イエバエグルタチオンS-トランスフェラーゼのカルコンによる阻害およびそのアイソザイムのラットとの比較

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Original Article

Inhibition of Housefly Glutathione *S*-Transferase by Chalcone and Comparison of Its Isozymes with Rat

Toru MIYAMOTO and Izuru YAMAMOTO

Department of Agricultural Chemistry, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156, Japan

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2-, 3- and 4-Chloro-4′-phenylchalcones were weakly inhibitory against glutathione *S*-transferases (GST) from resistant (R) and susceptible (S) housefly abdomens. However, each of glutathione (GSH) conjugates of the above chalcones inhibited strongly both GSTs in an incompetitive fashion with respect to CDNB or DCNB. The inhibitory activity was in the order of 2-, 4- and 3-chloro-compounds which was similar to the case of mouse liver GST. These facts indicated that the rate of GSH conjugation was slow *in vitro* insect system. On the other hand, the above chalcones increased the insecticidal activity of diazinon to R-fly in the order of 2-, 4- and 3-chloro-compounds. Diazinon is known to be more detoxified in R-fly than in S-fly by GST. Therefore, it is conceivable that such synergism is due to the conversion of the chalcones to the GSH conjugates by GST and the inhibition of GST by the conjugates, which decrease the detoxication of diazinon. There were differences between rat and housefly GSTs and between R- and S-fly GSTs. The affinity of CDNB was higher to R-fly GST than to S-fly GST, but both were far low as compared with the affinity to rat GST. On the other hand, the affinity of DCNB to GSTs was generally lower than that of CDNB and there was not so much difference between housefly and mouse or rat GSTs, although R-fly GST gave the lowest affinity. Comparison of subunits on sodium dodecylsulfate polyacrylamide gel electrophoresis suggested that fly GST was qualitatively different from rat GST. There was a minor difference between GSTs of R- and S-flies.

INTRODUCTION

The cause of insect resistance to organophosphorus insecticides is attributable to the increasing detoxication, the decreasing activation, the decreasing sensitivity of acetylcholinesterase (AChE) and perhaps decreasing sensitivity of acetylcholine receptor. The activated form of organophosphorus insecticides is usually the oxon. However, in the case of *S*-alkyl phosphoroinsecticides such as profenofos and prothiophos, which are effective even to resistant housefly strains, the oxon forms are not inhibitory to AChE *in vitro*. A new type of activation mechanism was provided by us[1] and others,[2-4] which involves the production of an unstable sulfoxide intermediate that inhibits AChE. Furthermore, there was an evidence that the glutathione (GSH) conjugate *via* the sulfoxide inhibits AChE.[5] These mechanisms serve to explain why such insecticides are effective to the resistant strains and modify our view that the GSH conjugation is a detoxication. Also, the evidence for the role of the GSH conjugate of several chloro-substituted 4′-phenylchalcones in inhibiting glutathione *S*-transferase (GST) was provided by us.[6]

In the present paper, we attempted an approach to increase the insecticidal activity of an insecticide, diazinon, by using the above chalcones and information. Also, difference of GST of rat and resistant (R) and susceptible (S) houseflies was studied using the chalcones as probes from the standpoint of selective toxicity.

MATERIALS AND METHODS

1. Chemicals

2-, 3- and 4-Chloro-4′-phenylchalcones were prepared by Claisen-Schmidt reaction of 4-acetylbiphenyl with chloro-substituted benzaldehydes and purified by recrystalization from ethanol. The GSH conjugates were prepared by reaction of corresponding chalcones with reduced GSH in borate buffer (pH 9.2) under inert atmosphere and purified by SEP-PAC C_{18} (Waters Co.) column chromatography as described in previous report.[6,7]

GSH was obtained from Sigma Chemical, CDNB (1-chloro-2,4-dinitrobenzene), DCNB (1,2-dichloro-4-nitrobenzene) as substrate were purchased from Tokyo Kasei and epoxide-activated sepharose 6B was purchased from Pharmacia. Other chemical and biochemical reagents were supplied from Aldrich Chemical, Tokyo Kasei, Wako Pure Chemical and Whatman International.

