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Glutathione-Dependent Degradation of Parathion and its Significance for Resistance in the Housefly

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Glutathione-dependent degradation of parathion was studied in six strains of houseflies to find out whether it might be important as a cause of resistance. When supernatant fractions of high-speed centrifuged homogenates were fortified with glutathione and incubated with parathion, water-soluble products were formed. The rate of parathion detoxication was highest in a malathion-resistant strain (c. 4 μg parathion degraded per abdomen per hour), lowest in a susceptible strain, and intermediate in some other organophosphate-resistant strains. In one of the latter strains, E_1, the gene for glutathione-dependent degradation is located on the second chromosome, closely linked with gene cm^+. This is the same chromosome on which gene *a* for low ali-esterase activity and hydrolytic detoxication of paraoxon is located. It is not likely that the gene for glutathione-dependent degradation is identical with gene *a*, since it is also present in strain Nic which lacks gene *a*, and, therefore, the presence of a separate gene which is called gene *g* is postulated.

Since the malathion-resistant strain was only 4-fold resistant to parathion, the glutathione-dependent degradation seems to confer only little resistance, at least to this insecticide. In three of the strains the products were identified. Three labeled products were formed from ethyl-labeled parathion: ethylglutathione, diethylphosphorothionic acid, and desethylparathion.

INTRODUCTION

Organophosphorus insecticides can be metabolized in insects by a number of different enzymes. In many insecticide-resistant strains increased activity of one or more of these enzymes has been found. In the housefly, *Musca domestica* L., mixed function oxidases as well as hydrolytic enzymes have been shown to be involved in resistance. In strains resistant to parathion, diazinon, and related compounds, paraoxon is hydrolyzed into diethylphosphoric acid and *p*-nitrophenol (1). The hydrolytic enzyme is produced under the control of gene *a* on the second chromosome, replacing an ali-esterase produced under the control of its wild-type allele. Mixed-function oxidases can also cause parathion resistance by attacking paraoxon, producing desethylparaoxon. This is also under the influence of a gene on chromosome 2 (2–4).

Lewis (5), in studying metabolites from diazinon in a number of substrains with different chromosomes from a resistant strain, reported the presence of a glutathione (GSH)-dependent detoxication enzyme, causing desethylation of diazinon. This enzyme was found in the substrain with chromosome 2 from the resistant strain, and assuming that this chromosome carried only one gene for resistance, he inferred that this detoxication enzyme derived from gene *a*.

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This would mean that the hydrolytic activity for paraoxon and diazoxon as well as the GSH-dependent activity on the thiono compounds were due to gene *a*.

An alternative explanation is, of course, that there is another gene on chromosome 2 responsible for the GSH reaction. Hoping to clarify this point we started a study of GSH-dependent degradation of parathion in a number of strains with and without gene *a*. Although the method for the estimation of activity, as will be shown below, is only semi-quantitative, it enabled us to establish that there are large differences in activity between different strains, and to study its inheritance and the detoxication products formed. Since it appeared that this type of detoxication causes only a low degree of resistance, a further study to develop a more refined method was not made.

MATERIALS AND METHODS

Strains of Flies

Since a major point in this study is to establish whether there is a relation between GSH-dependent activity and gene *a* for low esterase activity, several strains with and without this gene have been studied. Flies were used 1–4 days after emergence.

Strains with high ali-esterase activity comprised: acr, a very susceptible marker strain, with markers *acv; cm; ro* (chromosome 1; 2; 3); and Fc and Nic, resistant to diazinon and related compounds. In both strains microsomal oxidation is a resistance mechanism (2).

Strains with low ali-esterase activity comprised the following:

E_1, resistant to diazinon and related compounds. In this strain the hydrolytic degradation (phosphatase action) of paraoxon has been studied (1). This is thought to be produced by a particular gene *a* allele;

29, obtained from Dr. R. M. Sawicki, Rothamsted Experimental Station, England. This is one of the strains used by Lewis on which he based the relation between gene *a* and glutathion-dependent degradation. It is a substrain from the diazinon-resistant SKA strain with chromosome 2 derived from this strain (6);

G, malathion resistant. In this strain another gene *a* allele is a cause of resistance (7).

Chemicals

[1-^{14}C]Ethyl-labeled parathion (*O,O*-diethyl-*O*-(*p*-nitrophenyl)-thionophosphate) was obtained from Amersham Radiochemical Centre. Activity 67,600 dpm per μg. It was used either undiluted or diluted with unlabeled parathion; glutathione, reduced, from Calbiochem; and albumin, bovine, fraction V, from Nutritional Biochem. Corp.

