Journal of Chromatography, 355 (1986) 211–220
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 300

INTERACTION OF AMINO ACIDS AND GLUTATHIONE WITH THE FUNGICIDE 1-PHENYL-2-NITRO-3-ACETOXYPROP-1-ENE STUDIED BY CHARGE-TRANSFER CHROMATOGRAPHY

GÁBOR GULLNER* and TIBOR CSERHÁTI
Plant Protection Institute, Hungarian Academy of Sciences, Herman O. u. 15, 1022 Budapest (Hungary)
and
ATTILA KIS-TAMÁS and GYULA MIKITE
EGIS Pharmaceutical Works, P.O.B. 100, 1475 Budapest (Hungary)
(First received August 6th, 1985; revised manuscript received October 17th, 1985)

SUMMARY

Reversed-phase charge-transfer thin-layer chromatography has been applied to study the interaction of the fungicide 1-phenyl-2-nitro-3-acetoxyprop-1-ene with amino acids and glutathione. The fungicide interacts preferentially with thiol amino acids and glutathione, but weak interactions with other amino acids were also observed. Our results confirm that the fungicide can act by blocking the sulfhydryl groups of essential proteins in fungi. The interaction with thiol amino acids involves molecular complex formation, but that with glutathione is of covalent character. The pH value has no significant effect on the interaction.

INTRODUCTION

Charge-transfer interactions have been often applied in thin-layer chromatography (TLC) in order to obtain better separation[1,2], or to detect certain compounds[3]. TLC can also be used to study charge-transfer interactions of biologically important compounds. Slifkin *et al.*[4] investigated purines, pyrimidines, nucleotides, nucleosides and amino acids with different charge acceptors by TLC either by spotting their mixtures on standard silica gel plates or with the acceptor as part of the stationary phase. The binding constant $B = (R_F - R_F')/R_F \times 100$, where R_F is the value in the absence of acceptor and R_F' the value in the presence of the acceptor, was proposed as the measure of molecular interaction[2]. However, this parameter is asymmetric, taking values from $-\infty$ to 100 (ref. 4). The weak interactions of amino acids, including charge-transfer phenomena were demonstrated, also on Sephadex gels with a variety of cross-linked electron acceptors[5].

The derivatives of 1-phenyl-2-nitropropane-1,3-diol diacetate (Fenitropan®) are effective antifungal agents[6]. On the basis of quantitative structure–activity relationship studies the bioactivity of these compounds is mainly determined by the presence (or possible formation) of the double bond between carbon atoms 1 and 2

0021-9673/86/$03.50

of the alkyl chain, suggesting that they exert their antifungal action via the unsaturated form[7]. The activities of various bioactive α,β-unsaturated nitro compounds have been recognised as due to their reactivity towards sulfhydryl groups of biomolecules[8–12]. The first decomposition product of fenitropan is 1-phenyl-2-nitro-3-acetoxyprop-1-ene, which also showes marked antifungal activity. Recent studies showed that this compounds reacts readily with some thiols[13]. It is supposed that the fungicide blocks the sulfhydryl-containing systems essential to normal metabolism of fungi.

The aim of our present work was to study the interaction of 1-phenyl-2-nitro-3-acetoxyprop-1-ene with amino acids and glutathione applying charge-transfer reversed-phase thin-layer chromatography (RP-TLC). This procedure is based on the fact that the more hydrophilic amino acids and glutathione can lessen the lipophilicity of the more hydrophobic fungicide if they interact with it. The difference between the lipophilicities determined in additive free eluent and in eluents with additives, $R_M - R'_M$ is related to complex stability[14].

