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Correlation between Plant Growth Regulator Release Rate and Bioactivity for the Series of Newly Synthesized Phytoactive Polymers

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Abstract

Phytoactive polymers are high molecular weight systems in which a plant growth regulator (PGR) unit is attached to the polymeric chain by a hydrolyzable chemical bond. The release rate of the PGR is linked to the biological activity of the phytoactive polymer and can be controlled by properties inherent in the whole macromolecular system. In this study the correlation of biological activity and plant growth regulator hydrolytic release rate was investigated for the series of newly synthesized 2,4-dichlorophenoxyacetic acid (2,4-D) polymeric esters. The polymers synthesized differ in their molecular weight, side group structure, and 2,4-D residue content. The influence of these polymer characteristics on the 2,4-D hydrolytic release was investigated, and it was demonstrated that hydrolysis rate substantially depends on the polymer molecular weight, side group structure, and 2,4-D residue content. It was also demonstrated that phytoactive polymer bioactivity depends on the hydrolysis rate of the polymers, and in dependence of this parameter can provide stimulating or inhibiting activity. Biological activity was illustrated by the elongation of wheat and barley coleoptiles.

Key words: Phytoactive polymer systems; Structure–bioactivity correlation; Controlled release; 2,4–Dichlorophenoxyacetic acid

Introduction

Creation of highly effective biologically active polymeric systems is mainly determined by the understanding of the interconnection between their structure and biological activity. This also refers to systems with controlled release of a biologically active substance, which display certain advantages over conventional plant growth regulator (PGR) formulations because of their prolonged action, improved efficiency, and greater safety with reference to non-target organisms (Tsatsakis and Shtilman 1994). This research focuses on the

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investigation of the interconnection between biological activity and hydrolysis ability for a number of polymeric esters of the PGR 2,4-dichlorophenoxyacetic acid (2,4-D).

The phytoactive polymers (polymeric derivatives of PGRs) were selected as the model system to study the bioactivity–structure relationship because their biological activity is connected directly to the destruction (mainly by hydrolysis) of the bond between the low molecular weight PGR and the polymer-carrier, and it is not significantly affected by any other aspects of interaction between the macromolecular system and the living organism (Shtilman 1993; Tsatsakis and Shtilman 1994; Shtilman 1995; Tsatsakis and Shtilman 1993). The opposite is the case for polymeric drugs used for parenteral injection, where the polymer affinity to cell membranes or the polymer redistribution in the organism's tissues via endocytosis and exocytosis is extremely important in their bioactions.

In the case of phytoactive polymers, bioactivity is evident after the release of the regulator from the polymer system and its penetration into plant tissues. Correspondingly, the level of biological activity of such macromolecular systems is correlated with the rate of hydrolytic release of the low molecular weight regulator, which in turn is determined by the chemical structure of the phytoactive polymer (Tsatsakis and Shtilman 1994; Shtilman 1995; Tsatsakis and Shtilman 1993; Allan and others 1979, 1992; McCormick and others 1983).

The *in vivo* determination of the hydrolysis rate of the phytoactive polymers after the application on plant substrates (leaves, seeds, and so on) is very difficult, mainly because of extremely low concentrations and doses of polymers applied. Therefore, the correlation of biological activity with the rate of polymer hydrolysis was studied using model conditions (Tsatsakis and Shtilman 1994; Shtilman 1995; Tsatsakis and Shtilman 1993; Allan and others 1979, 1992; McCormick and others 1983; Tsatsakis and others 1995).

The elucidation and understanding of the parameters determining the bioactivity prediction contributes substantially to the development of new highly effective plant growth regulators. These new formulations could possess unique combinations of useful properties like water solubility, a stimulating effect in a wide range of concentrations and doses; high pattern of activity; and the absence of phytotoxicity and toxicity in warm-blooded animals and humans (low risk of side effects).