The following compounds, used as references for identification of the products, were synthesized in our laboratory:

Desethylparathion, sodium salt (*O*-ethyl *O*-hydrogen *O*-(4-nitrophenyl) phosphorothionate, sodium salt) was prepared by refluxing equimolar quantities of sodium thiophenolate and parathion in ethanol for 1.5 hr. After standing overnight and refluxing for another 2 hr the solution was treated with activated charcoal resulting in a clear yellow solution. The solvent was evaporated under vacuum and the partially crystallized oil was recrystallized from ethanol–hexane. After several recrystallizations a nearly white solid was obtained melting at 66–68°C.

Anal. Calcd for $C_8H_9O_5NaNSP.\frac{1}{2}H_2O$: C 32.66%; H 3.43%. Found: C 32.69%; H 3.57%.

DEPTA, *O,O*-diethyl phosphorothioic acid, was obtained by hydrolysis of triethylthionophosphate with potassium hydroxide (8).

S-ethyl glutathione was synthesized by reacting glutathione with ethyl iodide in a solution of sodium hydroxide in aqueous ethanol (9). Addition of ethanol after several hours of shaking did not provide a solid precipitate. Therefore, the ethanol was stripped off and the residue was adjusted to pH 3 with hydrogen iodide and sodium hydroxide (10).

TABLE 1

Influence of pH on glutathione-dependent parathion degradation[a]

Number of abdomens	Phosphate buffer				Tris buffer			
	pH				pH			
	6.5	7.0	7.5	8.0	7.2	8.1	8.6	9.1
0	0.007	0.019	0.023	0.012	0.010	0.016	0.020	0.023
1	0.114	0.355	0.375	0.750	0.016	0.246	0.252	0.308
5	0.220	0.725	0.810	1.700	0.021	0.700	0.704	1.100

[a] Activity in μg of parathion degraded per hour. The soluble fraction of the indicated amount of fly material, strain E_1, was incubated for 2 hr at 27°C in 2 ml of 0.15 *M* buffer solution with 0.075% albumin fraction V, 4 mg glutathione, and 10 μg parathion.

The product was recrystallized from water and ethanol giving a white solid melting at 192°C (d).

Anal. Calcd for $C_{12}H_{21}N_3O_6S$: C 42.98%; H 6.31%. Found: C 42.76%; H 6.34%.

Measurement of Enzymatic Activity

Optimal conditions for the GSH-dependent parathion reaction have not been fully studied. As will be discussed later it is possible that more than one enzyme with different optimal conditions is present, or that the requirements for different strains might not be the same.

Since the soluble fraction was used for the experiments, we started with the same conditions as used for obtaining microsomes (11): maceration of the fly material in Potter–Elvehjem tubes in 0.15 *M* phosphate buffer pH 7.5 with 0.25 *M* sucrose and 1.5% bovine albumin fraction V, centrifugation at 18,000*g* for 30 min and 150,000*g* for 1 hr and incubation of the supernatant fraction. Experiments with strain E_1 showed that the parathion degradation could be increased considerably by several alterations of these conditions. Table 1 shows the effect of buffer, pH and enzyme concentration. Figure 1 shows the effect of albumin fraction V on activity. Experiments in which whole flies were compared with abdomens showed equal or higher activity of the latter in strain G and E_1 and somewhat lower activity in strain Nic. Table 2 shows the influence of GSH con-

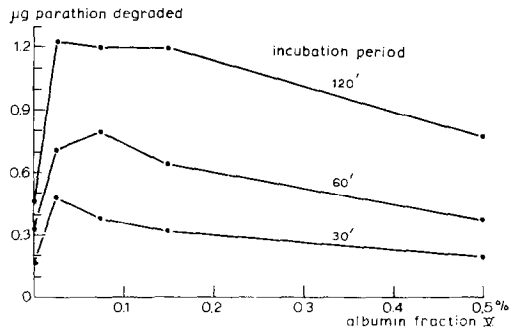

FIG. 1. *Influence of albumin fraction V concentration on GSH-dependent parathion degradation. Incubation of soluble fraction of 0.5 abdomens of strain G for the indicated periods in 2 ml 0.15 M phosphate buffer, pH8, with 0.25 M sucrose, 4 mg glutathione and 10 μg parathion.*

centration, amount of fly material and incubation time. On the basis of these observations the following conditions were adopted for most of the experiments. Abdomens (equal numbers of males and females) were homogenized in 0.15 *M* phosphate buffer, pH 8.0, with 0.25 *M* sucrose and centrifuged at 150,000*g* for 30 min. Parathion, 10 μg, was introduced into an incubation tube in acetone and the acetone was evaporated. One milliliter of the supernatant fluid and 1 ml phosphate buffer 0.15 *M* pH 8.0 with 4 mg GSH and 0.15% albumin fraction V were pipetted into the tube and incubated at 27°C for 2 hr.