EXPERIMENTAL

Amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical purity and used as supplied. DC-Alufolien Kieselgel 60 F_{254} plates (Merck, F.R.G.) were applied. The plates were impregnated overnight with 5% paraffin oil in *n*-hexane. The *n*-hexane was evaporated at room temperature, and 2 μl of the solution of the fungicide in acetone (5 mg/ml) were spotted on the plates, which were developed with water–methanol (1:1) without and with dissolved amino acids or glutathione. Each eluent contained only a single additive. The concentration of the amino acids in the eluents was 0.01 *M*; that of cysteine, homocysteine and glutathione was varied in the concentration range 0.0001–0.1 *M*. In some cases we applied higher amino acid concentration than 0.01 *M*. For studying the effect of the pH value on the interactions between the fungicide and cysteine, homocysteine and glutathione, the concentration of the additives was set to 0.05 *M*, and they were dissolved in 1:1 mixtures of Britton–Robinson buffers and methanol. The spots were detected under a UV lamp at 254 nm.

The migration distances of amino acids were determined in separate experiments at 0.01 *M* amino acid concentration and in the range $3.16 \cdot 10^{-4}$–10^{-2} *M* for cysteine, homocysteine and glutathione. The amino acids and glutathione were detected by the traditional ninhydrin method, and the plates were evaluated by Shimadzu CS-930 dual-wavelength chromato-scanner at 510 nm.

Because of the insufficient migration of Lys, Arg and His in the aforementioned system we changed the experimental design. The interaction of the fungicide with Lys, Arg and His was studied by spotting these amino acids on the plate and by dissolving the fungicide in the eluent. To check the applicability of this chromatographic system for the detection of the amino acid–fungicide interaction, cysteine (which showed marked complex-forming capacity in the previous system) was also spotted on the plates. Lys, Arg, His and Cys were dissolved in water at a concentration of 2 mg/ml, and 3 μl of each solution was spotted on reversed-phase plates. Water–methanol 1:1 and 1:4 mixtures served as eluents without and with dissolved 0.01 *M* 1-phenyl-2-nitro-3-acetoxyprop-1-ene. The front of the dissolved fungicide

was detected by a UV lamp at 254 nm, and the amino acid spots by ninhydrin reagent.

From the R_F values obtained in the additive-free eluent and the R'_F values obtained in eluents containing various additives, three parameters characterizing the strength of interaction were calculated: (a) $R_M - R'_M$, (b) binding constant B, and (c) $(R'_F - R_F)/(1 - R_F) \times 100$. Applying the binding constant B we obtained negative values on an asymmetric scale. This is why we propose the parameter c. In the case of enhanced migration caused by any additive this parameter takes values from 0 to 100. Linear correlations were calculated between these parameters and the logarithm of the concentration of the thiol amino acids and glutathione.

To investigate the interaction of the fungicide with glutathione, various amounts (0.25–100 μg) of the fungicide were spotted on the plates. Water–methanol (1:1) containing 0.0004 M glutathione was used as eluent. The plates were evaluated by Shimadzu CS-930 dual-wavelength chromato-scanner at 310 nm (absorption maximum of the fungicide).

All experiments were performed with five independent parallel determinations.

RESULTS AND DISCUSSION

Table I shows the retention of the amino acids dissolved in the eluent, that of

TABLE I

EFFECT OF THE AMINO ACIDS ON THE LIPOPHILICITY OF 1-PHENYL-2-NITRO-3-ACETOXYPROP-1-ENE AT 0.01 M CONCENTRATION AND THE MIGRATION OF THE DISSOLVED AMINO ACIDS

