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The binding of amino acids to the herbicide 2,4-dichlorophenoxy acetic acid

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Summary. The interaction of amino acids with the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was studied by charge-transfer chromatography carried out on diatomaceous layers covered with different amount of 2,4-D and the effect of salts on the strength of interaction was elucidated. It was established that Arg, His, Lys, Orn, Phe and Trp binds to 2,4-D, the binding process is of saturation character. Principal component analysis proved that the concentration of 2,4-D exerts the highest impact on the interaction and the effect of salts is of secondary importance. The results suggest that these amino acid residues may account for the binding of 2,4-D to proteins and can play a considerable role in the detoxification processes by forming conjugates with 2,4-D.

Keywords: Amino acids – 2,4-Dichlorophenoxyacetic acid – Polar interaction – Charge-transfer chromatography

Introduction

The herbicides 2,4-chlorophenoxyacetic acid (further 2,4-D) showing auxin-like activity has been intensively used to control the growth of grass and broad-leaf weeds in many crops such as rice (Leganès and Fernández-Valiente, 1992), winter wheat (Ogg and Young, 1991; Heering and Peeper, 1991), bermudagrass (Johnson and Murphy, 1991), etc. The molecular basis of the mode of action of phenoxyacetic acid herbicides is not entirely understood. It was assumed that they influence proton efflux from the plasma membrane (Barnwell and Cobb, 1993), and they are uncouplers of the oxidative phosphorylation and modify the structure of thylakoid membranes. Besides, its intended effects, 2,4-D has marked side effects too. Thus, it inhibits the germination of sporangiospores of Mucor piriformis and conidia of Botrytis cinerea and Penicillium expansum (Michailides and Spotts, 1991). The fate of 2,4-D in living organisms has been vigorously discussed. It has been many times indicated that 2,4-D readily binds to cytochrome P450 (Kelly

et al., 1992), and cytochrome P450 decomposes 2,4-D (Topal et al., 1993). The herbicide 2,4-D influenced not only the activity of cytochrome P450 but also other enzymes such as hydroxylase, N-demethylase (Mougin et al., 1991), phenylalanine amonialyase (Hoagland, 1990), and nonspecific esterase (Kao et al., 1995). To the best of our knowledge the amino acid residues of enzymes responsible for the binding of 2,4-D have not been studied in detail. The fact that conjugates of 2,4-D and amino acids were found in perennial Glycine species makes probable the direct interaction of 2,4-D with amino acids (White et al., 1990).

Chromatographic methods have been extensively used for the study of various molecular interactions of biochemical and biophysical importance (Cserháti and Valkó, 1994). The application of thin-layer chromatography (TLC) offers considerable advantages: the method is relatively rapid, allows the simultaneous determination of more interactions on one plate, it does not need complicated instrumentation and the amount of the interacting molecules required for the investigation is relatively low. However, TLC has marked drawbacks too: the stoichiometry of the complex cannot be determined and only the relative strength of the interaction can be calculated. TLC can be employed for the study of molecular interactions in two different manners:

a. One of the molecules is spotted onto the plates and the other interacting molecule is added to the mobile phase (Cserháti and Forgács, 1997). The dependence of the retention of the solute on the concentration of the interacting molecule in the mobile phase is related to the strength of interaction.

b. One of the compounds is mixed with the support and the retention of the other molecule is measured on this modified support (Hadzija et al., 1987). The dependence of the retention of the solute on the concentration of the interacting compound in the stationary phase is related to the strength of interaction.

Principal component analysis (PCA) (Mardia et al., 1979) has been frequently used in chromatography for evaluation of large retention data matrices (Karsnas and Lindblom, 1992) and for the elucidation of the relationship between retention and physiochemical parameters of solutes (Kaliszan et al., 1990). As the visual evaluation of the multidimensional matrices of PC loadings and variables is difficult, the dimensionality of the matrices can be reduced to two by the nonlinear mapping technique (Sammon, 1969), varimax rotation and cluster analysis (Willett, 1987).

The objectives of the study were the assessment of the interaction of amino acids with 2,4-D, the determination of the effect of various salts on the strength of interaction using PCA followed by two-dimensional nonlinear mapping, and the elucidation of the molecular substructures of amino acids accounting for the interaction.

