption was that the more damaged is the membrane the stronger chlorophyll decreases in the tissue.

The aminophosphonates studied differed in their substituents at the carbon, nitrogen and phosphorus atoms.

Methods

Aminophosphonates studied were synthesized in Department of Organic Chemistry, Biochemistry and Biotechnology, Technical University, Wrocław. Synthesis protocol as well as spectral data of

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aminophosphonates were given earlier (Wieczorek *et al.*, 2000; 2001). The general structure of the aminophosphonates and particular substituents are shown in Table I.

Fresh heparinized pig blood was used in the hemolytic experiments. Erythrocytes (RBC) were washed four times in the phosphate buffer of pH 7.4 and incubated in it, after adding aminophosphonates, at 37 °C for 4 h. The hematocrit was 4%. Percent of hemolysis was measured with 1 ml samples taken after 0.5 h of incubation. They were centrifuged and the hemoglobin content in the supernatant was measured at 540 nm. The concentrations of aminophosphonates were determined causing 50% hemolysis (C_{50}). All compounds were dissolved in ethanol; the concentration of ethanol in the samples did not exceed 1%.

Fluidity experiments were done on erythrocyte ghosts, prepared according to Dodge *et al.* (1963), and subjected to the action of the compounds studied at a concentration of 17 μM. Fluorescent probe TMA-DPH {[1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene] *p*-toluenesulfonate} was purchased from Molecular Probes Inc. (Eugene, Oregon, USA) and used at 1 μM concentrations. The measurements were performed with a SFM 25 spectrofluorometer (KONTRON, Zurich, Switzerland). The polarization coefficient P was calculated according to the formula (Lakowicz, 1983; Campbell and Dwek, 1984; Lentz, 1988):

$$P = (I_{II} - GI_{\perp})/(I_{II} + GI_{\perp}) \quad (1)$$

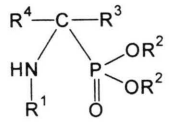
where I_{II} – intensity of fluorescence emitted in direction parallel to the polarization plane of the exciting light, $I_{\perp}$- intensity of fluorescence emitted in the direction perpendicular and G – is a factor used to correct for inability of the instrument to transmit differently polarized light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

All reagents used were of analytical grade

Cucumber (*Cucumis sativus* cv 'Wisconsin') was grown under white light at a constant fluence of 150 μmol m^{-2} s^{-1}. Cotyledons from 7-d-old seedlings were used for experiments. Discs of 7 mm diameter were cut avoiding the midrib with a brass cork borer. The discs were rinsed in water and floated 24 h under constant light (fluorescent lamp Pila LF 36 W "Daylight", PAR 110 μE m^{-2} s^{-1}) on 0.25 mM and 0.5 mM aminophosphonate solutions. Conductivity of the treatment solution (100 mM K-phosphate buffer; pH 7.0) was assayed with the OK-102/1 conductometer (Radelkis, Budapest, Hungary).

Chlorophylls were extracted with 80% acetone (Lichtenthaler, 1987).

Table I. Structure and substituent groups of the compounds studied.



Compounds	R^1	R^2	R^3	R^4
1	n-C_4H_9	n-C_4H_9	C_2H_5	CH_3
2	i-C_3H_7	i-C_3H_7	C_2H_5	CH_3
3	n-C_4H_9	CH_3	C_2H_5	CH_3
4	n-C_4H_9	n-C_4H_9	n-C_4H_9	CH_3
5	n-C_4H_9	n-C_4H_9	C_2H_5	C_2H_5
6	i-C_3H_7	n-C_4H_9	n-C_4H_9	CH_3
7	n-C_4H_9	C_2H_5	n-C_4H_9	n-C_4H_9
8	n-C_4H_9	n-C_4H_9	-C_5H_{10}-	
9	n-C_4H_9	n-C_4H_9	n-C_5H_{11}	CH_3

Results and Discussion

According to the results of hemolytic experiments (see Table II) aminophosphonates can be roughly divided into two groups. Aminophosphonates of first group did not caused RBC hemolysis up to concentrations so high as 1 mM. These were compounds **1–3, 5** and **7**. The common feature of these compounds was small overall lipophilicity assumed as the sum of the number of methyl and methylene groups at the carbon, phosphorus and nitrogen atoms. Each of them has 9 to 16 such groups. The exception is compound **6** (15 lipophilic groups) which was found to hemolyze RBC. This compound has an isopropyl substituent at the P atom and it was shown earlier that this substituent

Table II. Values of 50% hemolysis of erythrocytes (C_{50}) and the coefficients of polarization found for 17 μM concentration of aminophosphonates and temperatures 25 °C (P_{25}) and 37 °C (P_{37}).

Comp.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
C_{50} [mM]	>1	>1	>1	0.25	>1	0.73	>1	0.62	0.32
P_{25}	0.326	0.296	0.348	0.357	0.349	0.289	0.347	0.302	0.290
P_{37}	0.274	0.270	0.322	0.304	0.305	0.273	0.312	0.271	0.261

may improve activity of a compound (Kleszczyńska and Sarapuk, 2001) which seems to be the case here. Other hemolysing compounds were nos. **4** and **9** (17 and 18 lipophilic groups, respectively) and no. 8 which is cyclic one. Apparently, the cyclopentane ring is responsible for activity in the latter case.

Lack of hemolytic effects does not mean that there is no interaction between aminophosphonates and RBC. Fluidity experiments performed on erythrocyte ghosts permitted a study of the intensity of this interaction. We have measured the polarization coefficients P (see Table II) of a fluorescent probe incorporated into ghosts membranes and treated with predetermined concentrations of aminophosponates at two different temperatures (25 °C and 37 °C). Increasing concentration of a compound caused an increase of disorder in ghost membranes. No qualitative difference was found between the data obtained for temperatures studied. It is noteworthy that hemolyzing compounds had higher disordering properties than the rest of the aminophosphonates studied. An exception was compound **4** which, from unclear reasons, had a relatively small influence on polarization coefficient.

Similar qualitative results were found by conductance studies. The most intensive electrolyte efflux from cucumber cotyledons was induced by compounds **4, 6, 8** and **9** (Table III). As expected, on the basis of the conductance experiments, chlorophyll content in cotyledons depended on the efficiency of a compound to damage membrane (Table III).

Summarizing, the experiments performed indicate the lipid phase of membranes as the site where aminophosphonates studied exert their action. This action was shown to depend mainly on overall lipophilicity of particular aminophosphonates and/or their stereochemistry. Similar findings were obtained with other compounds studied, including aminophosphonates (Gancarz and Dudek, 1996; Grzy[*et al.*, 2001; Kleszczyńska and Sarapuk, 2001).

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Table III. Values of conductance of effusate and chlorophyll content in cucumber (*Cucumis sativus* cv "Wisconsin") cotyledons under 0.25 mM and 0.5 mM concentrations of aminophosphonates.

Parameters		Compounds								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Conductance [$\mu S\ cm^{-1}$]	0.25 mM	131	53	83	202	34	82	55	328	206
	0.5 mM	196	66	149	256	36	333	93	344	259
Chlorophyll Content [%]	0.25 mM	75	97	87	54	76	70	86	19	24
	0.5 mM	55	93	72	29	41	19	73	4	4

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