Some aspects regarding the influence of the chemical structure of polymeric plant growth regulators in connection to hydrolysis (Tsatsakis and others 1995; McCormick and others 1988; Harris and others 1977; Tsatsakis and others 1992; Shtilman and others 1992; Shtilman and others 1996; Shtilman and others 1997) and conformation in water solution (Rizos and others 1998a, 1998b, 1998c) have been examined as well as the activity of those on different plant subjects (Tsatsakis and Shtilman 1994; Shtilman 1995; Tsatsakis and Shtilman 1993). However, a systematic investigation of the correlation between chemical structure and bioactivity has not yet been performed.

Materials and Methods

Chemicals and Reagents

Polymer esters of 2,4-D were synthesized by two methods designated method A and method B.

Method A. Polyvinyl alcohol (PVA) of grades 7/1, 16/1, and 40/2 (''Plastpolimer,'' Yerevan) prepared by alkaline hydrolysis and purified by methanol extraction was used. The initial content in acetate groups in these species was 0.78, 0.93, and 1.21, respectively. The molecular mass, measured viscosimetrically and calculated according the formula $[\eta] = 140 \times 10^{-3} M_w^{0.6}$, was found to be 12.0×10^3, 29.0×10^3, and 65.0×10^3, respectively (Rizos and others 1998b).

Polyvinyl esters were synthesized by acylation of PVA with acyl chlorides of 2,4-D in dimethylacetamide according to a procedure described elsewhere (Shtilman and others 1998; Shtilman and others 2002). By variation of 2,4-D acyl chloride concentration in the reaction mixture, the number of 2,4-D moieties coupled to the polymer was regulated. With the increase of the conjugated acidic residues content, infrared (IR) spectra of polyvinyl esters showed an increase in intensity of the bands at 1740 cm^{-1} in response to the ester carbonyl for of 2,4-D (Specord M-80, Germany). The IR spectra were acquired by the use of NaCl pressed samples. The amount of the 2,4-D residues content in the polymers was determined after ultraviolet (UV) absorption at $\lambda = 284$ nm of 2,4-D in aqueous medium and in dimethylsulfoxide (DMSO) for water-insoluble polymers (Specord UV-VIS spectrophotometer, Germany).

Method B. The copolymers of 2,4-D allylic ester and acrylic acid were synthesized in heptane, with AIBN as an initiator. The copolymer was isolated by filtration, followed by heptane wash and drying in a

vacuum to a constant weight. The copolymers with acrylamide and N-vinylpyrolidone were prepared in a similar way. Isopropyl alcohol was used as a solvent. The acrylamide copolymer was precipitated during the synthesis, whereas the N-vinylpyrolidone copolymer was precipitated in diethyl ether. To prepare copolymers with the desired molecular masses and well-known amount of 2,4-D residues, copolymerization was performed for various ratios of monomers. The resulting copolymers were subjected to fractional precipitation and the fractions precipitated were isolated by centrifugation. For the acrylic acid copolymers isopropyl alcohol was used as a solvent and heptane was used as the precipitating agent; for the acrylamide copolymer DMSO and isopropyl alcohol, and for the N-vinylpyrrolidone copolymers dioxane and diethyl ether were used, respectively.

The amnount of the 2,4-D residues in copolymers was calculated by UV-spectroscopy at 284 nm (Specord UV-VIS instrument). The molecular weight of the copolymers at low concentrations of the substitutes was measured by viscosimetry and calculated according to the equation $[\eta] = 6.31 \times 10^{-3} M_w^{0.8}$ for the acrylamide copolymer (in water) (Rizos and others 1998b); $[\eta] = 1.3 \times 10^{-2} M_w^{0.68}$, for the N-vinylpyrrolidone copolymer (in water) (Rizos and others 1998c); and $[\eta] = 76 \times 10^{-5} M_w^{0.5}$, for the acrylic acid copolymer (in dioxane) (Korshak and others 1980).

Hydrolysis Studies

A 0.01 g polymer powder sample with particle size of 0.1 mm or less was placed in a small jacket flask connected to a thermostat, and 0.5 ml of distilled water was added. The suspension was left at 50°C for 3 h with a magnetic stirrer. The polymer species with the low content in active ingredient (not more than 2.0–2.5 mol%) were dissolved, whereas the polymer species with a higher content in acid residues remained in the solid phase. The temperature in the jacket was brought to the desired values and 4.5 ml of a buffer solution at pH = 10.0 (Merck pH-standard solution) were added.