Materials and methods

Reversed-phase thin-layer chromatography

Diatomaceous earth was chosen as support because it has been previously proven that it has a very low retention capacity for amino acids (Gullner et al., 1989). DC-Alufolien

Kieselgur F_{254} ready made plates (Merck, Darmstadt, Germany) were impregnated by overnight predevelopment. The impregnating agent was methanol containing 1–7 w/v% 2,4-D in steps of 1 w/v%. After impregnation the plates were dried at room temperature. L-amino acids of analytical purity were purchased from Reanal Fine Chemicals (Budapest, Hungary) and were used without any further purification. Amino acids were dissolved in water : 2-propanol 3 : 1 at a concentration of 2 mg/mL, and 3 μL of the solutions was spotted onto the plates. Due to its low solubility, Tyr was omitted from the experiments. The aqueous eluent systems used for the study of the amino acid – 2,4-D interactions are compiled in Table 1. The end concentration of NaCl, KCl and Mg_2Cl in the mobile phase was 0.16 M in each instance. After development the plates were dried at room temperature and the spots of amino acids were detected with ninhydrin reagent (Stahl, 1962). Each experiment was run in quadruplicate. The retention of amino acids was characterized by the R_M values ($R_M = \log 1/R_f - 1$). When the coefficient of variation between the parallel determinations was higher than 5% the data were omitted of the following calculations. As only Arg, Phe, His, Lys, Orn and Trp had significant interaction with 2,4-D these amino acids were included in the following calculations.

Table 1. Charge-transfer chromatographic systems used for the study of the binding of amino acids to 2,4-dichlorophenoxyacetic acid (2,4-D)

No. of system	Concentration of 2,4-D in the impregnating agent (%)	Presence of		
		NaCl (0.16 M)	KCl (0.16 M)	$MgCl_2$ (0.16 M)
1	1	0	0	0
2	1	1	0	0
3	1	0	1	0
4	1	0	0	1
5	2	0	0	0
6	2	1	0	0
7	2	0	1	0
8	2	0	0	1
9	3	0	0	0
10	3	1	0	0
11	3	0	1	0
12	3	0	0	1
13	4	0	0	0
14	4	1	0	0
15	4	0	1	0
16	4	0	0	1
17	5	0	0	0
18	5	1	0	0
19	5	0	1	0
20	5	0	0	1
21	6	0	0	0
22	6	1	0	0
23	6	0	1	0
24	6	0	0	1
25	7	0	0	0
26	7	1	0	0
27	7	0	1	0
28	7	0	0	1

Data evaluation by traditional regression analysis (hypothesis testing analysis)

In order to test the validity of the hypothesis that these amino acids significantly bind to 2,4-D regression analysis was employed. The R_M values of amino acids were the dependent variables and the linear and quadratic forms of the concentration of 2,4-D in the impregnating agent were the independent variables. The inclusion of the quadratic form of the concentration of 2,4-D was motivated by the observation that at higher 2,4-D concentrations the change in the retention of amino acids observed was not so high than at lower 2,4-D concentrations. Equations were separately applied for the retention data of Arg, His, Lys, Orn, Phe and Trp. The significance level was set to 95%. Calculations were carried out with the software Drugidea (CompuDrug Ltd, Budapest, Hungary).

Data evaluation by multivariate mathematical-statistical methods (hypothesis generating method)

PCA was employed for the elucidation of the similarities and dissimilarities between the effect of salts on the binding strength of amino acids to 2,4-D and between the charge-transfer (variable A), Phe (var. B), His (var. C), Lys (var. D), Orn (var. E), Trp (var. F), the concentration of 2,4-D in the impregnating agent (var. G), the presence of eluent additive characterizing the overall effect of salts (var. H), the presence of NaCl (var. I), KCl (var. J), $MgCl_2$ (var. K), the charge of the cation (var. L), the hydrated ion radii of the cation (var. M) and the quadratic form of the concentration of 2,4-D in the impregnating agent (var. N). The observations were the chromatographic systems compiled in Table 1. The ratio of variance explained was set to 99.9%. In order to compare the efficiency of multivariate methods suitable for the reduction of the dimensionality of large data matrices, the matrix of principal component loadings was submitted to cluster analysis, varimax rotation around two axes and two-dimensional nonlinear mapping. The iteration of the two-dimensional nonlinear mapping was carried out to the point where the difference between the last two iterations was lower than 10^{-8}.