Amino acids	R'_F *of the fungicide**	$R_M - R'_M$**	R_F *of the amino acid front*
Glycine	0.26 and 0.92	0.03 and 1.51	0.76
L-Alanine	0.24	−0.01	0.76
L-Valine	0.26	0.02	0.82
L-Leucine	0.24	−0.01	0.79
L-Isoleucine	0.26	0.03	0.77
L-Serine	0.24	−0.01	0.77
L-Threonine	0.27	0.05	0.77
L-Cysteine	0.52 and 0.86	0.51 and 1.24	0.80
L-Cystine***	0.25	0	0.73
L-Methionine	0.26	0.01	0.81
L-Aspartic acid	0.27	0.05	0.82
L-Glutamic acid	0.27	0.04	0.84
L-Asparagine	0.26	0.03	0.74
L-Glutamine	0.25	0	0.76
L-Lysine	0.26	0.02	Does not migrate
L-Arginine	0.24	−0.02	Does not migrate
L-Phenylalanine	0.27	0.05	0.77
L-Tyrosine***	0.26	0.01	0.83
L-Histidine HCl	0.26	0.01	0.21
L-Tryptophan	0.24	−0.03	0.80
L-Proline	0.25	0	0.76
L-Hydroxyproline	0.24	−0.02	0.80

* R_F of the fungicide in additive-free eluent: 0.25.

** Average value of the results of five separate calculations.

*** To 10 ml of eluent both with and without additive 0.5 ml of 1 M sodium hydroxide was added.

the fungicide, and the changes in the lipophilicity of the fungicide caused by the amino acids. Cysteine caused the most marked change in the R_M value of the fungicide. A new weak spot also appeared at $R_F = 0.86$. The significant difference between the effect of cysteine and the other amino acids probably follows from the fact that the nucleophilicity of the mercaptide ion greatly exceeds that of the amino group in the amino acids[15,16]. Although the amino acids did not migrate as far as the solvent front, the R_F values of the amino acid fronts are much higher than those of the fungicide spot determined in additive-free eluent (except Lys, Arg and His). For this reason the observed difference in the complex-forming capacities of the amino acids can be considered as real ones. Owing to their low mobilities the effect of Lys, Arg and His was not detectable in this system.

Several minor effects were also observed when other amino acids were applied, but the original position of the fungicide spot did not change, even at higher amino acid concentrations (up to the solubility limits). Glycine caused a new spot to appeare at $R_F = 0.92$, with a streak between the two spots. In the cases of Val, leu, Ser, Thr, Met, Asn, Phe, Tyr, His, Trp and hydroxy-Pro, a tail stretching upwards from the original fungicide spot appeared. After the plates with Phe and Tyr were developed the fungicide spot became visible with a yellowish-brown colour. In the cases of the other amino acids, Ala, Ile, Asp, Glu, Gln, lys, Arg, Pro and cystine, no effect was observed.

We assume that the minor effects observed involve weak interactions. There exist several mechanisms of weak interactions, *e.g.* ion–dipole and dipole–dipole interactions, dispersion forces, hydrogen bonding, charge-transfer interaction and water-mediated adsorption including hydrophobic interactions[5]. The complexity observed may result from the interplay of various effects.

The results of the experiments carried out by spotting Lys, Arg, His and Cys on plates are listed in Table II. In water-methanol (1:1) the R_F values of these amino acids were identical in the absence and in the presence of the dissolved fungicide. However, in this system the Cys spot migrated over the fungicide front, *i.e.* in this case the applicability of the system to detect molecular interactions cannot be proved. In water–methanol (1:4) the mobility of the fungicide front was greater than that of

TABLE II

THE EFFECT OF 1-PHENYL-2-NITRO-3-ACETOXYPROP-1-ENE ON THE LIPOPHILICITY OF LYS, ARG, HIS AND CYS USING VARIOUS ELUENT SYSTEMS

Eluent	R_F *value*				
	Fungicide front	*Lys*	*Arg*	*His*	*Cys*
Water–methanol (1:1)	–	0.06	0.04	0.12	0.51
Water–methanol (1:1) containing 0.01 *M* fungicide	0.28	0.06	0.04	0.12	0.51
Water–methanol (1:4)	–	0.02	0.02	0.07	0.38
Water–methanol (1:4) containing 0.01 *M* fungicide	0.79	0.02	0.02	0.07	0.76

the Cys spot (a complex can thus be formed). The retentions of Lys, Arg and His did not change in the presence of the fungicide, whereas that of Cys was altered significantly. The fact that the interaction of Cys with the fungicide was also detected in this system proved that Lys, Arg and His are really unable to form complexes with the fungicide.