In experiments in which the activity of different strains was studied, 0.5–1 abdomen

TABLE 2

Influence of amount of fly material, GSH, and incubation time on parathion degradation[a]

Expt. number	GSH (mg per 2 ml)	Number abdomens per 2 ml	Incubation time (min)	Amount of parathion degraded	Activity (μg/abdomen/hr)
1	0	1	120	0.023	0.012
	0.5	1	120	0.111	0.056
	1	1	120	0.171	0.086
	2	1	120	0.223	0.112
	4	1	120	0.240	0.120
	4	1	60	0.164	0.164
	4	1	30	0.062	0.124
	4	1	15	0.037	0.148
	4	0.25	120	0.092	0.184
	4	0.5	120	0.140	0.140
2	4	1	120	0.385	0.193
	4	2	120	0.519	0.130
	4	5	120	0.758	0.076
	4	10	120	1.01	0.050

[a] Incubation in 2 ml 0.15 *M* phosphate buffer, pH 7.5, with 0.25 *M* sucrose. Soluble fraction of abdomens of strain E_1, no albumin added, 10 μg parathion.

per tube was used. If analysis of the products of the reaction was the purpose of the experiments, higher enzyme concentrations were required, but there is no proportionality between amount of fly material and activity.

Unchanged parathion was removed at the end of the incubation period by extraction with four 2-ml portions of chloroform. Samples of the water phase and the chloroform phase (the latter after careful evaporation of the solvent) were counted in a liquid-scintillation counter. Blanks consisted of tubes in which no GSH or no fly material was present.

Chromatographic Techniques

After extraction with chloroform the water phase of the incubates was analyzed on three different chromatographic systems for identification of the products.

1. Column chromatography on Dowex, as described by Plapp and Casida (12) with an HCl gradient of 0.01–1 *N* in 20% methanol.

2. Descending paper chromatography with an acetonitril–water–ammonia 80–18–2 mixture (Sawicki, personal communication). The water phase of the incubates was mixed with 2.5 times its volume of acetone, kept at −20°C and centrifuged to remove precipitated proteins and salts. The supernatant portion was applied to the paper (Whatman No. 1), and 15-cm chromatograms were obtained. Detection of reference DEPTA and desethylparathion was carried out by spraying with 2,6-dibromo-*N*-chloro-*p*-quinoneimine (13).

3. Column chromatography on Sephadex LH-20, as described by Mulder and Buytenhuys (14). A column of 90 × 1.5 cm was eluted with 60% acetone–water containing 0.05 *M* NaCl. The water phase of the incubate to be analyzed was treated with acetone as in method 2. Four milliliters were applied to the column, a flow rate of 48 ml per hour was used, and 2-ml fractions were collected.

Reference compounds were detected by estimation of phosphorus content in the case of DEPTA and desethylparathion (15) or with the ninhydrin reaction in the case of *S*-ethyl glutathione.

RESULTS

Glutathione-Dependent Activity in Different Strains and in Crossing Products of Strain E_1 and acr

Table 3 shows the amount of parathion degraded in the presence of GSH in different strains. Only one concentration of parathion, 1.7×10^{-5} *M*, has been used and it is not known, therefore, whether these data represent maximal degradation capacity. The strains have been arranged in order of increasing activity. The values shown have been obtained by subtracting the blanks, as explained in the legend of the table. Since these vary themselves between 0.02 and 0.05 μg the data on the strains with low activity are only approximate. The extreme difference between the results for strain acr at 0.5 abdomen may be due to an experimental error and was not found with 1 abdomen per tube. Despite these inaccuracies, it can

TABLE 3
Glutathione-dependent degradation of parathion by supernatant fractions of homogenates of different strains[a]

Strain	0.5 Abdomen per tube		1 Abdomen per tube	
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
acr	0.01	0.11	0.08	0.10
F_c	0.13	0.13	—	—
29	0.22	0.22	—	—
E_1	0.25	0.29	0.36	0.31
Nic	0.36	0.39	—	—
G	1.47	2.40	—	—
B_1 *cm*	—	—	0.06	0.06
B_1 *cm*$^+$	—	—	0.20	0.20