Stepwise regression analysis was employed for the selection of the substructure of amino acids accounting for their interaction with 2,4-D. Linear correlation was calculated between the R_M values of amino acids and the concentration of 2,4-D in the support (due to the saturation character of the relationship only the lower 2,4-D concentrations were included in the calculation). The slope value of the correlation was considered to be related to the relative strength of amino acid – 2,4-D interaction. The independent variables in the stepwise regression analysis were the pI value of amino acids, the pK value of the carboxyl and amino groups (Windhloz, 1983; Weast, 1986), and the z_1, z_2 and z_3 amino groups (Windhloz, 1983; Weast, 1986), and the z_1, z_2 and z_3 parameters characterizing the hydrophobicity, side chain bulk and steric parameters of amino acids (Jonsson et al., 1989). As the linear character of the relationship has not been previously proved the square of the independent variables was also included in the stepwise regression analysis. The relative strength of amino acid – 2,4-D interaction was the dependent variable. The number of accepted variables was not limited the acceptance level for the individual independent variables was set to 95% significance level. Software for PCA, nonlinear mapping and cluster analysis were prepared by Dr. Barna Bordás (Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary). Software for stepwise regression analysis was produced by CompuDrug Ltd (Budapest, Hungary). Multivariate mathematical-statistical softwares such as INSPECT (Springer Verlag, Berlin, Germany), PSWIN (Lancaster University Computer Centre, Lancaster, United Kingdom) are also commercially available.

Results and discussion

The retention of Phe, His, and Arg increased with increasing concentration of 2,4-D in the support (Fig. 1). However, the relationship shows marked

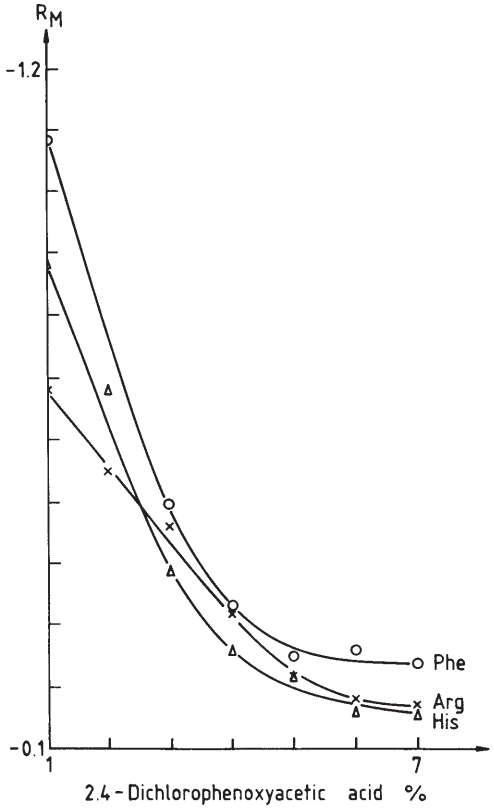

Fig. 1. Effect of the concentration of 2,4-D in the support on the retention of Arg, His and Phe. Mobile phase: 0.16 M KCl

nonlinearity. The increase of retention is quasi linear at lower 2,4-D concentrations (1–4%) and it is of saturation character at higher 2,4-D concentrations (5–7%). This result can be explained by the supposition that 2,4-D gradually covers the inert surface of diatomaceous earth particles. Higher coverage results in higher retention due to the enhanced number of 2,4-D molecules on the surface. When the surface is entirely covered, the probability of amino acid – 2,4-D interaction becomes constant and the retention does not change any more.