Figs. 1–3 show the concentration dependence of the effect of cysteine, homocysteine and glutathione on the chromatographic behaviour of the fungicide with respect to the three different parameters of complex strength. The densitograms of the plates developed in eluents containing cysteine, homocysteine and glutathione at various concentrations and sprayed by ninhydrin reagent are shown in Figs. 4–6. These figures clearly show that the position of the fronts of the thiol amino acids and glutathione does not depend on the concentration of these compounds. it means that the data of Figs. 1–3 can be used without correction for the concentration dependence of amino acid fronts.

The parameters of linear correlations between the logarithm of concentration of thiol compounds and the three parameters of complex strength are shown in Table III. Good correlations were found except in the case of glutathione. The lowest values of regression coefficients were found for the binding constant B probably owing to its inherent asymmetry mentioned above. These results show that the character of interaction is different in the case of glutathione.

The pH value of the eluent did not significantly influence the interactions between the fungicide and the thiol compounds (Table IV).

Our data show that the thiols decrease markedly the lipophilicity of the fungicide, resulting in strongly enhanced migration even at very low thiol concentrations.

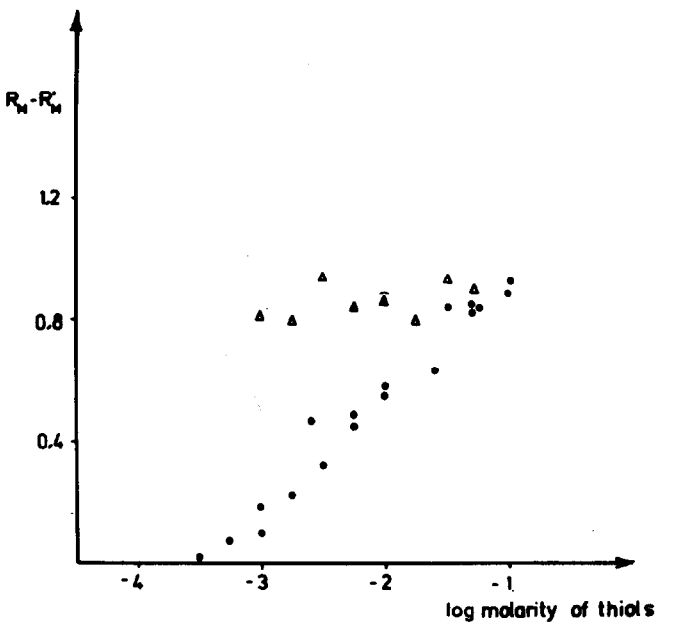

Fig. 1. The effect of some thiols on the lipophilicity of the fungicide characterized by the $R_M - R'_M$ parameter: ●, cysteine; ○, homocysteine; △, glutathione.

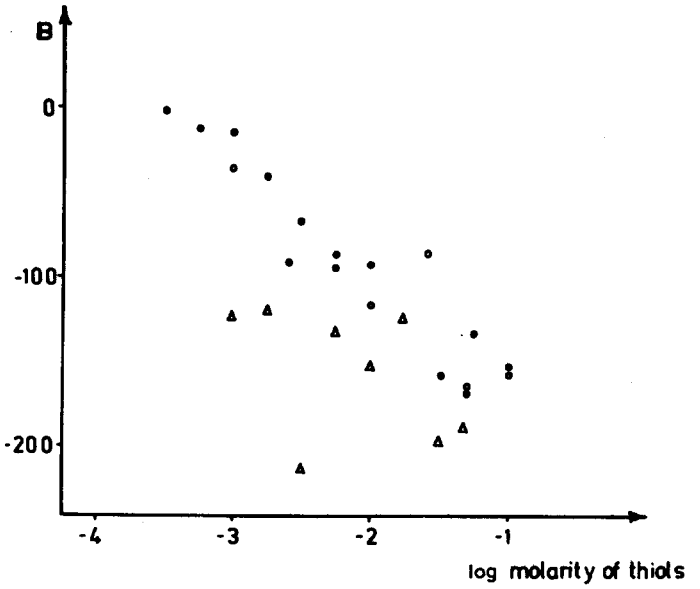

Fig. 2. The effect of some thiols on the lipophilicity of the fungicide characterized by the *B* binding constant: ●, cysteine; ○, homocysteine; △, glutathione.