The amount of acid released as a result of the hydrolysis was detected by high performance liquid chromatography (HPLC; LKB instrument equipped with a Bondapak C18 column from Waters). A mixture of ethanol, water, and acetic acid (145:130:15 volume ratios) was used as the eluent. The released acid was detected by an ultraviolet (UV) detector set at 284 nm at corresponding retention time. The hydrolysis capacity was estimated by measuring the effective rate constant corresponding to the initial moment of reaction (k_{ef}^{o}), calculated according to the second-order. The experimental accuracy was assessed in several series of experiments. The degree of hydrolysis versus time is a mean of five readings from parallel experiments and its variations ranged from 2.5% to 4.0% .

Biological Experiments

Seeds of wheat (*Triticum aestivum* L.), cv. *Virgina*, and barley (*Hordeum vulgare* L.), cv. *Hexatichum* were used in the experiments. Seeds were made aseptic with 70% ethanol, stirred for 15 min in 1.5 M sodium hypochlorite and washed several times with warm water. About 400 seeds were distributed between two sheets of wet filter paper on a plastic tray and germinated for 72 h (60 h under red light and 12 h in darkness) at 25°C in a plant growth cabinet.

Segments (10 mm long) were cut from 17–18-mm-long coleoptiles. The segments (15–20) were placed in sterile Petri dishes with 10 ml of solutions or suspensions of polymers containing 2,4-D in various concentrations and were incubated for 24 h at 25°C; distilled water was used as a control. The length of the coleoptiles was measured with accuracy of 0.5 mm. Because 2,4-D possessed the highest activity in the concentration range 10^{-6}–10^{-3}, the same concentration of low-molecular weight regulator was incorporated in the experiments.

Results and Discussion

A series of polyvinyl esters of the well-known plant growth regulator (PGR) 2,4-D (PRG of auxinic group), with considerable differences in side chain structure, molecular weight, and 2,4-D content, were synthesized by two different methods (Figure 1; Tables 1 and 2) and their bioactivity with respect to the rate of 2,4-D release by hydrolysis was investigated.

The standard auxin bioassay, that is, stimulation of the elongation of wheat coleoptile (*Triticum aestivum* L., cv. *Virgina*) and of barley (*Hordenum vulgare* L., cv. *Hexatichum*), was used to study the bioactivity of the polymeric esters gradually releasing 2,4-D by hydrolysis of the ester group (Shtilman and others 1997, 1998). It was found that the specificity toward bioactivity expression of the dose–response profiles depended markedly on the structure of the polymers (Table 1; Figures 2 and 3). Polymeric esters with initial constant rates of regulator release under alkaline conditions of 0.021–0.035 $Lmol^{-1}$ s^{-1} and content of 2,4-D 30.1–33.5