2. Housefly Strains

Yachiyo and Takatsuki strains were used as the R- and S-houseflies, respectively. Yachiyo strain which is multiply resistant was supplied from Dr. Motoyama of Chiba University and maintained in our laboratory as well as Takatsuki strain.

3. Enzyme Preparation

Crude rat cytosolic GST was prepared in 10% of cytosol in phosphate buffer (pH 7.4) in the usual ways from the liver of male Wistar rat (7 weeks, 180–200 g). Crude housefly GSTs were supplied from their abdomens. Flies 3 days after emergence were chilled (0–4°C) for 20 min, transferred from their cages to a precooled plastic bottle. Immediately after freezing with liquid nitrogen, the capped bottle was shaken vigorously until all fly body parts were separated. Body parts were then sieved through #1.7 brass screen (1.7-mm sieve) to retain most detached abdomens and thoraces. Detached heads, appendages and wings passed through the sieve. The thorax and abdomen were separated by hand. A batch of the abdomens was ground in a glass mortar with a pestle and homogenized with a teflon-glass Potter Elvehjem tube in two times volume of 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA (ethylenediaminetetraacetic acid) and 0.1 mM PMSF (phenylmethylsulfonyl fluoride). That is, the contents were homogenized for 30 sec, cooled for 1 min in an ice-bath and this cycle was repeated 5 times. After filtration through four layers of cheesecloth, the homogenates were centrifuged at $10,000\times g$ for 10 min and the supernatant was further centrifuged at $40,000\times g$ for 60 min to obtain the crude enzyme solution. The aliquots of rat cytosol and fly $40,000\times g$ supernatant were frozen immediately at −80°C after the preparation. The enzyme activity is expressed by nmole of GSH conjugate of DCNB per minute in protein milligram. The total protein content was measured by the method of Laury.

4. Inhibition for GSTs of Housefly and Rat by Chalcones[6,8]

The crude enzyme solution (0.2%, 1.45 ml) in 0.1 M sodium phosphate buffer (pH 7.0) containing 5 mM GSH was preincubated at 25°C for 1 min and to this was added chalcone in 25 μl of acetone, or just acetone (control). After 10 min, 25 μl of 60 mM DCNB in acetone was added to the mixture, and kept for another 10 min. The reaction was terminated with the addition of 3 ml of chloroform and vigorous vortexing. Following centrifugation ($1500\times g$, 10 min), the aqueous phase was transferred to UV cell and the absorbance was measured at 345 nm. From the difference in absorbance between two reaction mixtures with and without the inhibitor, the inhibitory percent was calculated. Inhibitory percent at 10^{-4}–10^{-8} M of inhibitors was the average of triplicate experiments. Inhibition of rat liver GST was measured in the same way as described previously.

5. Kinetics of GST Inhibition by the GSH Conjugate of Chloro-substituted 4′-Phenylchalcone[6]

Housefly crude enzyme solution (0.2%, 960 μl) containing 5 mM (or 1 mM) GSH was preincubated at 25°C for 20 sec. To this was added the GSH conjugate of a chalcone in 20 μl of methanol or just methanol (control), and the mixture was further preincubated at 25°C

for 40 sec. DCNB (or CDNB) in 20 μl of methanol (50, 25, 12.5, 8.75, 6.25 mM) was then added to measure the rate of DCNB (or CDNB) conjugation of GSH every 18 sec from 0.3 through 3.3 min by the change in the absorbance at 345 (or 340) nm according to the rate assay system with the U-3200 spectrophotometer. The Lineweaver-Burk plots in the presence of the GSH conjugates of the chalcones was made.