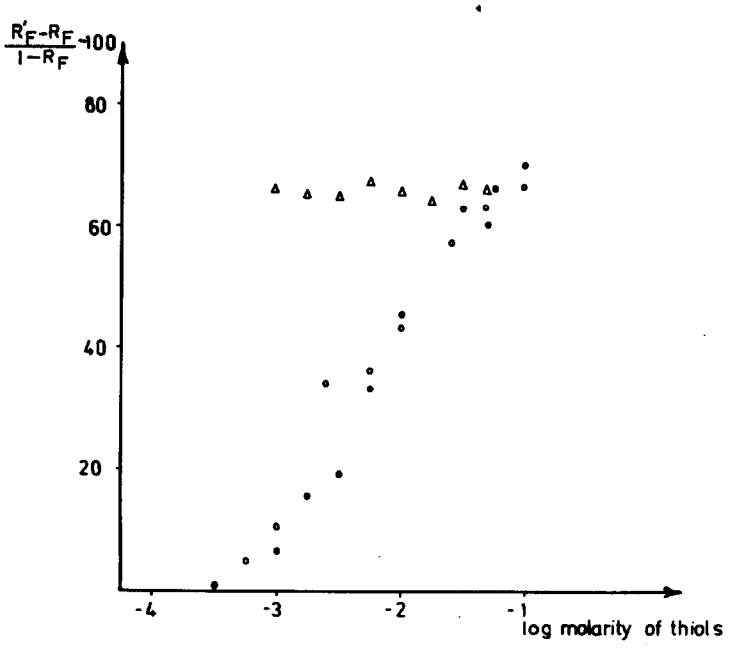

Fig. 3. The effect of some thiols on the lipophilicity of the fungicide characterized by the $(R'_F - R_F)/(1 - R_F) \times 100$ parameter: ●, cysteine; ○, homocysteine; △, glutathione.

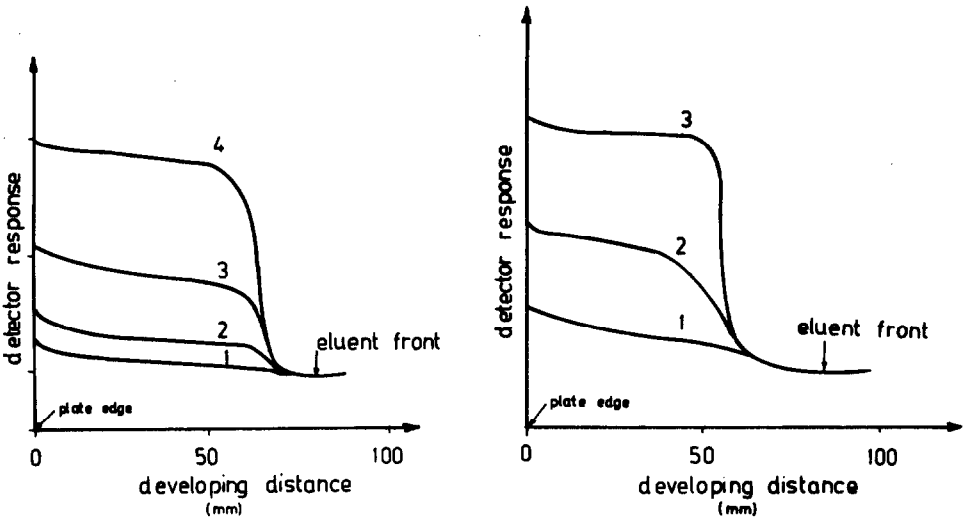

Fig. 4. Densitograms of plates developed in eluents containing various concentrations of cysteine and sprayed by ninhydrin reagent. Cysteine concentrations: 1 = $3.16 \cdot 10^{-4}$ *M*; 2 = 10^{-3} *M*; 3 = $3.16 \cdot 10^{-3}$ *M*; 4 = 10^{-2} *M*.