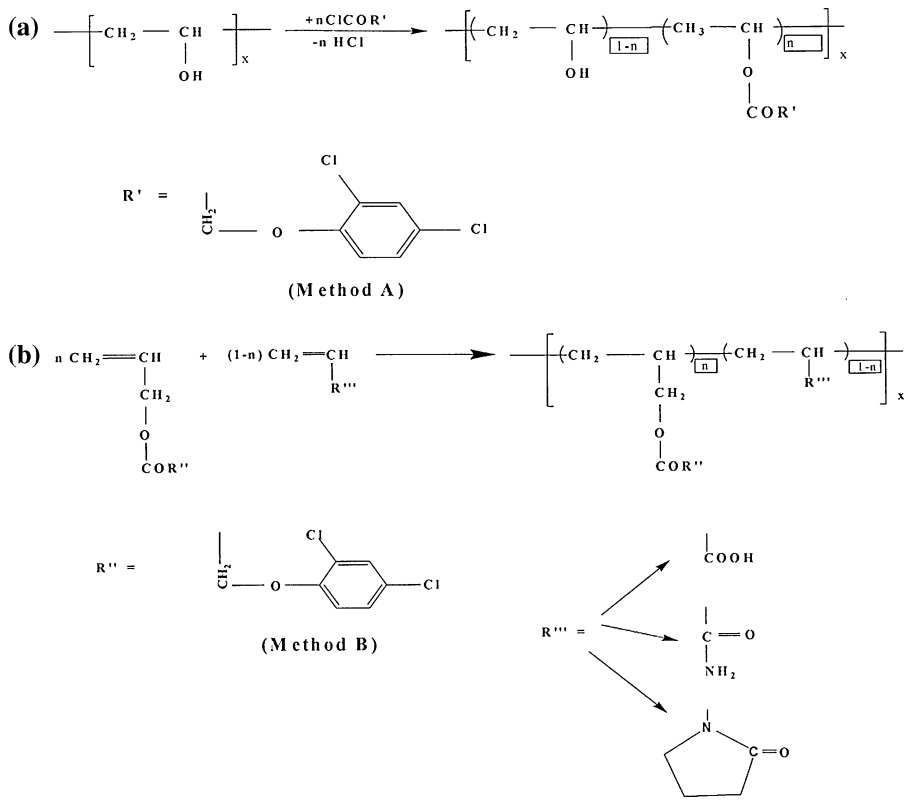

Figure 1. Chemical structures of polyvinyl alcohol esters of 2,4-D (a) and of the copolymers of allylic esters of 2,4-D with acrylic acid (PE1), acrylamide (PE2), and N-vinylpyrrolidone (PE3).

Table 1. Properties of the Initial Polyvinyl Alcohol and of the Synthesized Polyvinyl Esters of 2,4-Dichlorophenoxyacetic Acid (2,4-D) and Initial Hydrolysis Rate Constants of the Esters at pH 10, 90°C

Polyvinyl alcohol		Polyvinyl esters of 2,4-D			
		Content of 2,4-D units			
(M_w)[a] Daltons	([η])[b]	weight %	mol%	(k_{ef}^0)[c] $Lmol^{-1}$ s^{-1}	Designation
12×10^3	0.40	65.2	32.7	0.03	*1*I
		44.0	14.8	1.43	*1*II
		13.4	3.0	20.80	*1*III
29×10^3	0.65	66.0	33.5	0.03	*2*I
		41.3	13.2	0.55	*2*II
		12.4	2.8	11.90	*2*III
65×10^3	1.01	62.9	30.1	0.02	3I
		37.0	11.2	0.38	*3*II
		10.7	2.4	7.37	*3*III

[a] *Average molecular weight.*
[b] *Intrinsic viscosity of water solution at 25*°C, dLg^{-1}.
[c] *Initial hydrolysis rate constant.*

mol% (that is, 1I, 2I, 3I) did not show any considerable activity over the range of concentrations used (Figures 2 and 3, plot I, curves 1–3). Apparently the concentration of the regulator released was inadequate for the stimulation of the coleoptiles even for high polymer doses.

The dose–response profiles of the highly lyophilic polymeric esters shown in Table 1 (1III, 2III, 3III) with low content of 2,4-D, 2.4–3.0 mol% (hydrolysis rate constants in the range of 7.4–20.8 $Lmol^{-1}$ s^{-1}) were similar to that of the low molecular weight 2,4-D (Figures 2 and 3, plot III, curves 1–3 and plots I

Table 2. The Initial Hydrolysis Rate Constants (k_{ef}^{0}) for the Copolymers of Allylic Esters (AE) of 2,4-D with Acrylic Acid, Acrylamide, and N-vinylpyrrolidone (pH 10.0, 40°C)

Copolymers	Content of 2,4-D, mol%	Average molecular weight, M_w	k_{ef}^{0} $Lmol^{-1-1}$
AE 2,4-D & arylic acid (PE1)	0.68	25×10^3	0.29*
AE 2,4-D & arylamide(PE2)	0.51	26×10^3	8.42
AE 2,4-D & N-vinylpyrrolidone(PE3)	0.75*	27×10^3	5.95