6. Effects of Chloro-substituted 4'-phenylchalcones on the Insecticidal Activity of an Organophosphorus Insecticide Diazinon to the Resistant Housefly

Experiments carried out in 4 groups as follows: A group, chalcone free; B, C and D groups, 2-, 3- and 4-chloro-4'-phenylchalcones were given to the fly, respectively. In A group only acetone was given to the fly. In B, C and D groups, 5 μg of each chalcone in 1 μl of acetone was topically given to each abdomen of 10 resistant flies (mixed male and female, 3 to 5 days old). After 2 hr, the houseflies treated were released on the filter paper coated with diazinon (5 mg/55 cm^2 paper) in Petri dish to count the number of abnormal and dead houseflies every 1 hr from 1 through 8 hr. Triplicate insecticidal tests using two Petri dish per one group were run.

7. Purification Procedures for GST from Housefly and Rat

7.1 Treatment with DE-52 cellulose

A large excess, 100 mg dry gel/ml crude enzyme solution, of DE-52 cellulose powder was mixed with 2 ml of each crude enzyme solution in centrifuge tube, and occasionally stirred under ice-cooling. After 20 min, the mixture was centrifuged at 10,000$\times g$ for 10 min to obtain the supernatant. To each residual gel 2 ml of 0.1 M phosphate buffer (pH 7.0, containing 1 mM EDTA) was added and further centrifuged at 10,000$\times g$ for 10 min to get the supernatant which was combined with the former.

7.2 Precipitation and dialysis

Ammonium sulfate was little by little added with stirring for 30 min at 4°C to the combined supernatants from DE-52 cellulose powder, and the protein fraction precipitating between 40 and 80% saturation with ammonium sulfate was collected by centrifugation at 10,000$\times g$ for 30 min. The protein was dissolved in a small amount of 10 mM phosphate buffer (pH 7.0, containing 1 mM EDTA), and the solution was dialyzed overnight at 4°C against 3 l of the same buffer with three to four times.

7.3 Gel filtration by Sephadex chromatography

The above sample was then applied to and eluted from a Sephadex G-100 column (2.6$\times$ 30 cm) that had been equilibrated with 0.1 M phosphate buffer (pH 7.0, containing 1 mM EDTA). The elution was made at flow rate of 10 ml/hr to collect the fractions having GST activity.

7.4 Affinity chromatography

A GSH affinity column with a GSH linked to epoxy-activated Sepharose 6B was prepared according to the method by Simons & Vander Japt.[9] The sample was applied to the column (1.2$\times$22 cm) and several column volumes of 22 mM phosphate buffer (pH 7.0) was passed through the column. The buffer was changed to 50 mM Tris-HCl buffer (pH 9.6, 4°C), then to 50 mM Tris-HCl buffer (pH 9.6, 4°C) with 5 mM GSH, and finally to 50 mM Tris-HCl buffer (pH 9.6, 4°C) with 10 mM GSH to obtain 2 or 3 fractions having GST activity.

7.5 Sodium dodecylsulfate polyacrylamide gel electrophoresis[10,11]

To a mixture of 10 mM Tris(hydroxymethyl)-aminomethane [2-amino-2-(hydroxymethyl)-1,3-propanediol], 20% glycerol, 1% SDS [sodium dodecylsulfate] and 0.02% BPB [bromophenol blue], 1% 2-mercaptoethanol was added just before the addition of lyophilized GST preparation. For GST preparation in buffer solution, the concentrations of the above components was adjusted to have the same final concentrations. The overall mixtures contain 1–2 mg protein/ml which was gradually heated to 100°C and kept at 100°C for 1–2 min, and then submitted to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). A low molecular weight calibration kit from Pharmacia was treated in the same way as the reference. SDS-PAGE was carried out in a solution of 25 mM Tris (hydroxymethyl)aminomethane, 192 mM glycine and 0.1% SDS at 20 mA per one plate (gradually to 190 Voltage), using 5–20% continuous

polyacrylamide slab gel Pagel®, Atto Corporation, Japan. The protein was stained with Coomassie brilliant blue.