The parameters of the equations describing the dependence of the R_M values of Arg, His, Lys, Orn, Phe and Trp on the concentration of 2,4-D in the impregnating agent are compiled in Table 2. The relationship was in each instance significant the significance level being always over 99.9% (see $F_{calc.}$ values). The ratio of the change in the R_M value explained by the change in the concentration of 2,4-D varied between 79.4–45.43% (see r^2 values). The fact the R_M values significantly depend on the concentration of 2,4-D proves the validity of the hypothesis that Arg, His, Lys, Orn, Phe and Trp bind to 2,4-D. The path coefficients (b_i' values of Arg, His, Phe) and the not significant dependence of the R_M values of the quadratic form of the 2,4-D concentration (Lys, Orn, Trp) indicates that the relationship is of mainly linear character.

Table 2. Parameters of the equations describing the dependence of the R_M values of Arg, His, Lys, Orn and Trp on the concentration of 2,4-D in the impregnating agent (C %)

$$R_M = R_{M0} + b_1 \cdot C + b_2 \cdot C^2$$

Parameter	Arg	His	Lys	Orn	Trp	Phe
R_{M0}	−0.91	−0.77	−0.74	−0.88	−0.85	−1.22
$b_1 \cdot 10$	2.90	2.13	6.84	8.62	1.55	2.86
$s_{b1} \cdot 10^2$	5.91	6.08	1.47	1.50	2.46	5.40
$b_2 \cdot 10^2$	−2.58	−1.61	n.s.	n.s.	n.s.	−1.91
$s_{b2} \cdot 10^2$	7.09	7.94	–	–	–	7.42
b_1' (%)	57.47	62.40	–	–	–	67.24
b_2' (%)	42.53	37.60	–	–	–	32.76
$F_{calc.}$	27.96	20.11	21.65	33.11	39.63	55.92
r^2	0.6997	0.6167	0.4543	0.5508	0.5692	0.7941

R_{M0} lipophilicity value extrapolated to zero concentration of 2,4-D in the impregnating agent; b_1 and b_2 coefficients of regression indicating the effect of 2,4-D on the R_M value; s_{b1} and s_{b2} standard deviation of b_1 and b_2, respectively; b_1' *(%)* and b_2' *(%)* path coefficient indicating the relative impact of independent variables on the dependent variable without taking into consideration their original dimensions; $F_{calc.}$ calculated F value indicating the fitness of the equation to the measured values; r^2 coefficient of determination related to the variance explained by the independent variables. *n.s.* not significant.

The results of PCA are compiled in Table 3. Four principal components explain the overwhelming majority of variance. It means that the information content of the 14 original variables can be expressed by four background variables with only 7.58% loss of information. Unfortunately, PCA does not define the four background variables as concrete biochemical or biophysical entities, but only indicates their possibility as mathematical constructions. The loadings of amino acids, the concentration of 2,4-D in the impregnating agent and its logarithm are high in the first principal component. This finding indicates that the interaction mainly depends on the concentration of 2,4-D and the effect of salts and the physicochemical parameters of salts are of secondary importance. The results of methods reducing dimensionality entirely supports the conclusions drawn from the data in Table 2. The retention behaviour of amino acids and the linear and logarithmic forms of the concentration of 2,4-D are in a well defined cluster in both the two-dimensional nonlinear map (Fig. 2) and the map of the varimax rotation around two axes (Fig. 3). Similar conclusions can be drawn from the result of cluster analysis (Fig. 4). However, due to the different mode of calculation slight differences between the results can be observed. Theoretically each method (two dimensional nonlinear mapping, varimax rotation around two axis and cluster analysis) is similar, they reduce the dimensionality of the original data matrix. Our data prove that this theoretical principle is a correct one. This conclusion is based on a case study and not on theoretical principles, therefore its extension to other data matrices can be lead to serious misinterpretation of the results.

Chromatographic systems form distinct clusters according to the character of the salts on the two-dimensional nonlinear map of principal component

Table 3. Similarities and dissimilarities between the effect of salts on the binding strength of amino acids to 2,4-dichlorophenoxyacetic acid. Results of principal component (PC) analysis. Letters refer to variables in Materials and methods

No. of PC	Eigenvalues	Explained variance %	Cumulative variance %
1	6.58	46.97	46.97
2	3.09	22.09	69.06
3	1.88	13.41	82.47
4	1.39	9.95	92.42