* *Determined at 70°C.*

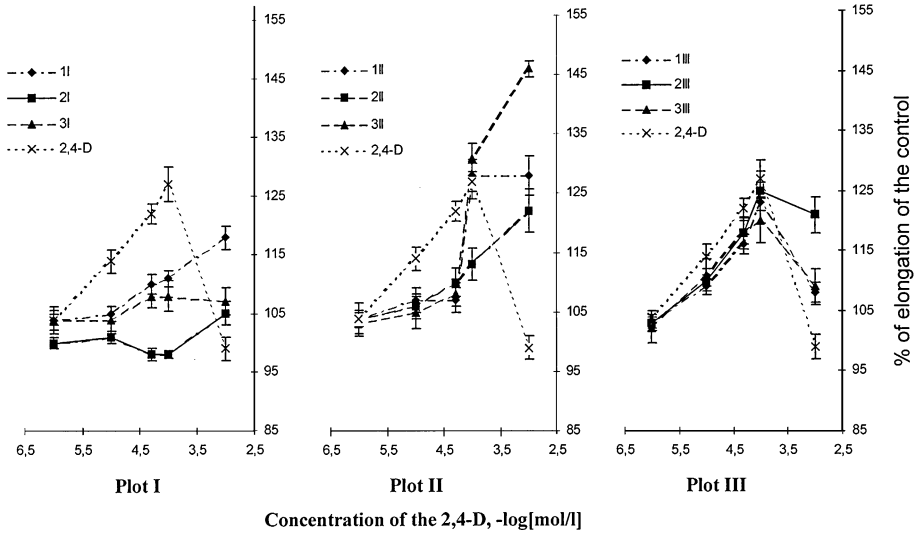

Figure 2. Mean values (± standard errors) of bioactivity expression versus concentration of the 2,4-D in standard wheat coleoptile elongation test for the nine polyvinyl esters of 2,4-D (Table 1) of different molecular weight and content of 2,4-D units (1I, 2I, 3I, 1II, 2II, 3II, 1III, 2III, 3III) and for the simple 2,4-D (plots I, II, and III).

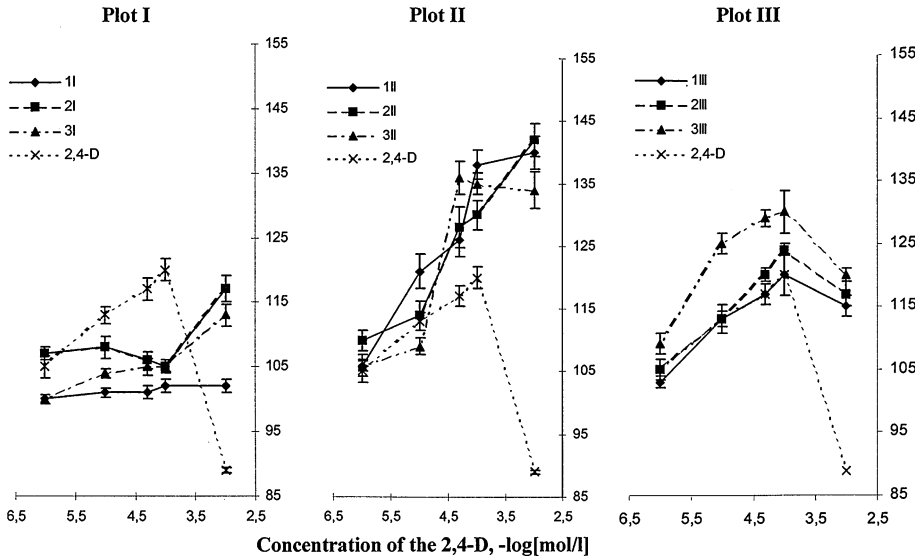

Figure 3. Mean values (± standard errors) of bioactivity expression versus concentration of the 2,4-D in standard barley coleoptile elongation test for the nine polyvinyl esters of 2,4-D (Table 1) of different molecular weight and content of 2,4-D units (1I, 2I, 3I, 1II, 2II, 3II, 1III, 2III, 3III) and for the simple 2,4-D (plots I, II, and III).

and II, curve 4). For these polymers, the level of stimulation depended on the concentration of the regulator.