RESULTS AND DISCUSSION

1. *Inhibitory Activity of 2-, 3- and 4-Chloro-4′-phenylchalcones against GSTs from Housefly Abdomens and Rat Liver*

2-Chloro-4′-phenylchalcone showed 50% inhibition of rat liver GST at 10^{-8} M order followed by 3-chloro-compound at 10^{-7} M order, whereas 4-chloro-compound was weakly inhibitory. The results was similar to the case of mouse liver GST. To the GSTs from housefly abdomens, the inhibitory activity of the chalcones was extremely weak, although 2-chloro-compound was the strongest as compared with 3- or 4- chloro-compound as shown in Table 1.

2. *Kinetics of GST Inhibition by the GSH Conjugates of Chalcones*

The Lineweaver-Burk plots were made. The K_m and V_{max} values for CDNB and DCNB against R- and S-fly abdomen GSTs were calculated from these plots as shown in Table 2. CDNB had higher affinity to R-fly GST than to S-fly GST and the highest affinity was seen to rat GST. On the other hand, the affinity of DCNB to GSTs was generally lower than that of CDNB and there was not so much difference between housefly and mouse or rat GSTs, although R-fly GST gave the lowest affinity. *In vitro* GSH conjugation of CDNB or DCNB was conducted in the presence of the GSH conjugates of chalcone to examine the mode of inhibition and to calculate the K_i value. These conjugates inhibited the GST in an incompetitive fashion with respect to CDNB or DCNB, respectively. As shown in Table 3, the inhibitory activity was in the order of 2-, 4- and 3-chloro-compounds which was similar to the case of mouse liver GST.

Table 1 Inhibitory activity of 2-, 3- and 4-chloro-4′-phenylchalcones against glutathione *S*-transferases from resistant (R) and susceptible (S) housefly abdomens and rat liver.

	% of inhibition against					
	R-fly	S-fly	Rat			
Inhibitor	at 10^{-4}	at 10^{-4}	10^{-5}	at 10^{-6}	10^{-7}	10^{-8}
2-Cl	53	38	90	83	59	34
3-Cl	45	23	81	60	30	23
4-Cl	28	28	59	44	34	18

2-, 3- and 4-Cl: 2-, 3- and 4-chloro-4′-phenylchalcones.

Table 2 Michaelis constant and maximum velocity of glutathione conjugation of CDNB (or DCNB) by glutathione *S*-transferases (GST) from resistant (R) and susceptible (S) housefly abdomens and mouse and rat livers.

	R-fly	S-fly	Mouse	Rat
CDNB				
K_m, mM	0.18	0.25		0.06[b]
V_{max}, unit[a]	752	690		
DCNB				
K_m, mM	2.5	1.2	1.2	1.1[b]
V_{max}, unit[a]	637	207		

[a] One unit is 1 nmol conjugation/min·mg protein for fly GST.

[b] Michaelis constant of the glutathione conjugation by purified GST A from rat liver. Data from Ref. 8).

Table 3 K_i values of glutathione (GSH) conjugates of chalcone against glutathione *S*-transferases (GST) from resistant and susceptible housefly abdomens.

Housefly strain	Substrate	K_i (μmol) GSH conjugate of 2-Cl	3-Cl	4-Cl
Resistant	CDNB	0.33	7.8	0.86
	DCNB	0.65	2.1	1.1
Susceptible	CDNB	0.41	29	1.2
	DCNB	1.4	7.2	2.2

2-, 3- and 4-Cl: 2-, 3- and 4-chloro-4′-phenylchalcones.