Fig. 5. Densitograms of plates developed in eluents containing various concentrations of homocysteine and sprayed by ninhydrin reagent. Homocysteine concentrations: 1 = $3.16 \cdot 10^{-4}$ *M*; 2 = $2.5 \cdot 10^{-3}$ *M*; 3 = 10^{-2} *M*.

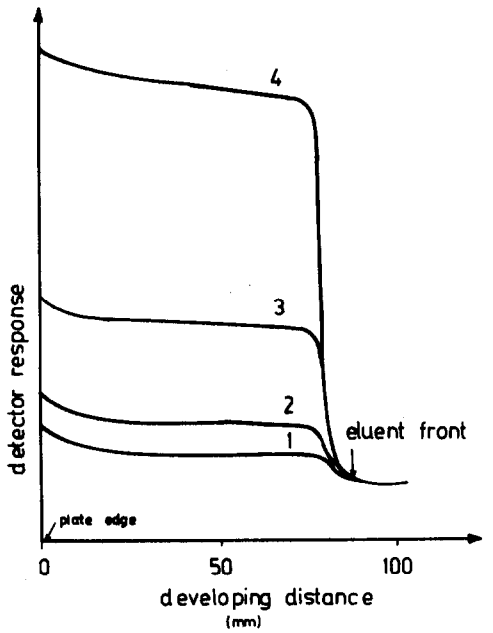

Fig. 6. Densitograms of plates developed in eluents containing various concentrations of glutathione and sprayed by ninhydrin reagent. Glutathione concentrations: 1 = $4 \cdot 10^{-4}$ *M*; 2 = 10^{-3} *M*; 3 = $3.16 \cdot 10^{-3}$ *M*; 4 = 10^{-2} *M*.

TABLE III

PARAMETERS OF THE CORRELATIONS BETWEEN THIOL CONCENTRATION (x) AND THREE PARAMETERS OF COMPLEX STRENGTH (y)

y parameter	*Thiol*	*n*	*a*	*b*	s_b	*r*	*t*
$R_M - R'_M$	Cysteine	10	1.36	0.41	0.02	0.9916	21.31
	Homocysteine	8	1.26	0.35	0.03	0.9777	11.32
	Glutathione	8	0.98	0.05	0.04	0.4787	1.33
B (binding constant)	Cysteine	10	−238.21	−69.89	6.26	0.9694	11.17
	Homocysteine	8	−219.45	−59.17	11.31	0.9057	5.23
	Glutathione	8	−216.95	−28.64	22.82	0.4559	1.26
$\frac{R'_F - R_F}{1 - R_F} \times 100$	Cysteine	10	103.72	31.24	1.81	0.9869	17.31
	Homocysteine	8	99.37	28.17	2.15	0.9829	13.09
	Glutathione	8	66.45	0.16	0.56	0.1177	0.29

The higher the concentration of cysteine and homocysteine the further the spot migrated. This phenomenon points to molecular complex formation. It is possible that the conjugated π electron system of the fungicide acts as an electron acceptor in this interaction. The effect of the two thiol amino acids was similar.