Figures 2 and 3 illustrate that at high concentration of the regulator released from the polymer (that is, the regulator concentration is close to that of the introduced 2,4-D) the decrease in the rate of the regulator release does not influence the polymer's bioactivity.

If the rate of regulator release is low, high concentrations of the polymer produced shows insignificant inhibition of coleoptile elongation, in

comparison to the similar dose of the low-molecular-weight 2,4-D (Figures 2 and 3, plots I and II and curve 4).

The polymers (Table 1, 1II, 2II, 3II) containing 11.2–14.8 mol% regulator units and possessing values of initial hydrolysis rate constants (k_{ef}^{0}) from 0.38 to 1.43 $Lmol^{-1}$ s^{-1} exhibit optimum dose–response profiles. These polymers stimulated coleoptile elongation over a wide range of concentrations, and the level of the stimulation was not decreased by high polymer concentrations. In addition, the polymer concentrations for optimum stimulation were much higher than those of free 2,4-D (Figures 2 and 3, plot II).

It was interesting to determine whether a similar correlation between hydrolysis rate and bioactivity could be found for other phytoactive polymeric structures with the same regulator. We studied copolymers of allylic ester of 2,4-D (AE 2,4-D) with acrylamide, N-vinylpyrrolidone and acrylic acid, and polymeric esters PE1, PE2, and PE3 (Table 2).

The copolymers were synthesized by radical polymerization as described above (Shtilman and others 1997). The selection of these polymers enabled us to elucidate the effect of the type of side - lyophilizing group on the hydrolysis and bioactivity of the polymers. To minimize the influence of compaction into a macromolecular coil (for example, this was observed when side aromatic groups were introduced into water-soluble polymers (Rizos and others 1998a), polymeric esters with low content of 2,4-D were used. The polymers were water soluble with close molecular masses, and they contained the regulator in almost equal amounts (0.51–0.75 mol%) (Table 2). The effect of the side-lyophilizing group upon the hydrolysis of the polymers can be assessed by studying the ability for hydrolysis of the polymeric esters.

Table 2 shows that the values of the k_{ef}^{0} for the derivatives with acrylamide, and N-vinylpyrrolidone are similar. This indicates that the structure of the side groups (primary and cyclic amide) and the difference in the size of the macromolecular coil insignificantly affect the hydrolysis rate of 2,4-D.

The values of k_{ef}^{0} for the acrylic acid copolymer (salt form), under the conditions of alkaline hydrolysis at 343 K were found to be remarkably lower than the values of the other copolymeric esters. The effect of low values of k_{ef}^{0} for the acrylic acid copolymers is given in Figure 4. The lower rate of hydrolysis of the acrylic acid copolymers in alkaline solution in comparison with vinylpyrrolidone and acrylamide polymers may conceivably be attributed to the influence of the negatively charged carboxylate group. The latter is present in neighboring units of the copolymer and hampers the diffusion of OH. The considerable difference in the values of k_{ef}^{0} for the copolymer of acrylic acid and the other polymers predicts the variations in dose–response profiles for these polymers.