3. *Insecticidal Activity of Diazinon Synergized by Chloro-substituted 4′-phenylchalcones*

As shown in Fig. 1, no change occurred in all groups until 2 hr after the houseflies were released on the filter paper coated with diazinon, but after 3 hr the slight abnormality was observed. Particularly, in B and D groups, 3 and 4 flies showed heavy abnormality, re-

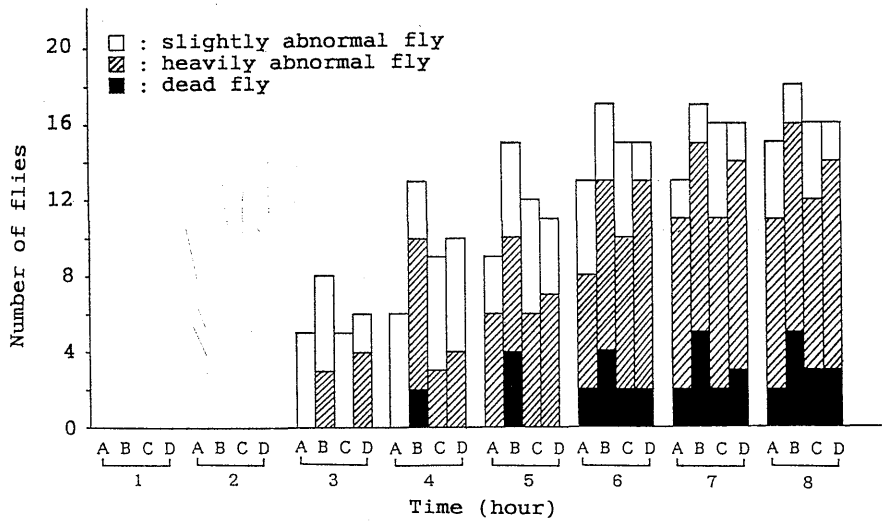

Fig. 1 Effects of chloro-substituted 4′-phenylchalcones on the insecticidal activity of an organophosphorus insecticide diazinon to the resistant housefly.

Experiments carried out in 4 groups as follows: A group, chalcone free; B, C and D groups, 2-, 3- and 4-chloro-4′-phenylchalcones, respectively. In B, C and D groups, 5 μg of each chalcone was topically given to each abdomen of 10 flies (mixed male and female, 3 to 5 days old). After 2 hr, the flies treated were released on the filter paper coated with diazinon (5 mg/55 cm^2 paper) in Petri dish to count the number of abnormal and dead houseflies every 1 hr from 1 through 8 hr.

spectively. After 4 hr, the abnormality increased in chalcone treated groups. That is, the number of abnormal fly was 13 (slight, 3; heavy, 8; dead, 2), 9 (slight, 6; heavy, 3), and 10 (slight, 6; heavy, 4) in B, C and D groups, respectively. After 5 hr, the number of the heavily abnormal flies further increased in all groups, and also dead flies increased in group B. The trend continued until 8 hr. In brief, when each chalcone was previously given to flies, the number of abnormal houseflies was increased at any time in the order of 2-, 4- and 3-chloro-4′-phenylchalcones.

It has been known that diazinon is detoxified in houseflies by their GST, particularly in R-flies. Therefore, chalcone would be involved in the interaction with GST. We have already revealed in mouse that the *in vitro* GST inhibition by chalcones was mainly due to their activated form, the GSH conjugates of chalcones, and that overall GST inhibition depended on the rate of GSH conjugation and the intrinsic activity of the conjugate in the previous paper.[6] In this study, as shown in Tables 1 and 3, housefly GSTs were poorly inhibited *in vitro* by chalcones, but strongly by the GSH conjugates. This is probably due to the slow conversion of chalcones to the GSH conjugates *in vitro*. On the other hand, *in vitro* inhibitory activities of the conjugates against GST were increased in the order of 2-, 4- and 3-chloro-4′-phenylchalcones, and the order just coincided with the order of the synergistic effect of chalcone on the insecticidal activity of diazinon. Therefore, chalcones seemed to be converted to the conjugates effectively *in vivo*. From these facts, it is conceivable that chalcones after conversion to the GSH conjugates inhibit the GST, thus synergizing diazinon activity.