However, the effect of glutathione was strikingly different. The R'_M value of the fungicide–glutathione complex was independent of the glutathione concentration. At low glutathione concentrations (up to 0.001 *M*) the original fungicide spot was also observed beside the spot of the complex with a streak between the two spots. Some densitograms showing the effect of the amount of fungicide on the interaction at constant glutathione concentration are collected in Fig. 7. The quantitative evaluation of these plates developed with an eluent containing 0.0004 *M* glutathione (Fig. 8) showed that at low amounts of the fungicide (up to 4 μg) all of the material applied was accumulated in the upper spot (fungicide–glutathione complex). As the amount of the applied fungicide was increased the amount of the upper spot became constant,

TABLE IV

EFFECT OF THE pH VALUE OF THE ELUENT ON THE INTERACTIONS OF FUNGICIDE WITH THREE THIOL COMPOUNDS

pH	*Cysteine*	*Homocysteine*	*Glutathione*
2.55	0.76 ± 0.06	0.78 ± 0.02	0.89 ± 0.02
4.45	0.70 ± 0.03	0.76 ± 0.03	0.83 ± 0.01
6.25	0.72 ± 0.03	0.74 ± 0.06	0.86 ± 0.04
7.55	0.78 ± 0.05	0.76 ± 0.05	0.85 ± 0.05
8.10	0.68 ± 0.03	0.72 ± 0.06	0.88 ± 0.02
9.40	0.72 ± 0.02	0.77 ± 0.03	0.85 ± 0.06
11.54	0.76 ± 0.04	0.78 ± 0.03	0.87 ± 0.03

and the original fungicide spot and the streak simultaneously appeared. At higher applied quantities only the amount of the fungicide spot increased. These data make it probable that this interaction is of covalent character.

Our data can not evidence that the interactions studied are due to charge-transfer complexing. "Charge-transfer" RP-TLC was considered as a method to detect any type of weak interactions.

The results suggest that the fungicide interacts most markedly with thiols, which confirms our earlier assumption concerning its mode of action. We found that the charge-transfer RP-TLC is a suitable tool for studying these interactions.

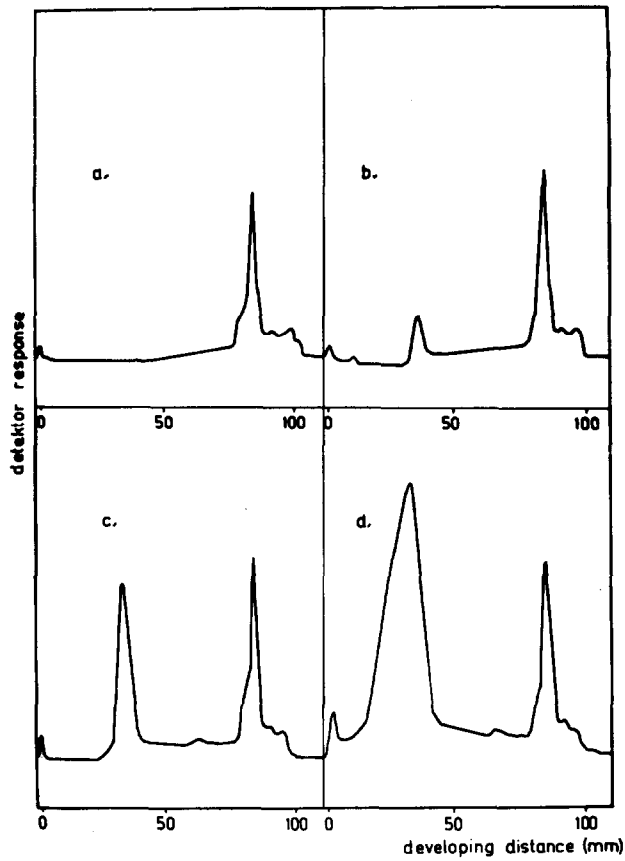

Fig. 7. Densitograms of various amounts of the fungicide developed in water–methanol 1:1 mixture containing glutathione at 0.0004 *M* concentration. Fungicide amounts: (a) 2.5 μg; (b) 5 μg; (c) 10 μg; (d) 25 μg.