	Principal component loadings No. of principal component			
Variable	1	2	3	4
A (Arg)	*0.90*	−0.17	0.03	0.07
B (Phe)	*0.86*	0.44	0.07	−0.09
C (His)	*0.94*	−0.08	−0.02	0.09
D (Lys)	*0.89*	−0.28	0.05	0.05
E (Orn)	*0.88*	0.14	−0.02	0.10
F (Trp)	*0.74*	0.49	−0.11	−0.06
G	*0.79*	0.47	0.08	−0.14
H	0.46	*−0.83*	−0.09	−0.27
I	0.07	0.11	0.27	*0.95*
J	−0.33	0.30	*0.69*	*−0.55*
K	−0.19	0.42	*−0.87*	−0.13
L	−0.40	*0.77*	−0.48	0.08
M	−0.44	*0.70*	*0.54*	0.09
N	*0.83*	*0.51*	0.07	−0.15

G concentration of 2,4-D in the impregnating agent, *H* presence of eluent additive characterizing the overall effect of salts, *I*, *J*, and *K* presence of NaCl, KCl, and $MgCl_2$, respectively, *L* charge of the cation, *M* hydrated ion radii of the cation, *N* logarithm of the concentration of 2,4-D in the impregnating agent.

variables (Fig. 5). This finding indicates that the effect of salts is selective and slightly influences the strength of the amino acid – 2,4-D interaction. The points representing low concentrations of 2,4-D (1–4%) are far from each other while the other points (5–7% of 2,4-D) are very near to each other. This results entirely supports of our previous conclusions that the interaction is of saturation character and the retention of amino acids on 2,4-D impregnated surface is nonlinearly related to the concentration of 2,4-D.

Stepwise regression analysis did not found any significant relationship between the relative strength of amino acid – 2,4-D interaction and the physicochemical parameters of amino acids. We assume that more than one molecular substructure of amino acids accounts for the binding to 2,4-D and the effect observed is the results of the interplay of various hydrophobic and hydrophilic interactive forces.

It can be concluded from the data that Arg, Phe, Lys, Orn, His and Trp readily bind to 2,4-D, the presence and character of salts in the environment

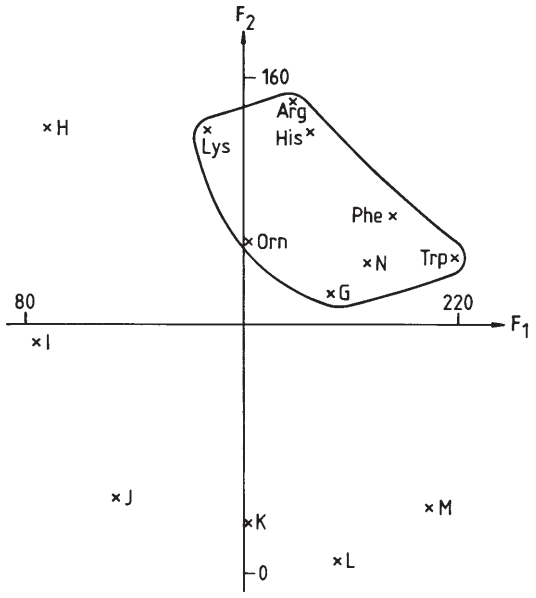

Fig. 2. Similarities and dissimilarities between the binding of amino acids to 2,4-D. Two dimensional nonlinear map of principal component loadings. Number of iterations, 109; maximum error, $4.86.10^{-2}$. For symbols see Materials and methods

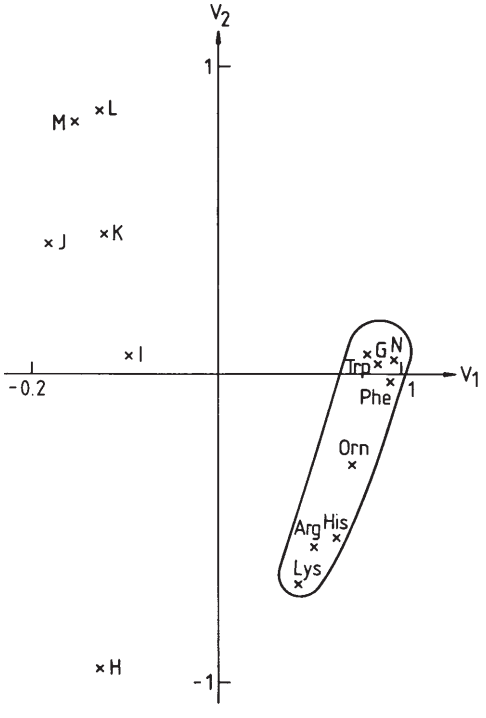

Fig. 3. Similarities and dissimilarities between the binding of amino acids to 2,4-D. Map of the varimax rotation of principal component loadings around two axes. For symbols see Materials and methods