4. *Purification of GSTs from R- and S-Houseflies and Rat*

The results of purification are summarized in Tables 4, 5 and 6. Up to the step of gel filtration, the extent of purification was poor. Recovery and level of purification were: from R-fly, 18% yield (0.7-fold); S-fly, 55% yield

Table 4 Purification of glutathione *S*-transferase from the Yachiyo strain of resistant housefly.

Procedure	Vol. (ml)	Units/ ml	Total units	Protein (mg/ml)	Units/ mg	Yield (%)	Purification (fold)
40,000×*g* sup.	72	50.0	3600	34.48	1.45	100	1
DE-52 cellulose	97	27.5	2668	18.18	1.51	74	1.04
Ammonium sulfate	45	24.4	1097	14.11	1.73	30	1.19
Sephadex G-100	54	11.8	635	11.21	1.05	18	0.72
Affinity rinsed	68	1.8	119	7.63	0.23	3	0.16
eluted I	19	10.5	194	0.05	210.00	5	144.83
eluted II	8	10.8	86	1.08	10.00	2	6.90
eluted III	24	6.0	144	1.47	4.08	4	2.81

One unit is defined as 1 nmol of GSH conjugate of CDNB per minute at 25°C.

Table 5 Purification of glutathione *S*-transferase from the Takatsuki strain of susceptible housefly.

Procedure	Vol. (ml)	Units/ ml	Total units	Protein (mg/ml)	Units/ mg	Yield (%)	Purification (fold)
40,000×*g* sup.	28	23.75	665	27.93	0.85	100	1
DE-52 cellulose	38	11.75	447	11.68	1.01	67	1.19
Ammonium sulfate	17	21.75	370	29.95	0.73	56	0.86
Sephadex G-100	88	4.13	363	3.21	0.78	55	0.92
Affinity rinsed	92	3.25	299	0.94	3.42	45	4.02
eluted I	20	7.00	140	0.08	87.50	21	102.94
eluted II	4	5.25	21	0.38	13.82	3	16.25

One unit is defined as 1 nmol of GSH conjugate of CDNB per minute at 25°C.

Table 6 Purification of glutathione *S*-transferase from rat liver.

Procedure	Vol. (ml)	Units/ ml	Total units	Protein (mg/ml)	Units/ mg	Yield (%)	Purification (fold)
40,000×*g* sup.	30	35.00	1050	17.31	2.02	100	1
DE-52 cellulose	38	30.00	1140	6.46	4.64	108	2.30
Ammonium sulfate	14	31.25	438	11.40	2.74	42	1.36
Sephadex G-100	42	8.75	368	2.28	3.84	35	1.90
Affinity rinsed	18	6.75	122	1.15	5.87	12	2.91
eluted I	14	4.25	60	0.20	21.25	6	10.52
eluted II	7	18.25	128	1.08	16.90	12	8.37
eluted III	4	4.00	16	0.74	5.41	2	2.68

One unit is defined as 1 nmol of GSH conjugate of CDNB per minute at 25°C.

(0.9-fold); rat, 35% yield (1.9-fold). However, from houseflies relatively good results were obtained at the final step of purification by affinity chromatography. Three GST active fractions from R-fly: I (145-fold), II (7-fold) and III (3-fold); two fractions from S-fly: I (103-fold) and II (16-fold). On the other hand, three fractions from rat liver were obtained: I (11-fold), II (8-fold) and III (3-fold).

5. *Subunits of Various GSTs*

The results of SDS-PAGE are summarized in

Table 7 Comparison of the subunits of glutathione *S*-transferase from resistant (R) and susceptible (S) housefly abdomens and rat liver.