[a] Activity in μg of parathion degraded per hour. Incubation in 2 ml 0.15 *M* phosphate buffer, pH 8, containing 1.5 mg albumin fraction V, 4 mg glutathione, and 10 μg parathion. Incubation for 2 hr at 27°C. The small activity found without glutathione has been subtracted. It varied between 0.02 and 0.05 μg, not significantly higher than the controls without fly material. B_1 *cm* and B_1 *cm*$^+$ indicate flies from a cross between strains acr and E_1. F_1 flies were backcrossed to the acr strain and the progeny was sorted out according to the eye color marker on chromosome 2. Experiment 3: backcross between F_1 ♂ and acr ♀ (no crossing-over possible). Experiment 4: backcross between F_1 ♀ and acr ♂ (cross-over between *cm* and GSH-dependent factors possible).

be concluded that strain 29 and E_1 have an activity of at least two to three times that of the susceptible strain, Nic approximately four times, and G over 14 times. For one of the strains, E_1, appropriate crosses with the susceptible strain provided material sorted out for the marker gene *cm*. As explained in the legend to the table, two backcross groups are tested. Of these the one denoted as *cm* has the two marked (2nd) chromosomes from the susceptible strain, in the other one, *cm*$^+$, one of these chromosomes is derived from strain E_1. The other chromosomes from strain E_1 will be present at random in 50% of the *cm* and *cm*$^+$ individuals (in the heterozygous condition). The results of Expt. 3 in which F_1 males were backcrossed show that the activity in the *cm* groups is low, just as in the susceptible strain, that in the *cm*$^+$ group is high, about intermediate between the parent strains. This is in accordance with expectation if the gene(s) for GSH-dependent activity are on the 2nd chromosome. Experiment 4, in which F_1 females were backcrossed, provides a similar set of data, but in this case crossover is possible. Since the data are exactly the same as those of Expt. 3, it can be concluded that no appreciable crossover between the marker gene *cm* and the gene(s) for activity has taken place.

Identification of the Products

Application of column chromatography on Dowex showed two peaks of ^{14}C activity. The second peak cochromatographed with DEPTA. Application of desethylparathion as a reference compound showed that this could be eluted only with high concentrations of HCl which caused difficulties in the scintillation counter.

The paper chromatographs showed three radioactive spots with R_f of 3, 57, and 83. The product with R_f 57 cochromatographed with DEPTA, that of R_f 83, with desethylparathion. This method is a rapid and convenient one, but in our hands separation of the products was not always satisfactory. Moreover, one of the products hardly moved from the origin. We used the method for rapid quantitative evaluation of the three products produced by different strains, but for precise identification the Sephadex LH-20 column was used.

The Sephadex column showed three distinct peaks of ^{14}C activity (Fig. 2). The first radioactive peak did not coincide precisely with that of ninhydrin-positive material when relatively small amounts (0.5 mg) of ethyl glutathione were added as reference. The two curves became nearly identical when this amount was increased to 4 mg, which made it likely that the small discrepancy was due to other ninhydrin-positive material present in the fly extracts. The second peak cochromatographed with DEPTA, the third with desethylparathion.

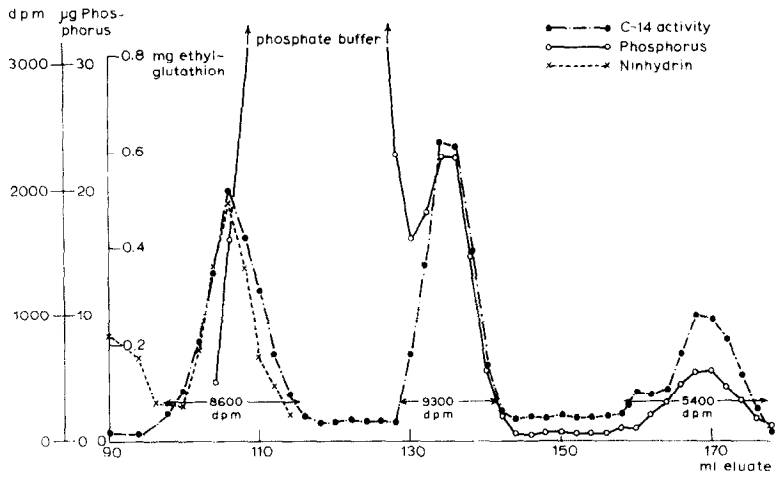

FIG. 2. *Chromatogram on Sephadex LH-20 of water phase of incubate of strain 29. These results are from the second experiment described in Table 4. See legend of this table and text for explanation of the technical data.*

TABLE 4

Relative amount of different products produced by GSH-dependent enzymes

Strain	29		Nic	G	G		G
Conditions of incubation[a]	I		I	I	II		II
Separation method	Paper	Column	Paper	Paper	Paper	Column	Column
Amount of parathion degraded (µg/tube)	0.6		0.6	1.7	5.4		3.0
Percentage of total radioactivity found in							
Peak I (ethylglutathion)	30	37	17	15	9	10	14
Peak II (DEPTA)	44	40	61	73	81	78	68
Peak III (desethylparathion)	26	23	22	12	10	12	18