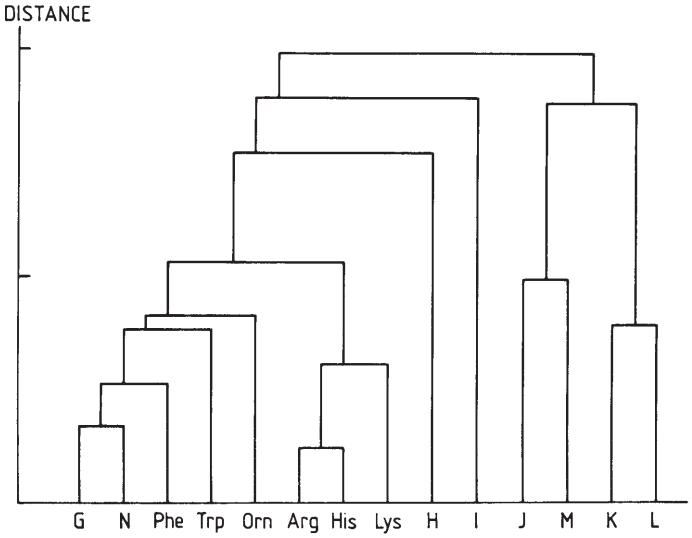

Fig. 4. Similarities and dissimilarities between the binding of amino acids to 2,4-D. Cluster analysis of principal component loadings. For symbols see Materials and methods

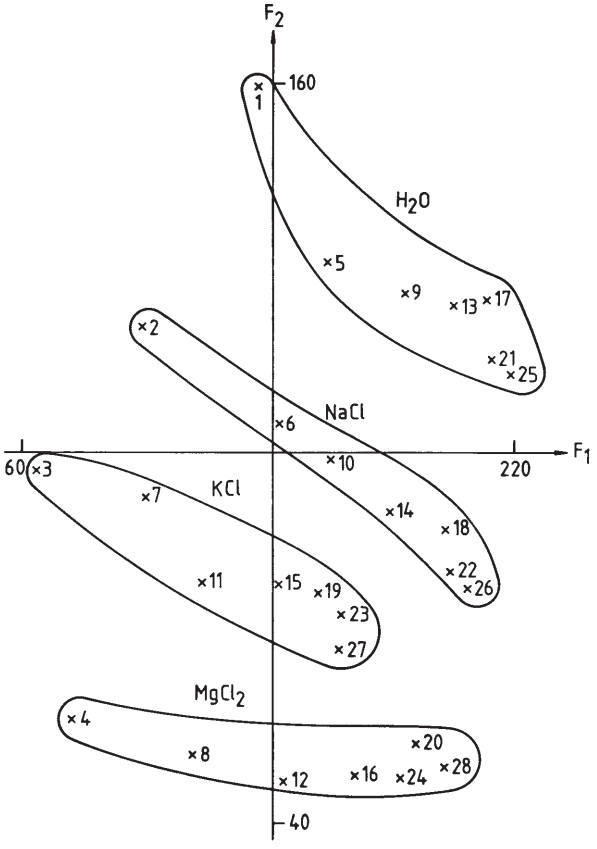

Fig. 5. Similarities and dissimilarities between the chromatographic systems. Two-dimensional nonlinear map of principal component variables. Number of iterations, 109; maximum error, $3.77.10^{-2}$. Numbers refer chromatographic systems in Table 1

exert a secondary effect on the strength of interaction. The results suggest that these amino acids play a considerable role of binding 2,4-D to proteins and the amino acid 2,4-D conjugates can actively influence the de-toxification processes.

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