Origin	GST fraction	Subunit (*MW*)
Rat	I	Not visible
	II	28,000 (Yc) 25,000 (Ya)
	III	28,000
R-fly	I	>30,000 <25,000 <25,000 (Ya?)
	II	>30,000 <25,000 <25,000 (Ya?) 14,000
	III	<25,000
S-fly	I	Not visible
	II	>30,000 28,000 (Yc?) <25,000

Table 7. Fraction II from rat gave two bands which corresponded to Yc (28,000) and Ya (25,000) of the data of Jakoby *et al.*[10] Fraction II from R-fly gave four bands: first, at a little over 30,000; second and third, at a little below 25,000, one of which might be Ya; fourth, at about 14,000. From fraction II of S-fly, three bands were detected: two were the same as found from R-fly (at a little over 30,000 and a little below 25,000), but the remaining one corresponded to Yc (28,000) from rat. It has been well established that the cytosolic GST of rat liver are dimeric proteins that arise from the combination of four different subunits, Ya, Yc, Yb_1 and Yb_2, that is, YaYa, YaYc, YcYc, Yb_1Yb_1, Yb_1Yb_2, Yb_2Yb_2 isozymes (now called 1-1, 1-2, 2-2, 3-3, 3-4 and 4-4, respectively).[10-12] Although we could not detect all the subunits of rat GST in our experiments, comparison of the subunits suggested that housefly GST was qualitatively different from rat GST. There was a minor difference between GSTs of R- and S-flies.

Clark *et al.*[13,14] purified GSTs from several strains of housefly to a high degree of homogeneity by a procedure involving affinity chromatography. Molecular weight of the subunits was recorded as 20,000, 22,000 and 23,500, based on the subunit molecular weights Ya, Yb and Yc from rat liver GST assigned as of 22,000, 23,500 and 25,000 by Bass *et al.*[11] However, Jakoby *et al.* modified the molecular weights of the subunits Ya, Yb and Yc to 25,000, 26,500 and 28,000, respectively. Our subunit 28,000 from S-fly corresponded to Yc and seemed different from the subunit (23,500 =Yb) described by Clark *et al.* from S- and R-flies. Although the molecular weights of our subunits from houseflies seemed very close to Ya and Yc from rat liver GST. More detailed study will be required for the exact identification and for correlating our finding to selective toxicity.

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要　約

イエバエグルタチオン *S*-トランスフェラーゼのカルコンによる阻害およびそのアイソザイムのラットとの比較

宮本　徹，山本　出

4′-Phenylchalcone の 2-, 3-, 4-塩素置換体の抵抗性 (R) および感受性 (S) イエバエ腹部グルタチオン *S*-トランスフェラーゼ (GST) に対する阻害は弱かった．しかし，これらグルタチオン (GSH) 抱合体は両 GST を CDNB や DCNB に関し非拮抗的に強く阻害した．阻害活性はマウス肝 GST の場合と同様 2-, 4-, 3-塩素置換体の順であった．この事実は *in vitro* では GSH 抱合化が遅いことを示唆している．他方，上記カルコンは 2-, 4-, 3-塩素置換体の順で有機リン殺虫剤ダイアジノンの R-イエバエへの殺虫力を増強した．ダイアジノンは R-イエバエで GST により解毒されることが知られているので，このカルコンの共力効果は，カルコンが *in vivo* で GST により GSH 抱合体に変換された後 GST を阻害し，これによりダイアジノンの解毒を減少させたことによると推察される．ラットとイエバエ間，また R-と S-イエバエ間で GST に差異があることを認めた．すなわち，CDNB についてみると親和性は S-イエバエ GST に対するより R-イエバエ GST に対して強かったが，両者ともラット GST への親和性に比べかなり低かった．DCNB の各種 GST への親和性は CDNB のそれより一般に低く，イエバエ，マウス，ラット間であまり大きな差はなかったが，とくに R-イエバエ GST への親和性は低かった．イエバエとラットの GST は SDS-電気泳動の subunit からみて質的に大きく異なっていた．また R-, S-イエバエの GST 間では若干の違いが認められた．