[a] I Incubation of supernatant of 10 whole flies in 2 ml phosphate buffer pH 7.3 with 2 mg GSH for 2 hr at 27°C. II Incubation of supernatant fraction of five abdomens in 2 ml phosphate buffer, pH 8.0, with 0.25 *M* sucrose and 0.075% albumin fraction V, with 4 mg GSH for 2 hr at 27°C. Separation between peak II and III on paper is poor, the values are only approximate.

The relative activity found in the three peaks and in different strains, separated by paper chromatography or in the Sephadex column is shown in Table 4. Since ethyl glutathione and desethylparathion each carry only one labeled ethyl group, their activity should be identical. It appears from this study that parathion is degraded in two ways: by removal of an ethyl- or of the *p*-nitrophenol group. The percentage of degradation by each route varies between different experiments, but suggests that desethylation

is relatively more prominent in strain 29 than in strain G. In view of the fact that the conditions used in these experiments are not optimal, further experiments will be needed to find out whether incubation conditions and strains used influence the relative importance of the two routes of degradation. It should be mentioned that method I was chosen to enable comparisons with work by Dr. Sawicki at Rothamsted Experimental Station.

DISCUSSION

The genetic experiments with strain E_1 confirm the observation of Lewis (5) that the factor(s) for GSH-dependent degradation are located on chromosome 2. As mentioned in the introduction, this is the chromosome on which gene *a* is located, which causes low ali-esterase activity and the presence of a hydrolytic enzyme attacking paraoxon (1). We now have to consider the evidence on the identity or nonidentity of the factors causing GSH-dependent degradation of parathion and hydrolytic degradation of paraoxon. As shown in this paper there is evidence for a rather close linkage between gene *cm* and the GSH factor(s). The same is true for gene *a* (17) and, therefore, the two factors must be identical or closely linked. There is ample evidence that the enzymes responsible for the two reactions cannot be identical, since the GSH-dependent reaction is found in the soluble fraction of homogenates (5, 18, 19), whereas the hydrolytic enzyme is known to predominate in the microsomes (20). Still they could stem from the same gene. We have found a GSH-dependent reaction in strain Nic, in which the enzyme hydrolyzing paraoxon is not present. Although it is not certain that the GSH-dependent enzyme in strain Nic and E_1 derive from the same gene, this finding makes it increasingly unlikely that gene *a* is involved. We, therefore, suggest that the factor on chromosome 2 causing GSH-dependent parathion degradation should be called gene *g*.

The genetic basis of the differences between the GSH-dependent degradation in different strains is not known. Whether the two reactions, desethylation and removal of *p*-nitrophenol, are due to the same gene and the same enzyme also needs further investigation.

An important question is the significance of GSH-dependent degradation for resistance to insecticides. Other resistance factors are known to be present in some of the strains and their relative importance should be considered. Resistance to parathion in strain G (topical application in 0.5 μl acetone) is only 4-fold (LD_{50} 0.06 μg per male fly) and its *in vitro* degradation capacity is c. 4 μg per abdomen per hour. This may indicate that the *in vitro* conditions are rather artificial or that the location of the enzyme is unfavorable for participation in the detoxication of an insecticide when it is applied topically. In strain E_1, with a 40-fold resistance to parathion, GSH-dependent parathion degradation *in vitro* is 0.5 μg per fly per hour.

In the same strain there is a hydrolytic capacity for paraoxon of 0.04 μg (1) and an oxidative degradation of 0.17 μg (3). Evidence has been presented indicating that the hydrolytic degradation, which has the lowest capacity, is still the more important one for resistance (3). From the fact that in all the resistant strains an elevated level of GSH-dependent detoxication is found, it can be concluded that at least for the insecticides with which the strains were selected (G: malathion; E_1, 29, F_c: diazinon; Nic: dithion) the mechanism must contribute, however little, to survival.

GSH-dependent degradation has been described for a number of mammals and insects (18). In insects evidence for the two reactions described in this paper has been presented: desethylation of diazinon by Lewis (5), DEPTA formation from diazinon by Yang *et al.* (19). From Fig. 1 in their paper a rate of 1 μg diazinon degraded per fly per hour can be calculated for their resistant strain. If we consider the influence of albumin and

Tris buffer as shown in our Fig. 1 and Table 1 this is of the same order of magnitude as the activity in strain G for parathion.

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