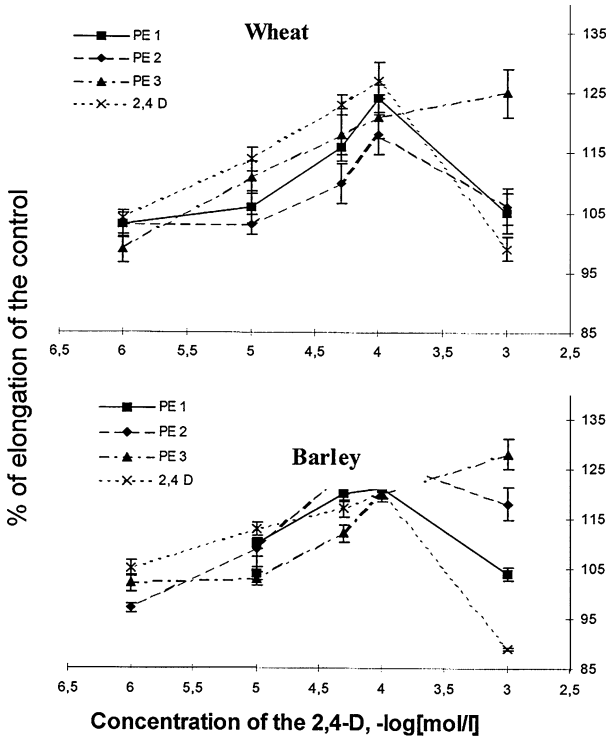

Figure 4. Mean values (± standard errors) of bioactivity expression versus concentration of the 2,4-D in standard barley and wheat coleoptile elongation test for the three allylic esters of 2,4-D with acrylic acid, acrylamide and N-vinylpyrrolidone (polymeric esters PE1, PE2, PE3), and for the simple 2,4-D.

Figure 4 clearly illustrates that for the acrylamide and N-vinylpyrrolidone copolymers whose hydrolysis proceeds at a high rate, the level of biological activity versus concentration is close to the corresponding pattern for 2,4-D. The region of stimulation is characterized by an extreme and shows a tendency to inhibition at concentrations exceeding the optimum values. Interestingly, the hydrolysis rate constants of those copolymers are comparable with the hydrolysis rates for polyvinyl esters of 2,4-D type III, which are characterized by low content of immobilized 2,4-D (Figures 2 and 3, plot III).

However, for the 2,4-D copolymer with acrylic acid whose hydrolysis proceeds at a lower rate, the bioactivity stimulation is observed in the entire range of the studied concentrations (that is, the onset of inhibition is not measured). The k_{ef}^{0} value of this polymer is comparable with the hydrolysis

rate constant of polyvinyl esters of 2,4-D with moderate k_{ef}^{0} values and a similar pattern of bioactivity (Figures 2 and 3, plot II).

The correlation between the hydrolysis rate constant and the level of biological activity for a wide range of concentrations of the above polymeric PGRs has been revealed.

The PGR 2,4-D displays herbicidal properties in high concentrations; however, it is a growth stimulator at low concentrations. This fact allowed us to observe the biological effect of the polymeric PRG in three ranges of activity

When the rate of hydrolysis is high, then the activity of a bioactive polymer is the same as for a pure low molecular weight substance (there is no effect of prolongation). If the rate of hydrolysis is low, then the bioactivity of a bioactive polymer is low, even at high concentrations of the applied polymer. Finally, if the rate of hydrolysis is intermediate, then good stimulation will be observed.

Conclusions

The results obtained in this investigation strongly indicate that the activity of the polymeric plant growth regulators is due to the release of the low-molecular-weight regulator. The regulator itself, when bound to the polymer, is inactive. The various polymeric forms of the same regulator can differ in their degree of activity and in dose response. The biological model revealed that the degree and the range of the polymer activity could be correlated with the parameters of hydrolysis.

This conclusion is crucial because (1) the values of the hydrolysis rate constant k_{ef}^{0} can be measured easily under laboratory conditions, (2) the hydrolysis rate constants of different polymers measured *in vitro* correspond to the real conditions with physiological pH and temperature and participation of enzymes in the hydrolysis reaction.

It is of great importance to know whether the hydrolysis of the polymer occurs inside the biological tissues after diffusion or in the extracellular space. Until now this question has remained unanswered because of the extremely low doses introduced into the system and the very low doses absorbed by the biological object.

The designed study clearly showed that elucidation of parameters for the prognosis of the biological activity for different polymeric derivatives of a plant growth regulator is possible and mainly concerns knowledge of hydrolysis constants measured *in vitro*. Our results confirm that on the basis of examination of hydrolysis and the physical-chemical properties of phytoactive polymers, the bioactivity could be predicted. The study assures the prospect of synthesizing phytoactive polymers with an optimum rate of low molecular weight regulator release and stimulation activity over a wide range of concentrations with the absence of inhibition. It is believed that the present *in vitro* method allows prediction of the biological activity of phytoactive polymer-based systems and can be applied in other controlled-release systems.

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