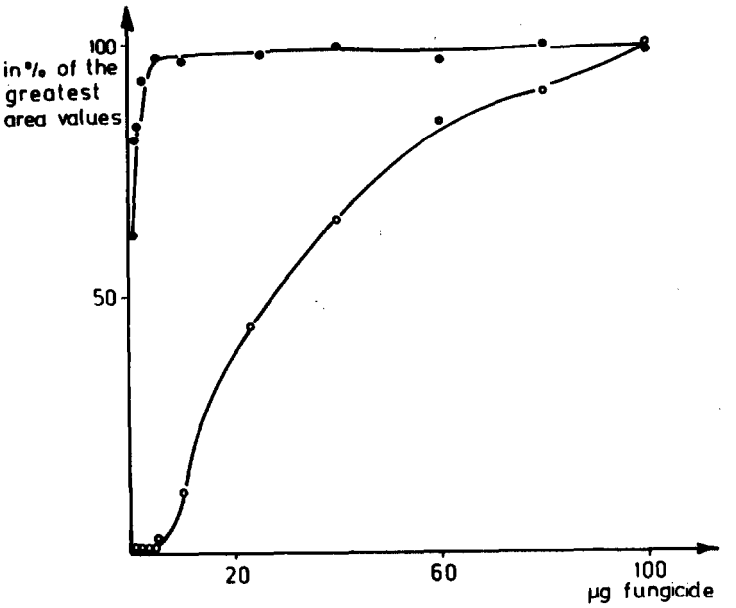

Fig. 8. Dependence of the amount of stock in the spots of the fungicide and of its glutathione complex on the amount of fungicide spotted on the plate, determined at 0.0004 *M* glutathione in the eluent: ○, fungicide; ●, fungicide–glutathione complex.

REFERENCES

1 H. Kessler and E. Müller, *J. Chromatogr.*, 24 (1966) 469.
2 R. G. Harvey and M. Halonen, *J. Chromatogr.*, 25 (1966) 294.
3 L. Fishbein and J. Fawkes, *J. Chromatogr.*, 22 (1966) 323.
4 M. A. Slifkin, W. A. Amarasiri, C. Schandorff and R. Bell, *J. Chromatogr.*, 235 (1982) 389.
5 J. Porath and B. Larsson, *J. Chromatogr.*, 155 (1978) 47.
6 Gy. Mikite, E. Jakucs, A. Kis-Tamás, F. Darvas and A. Lopata, *Pestic. Sci.*, 13 (1982) 557.
7 A. Lopata, F. Darvas, K. Valkó, Gy. Mikite, E. Jakucs and A. Kis-Tamás, *Pestic. Sci.*, 14 (1983) 513.
8 T. Zsolnai, *Biochem. Pharmacol.*, 5 (1961) 287.
9 T. Zsolnai, *Acta Phytopathol. Acad. Sci. Hung.*, 9 (1974) 143.
10 R. T. Louis-Ferdinand and G. C. Fuller, *Toxicol. Appl. Pharmacol.*, 16 (1970) 668.
11 W. Billard and E. Peets, *Antimicrob. Agents Chemother.*, 5 (1974) 19.
12 E. Šturdík, Ľ. Drobnica and Š. Baláž, *Coll. Czech. Chem. Commun.*, 48 (1983) 327.
13 A. Lopata, F. Darvas, Gy. Mikite, A. Kis-Tamás, G. Gullner and Gy. Josepovits, *Fifth European Symposium on QSAR, Bad Segeberg, F.R.G.*, Ab. vol. III/9, 1984.
14 T. Cserháti, É. Fenyvesi and J. Szejtli, *Acta Biophys. Biochim. Acad. Sci. Hung.*, 18 (1983) 60.
15 M. Friedman, J. F. Cavine and J. S. Wall, *J. Am. Chem. Soc.*, 87 (1965) 3672.
16 E. Šturdík, Ľ. Drobnica, Š. Baláž and V. Marko, *Biochem. Pharmacol.*, 28 (1979) 2525.