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INDUCIBILITY OF VARIOUS CYTOCHROME P450 ISOZYMES BY PHENOBARBITAL AND SOME OTHER XENOBIOTICS IN *DROSOPHILA MELANOGASTER*

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Abstract—The inducibility of cytochrome P450 isozymes has been investigated in the *Drosophila melanogaster* insecticide susceptible (Oregon R) and insecticide resistant (91R) strains. Both the level and induction kinetics of 7-ethoxycoumarine *O*-deethylase activity were stimulated by phenobarbital (PB) to a lower extent than that of aryl hydrocarbon hydroxylase in the Oregon R strain. The basal level of the cytochrome P450-linked activities in insecticide resistant flies was higher than that noted in susceptible ones. However, treatment with PB has increased levels of 7-ethoxycoumarine *O*-deethylase and aryl hydrocarbon hydroxylase activities more in susceptible flies than in resistant ones. In contrast to PB, the polycyclic aromatic hydrocarbon benzo[*a*]pyrene induced both activities in 91R flies to a greater extent than in Oregon R ones. The potent PB-like inducer in mice but not in rats 1.4-bis[2-(dichloropyridyloxy)]-benzene failed to induce the cytochrome P450 system in *D. melanogaster*, when triphenyldioxane (PB-like inducer in rats but not in mice) markedly affected this system in a PB-like manner. The SDS–PAGE followed by immunoblotting analysis using monoclonal antibodies 13-2e and 8-1d have shown that the level of the 56,000 and 54,000 Da insecticide resistance-related forms has increased in the susceptible strain by PB and some other PB like inducers. The relationship between these isozymes appearance and 7-ethoxycoumarine *O*-deethylase activity has been discussed.

Key words: Drosophila melanogaster; cytochrome P450; isozymes; phenobarbital induction

The genetically well-studied *Drosophila melanogaster* offers an excellent opportunity to comprehend the mechanisms of cytochrome P450 forms inducibility. The presence of an inducible cytochrome P450 system has been established in *D. melanogaster*, as well as genetic variations in metabolism [1, 2] and multiplicity of P450 forms [3].

The IR† strains (91R and Hikone R) have been shown to have a high level of MFO activities compared to the susceptible (IS) wild-type ones [1, 4, 5]. The enhanced level of dealkylation activities toward dimethylnitrosamine, aminopyrine, benzphetamine and, probably, 7-ethoxycoumarine was reported to correlate with insecticide resistance and high constitutive level of 56,000 and (or) 54,000 Da hemoproteins [4–7].

At present a large subset of P450 protein products have been reported in *Drosophila*. The occurrence of three microsomal P450 isozymes of 55,000, 52,000 and 50,000 Da was reported by Naquira *et al.* in a wild-type strain [3], whereas Hällström *et al.* [4] identified five microsomal hemoproteins of 58,000, 56,000, 54,000, 52,000 and 51,500 Da in flies of another IS strain. Two P450 subsets containing a number of forms with apparent molecular mass of 59,000 (P450A) and of 56,000 Da (P450B) were also reported by Waters and co-workers [5, 6]. The present paper deals with the inducibility of cytochrome P450-linked MFO activities and of cytochrome P450 forms by PB-like mammalian inducers in the IS and IR strains of *D. melanogaster*.

MATERIALS AND METHODS

Drosophila *strains and treatment.* The wild type Oregon R strain of *D. melanogaster* was obtained from the stock collection of Moscow State University. The DDT resistant 91R strain [8] was kindly provided by Dr F. E. Würgler (University of Zurich, Switzerland).

Flies were raised in the standard vials (75 mm long and 22 mm in diameter) containing 10 mL of standard nutrient medium (12.5 g agar, 96 g unrefined sugar, 24 g yeast, 72 g semolina and 4 mL of propionic acid per liter) at 12 hr light/dark cycle at 24 ± 1°.

Enzyme induction was performed by adding the inducing compounds into the nutrient medium. Agents were dissolved in DMSO and the stock solutions were thoroughly mixed with warm nutrient medium (50°). The final concentration of DMSO in the medium was less than 4%. Flies (5–6 days old) were fed with the medium containing inducers during 20 hr for BP, 24–72 hr for PB and 72 hr for other inducers. Flies of control groups were fed with the

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† Abbreviations: AHH, aryl hydrocarbon hydroxylase; BP, benzo[a]pyrene; TCPOBOP, 1.4-bis[2-(dichloropyridyloxy)] benzene; ECOD, 7-ethoxycoumarin *O*-deethylase; IR, insecticide resistant; IS, insecticide susceptible; MFO, mixed function oxidase; MoAbs, monoclonal antibodies; PB, phenobarbital; TPD, triphenyldioxane; TSO, *trans*-stilbene oxide; DDT, dichlorodiphenyltrichloroethane.

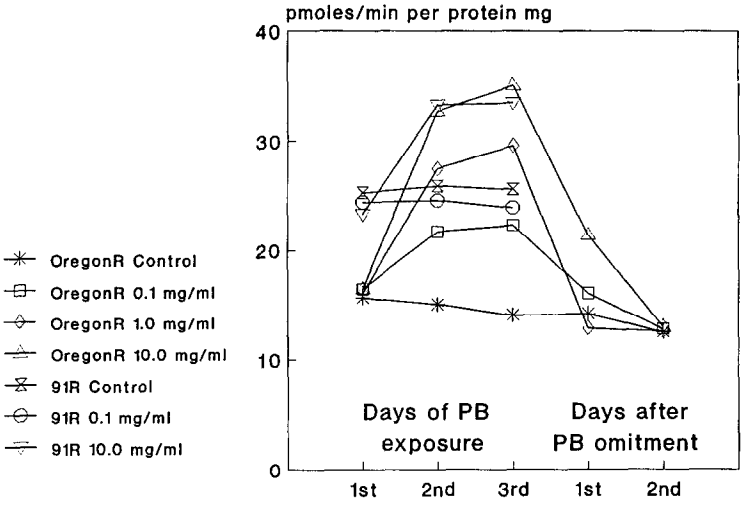

Fig. 1. The kinetics of ECOD activity in homogenates from 91R and Oregon R flies treated with PB. Data were measured as pmol of 7-hydroxycoumarin formed/min per mg protein; each value represents mean of at least four separate experiments.

medium containing a comparable concentration of DMSO.

Chemicals. The following inducers of cytochrome P450 were used: sodium PB and BP from Fluka AG (Buchs, Switzerland) TSO from the Sigma Chemical Co. (St Louis, MO, U.S.A.) TPD and TCPOBOP (both agents of 99.9% purity) were obtained from Dr Yu. Yu. Klyatsky at the Center of Development and Introduction of Molecular Diagnostic Methods (Moscow, Russia). MoAbs 13-2e and 8-1d were a generous gift of Dr L. C. Waters (Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.). 3,3-Diaminobenzidine was also from Sigma. Affinity-purified horseradish peroxidase-conjugated goat anti-mouse IgG were the commercial products of the Institute of Microbiology, Russian AMS (Moscow, Russia). All other chemicals and reagents were of the highest quality available.

Microsome preparation and enzyme assays. The whole body homogenates in 0.05 M Tris–HCl (7.5) with 150 mM KCl were used as a source of enzyme in the experiments with various dose and terms of PB exposure. In the other experiments, microsomes were isolated from imagoes whole body homogenates as described elsewhere [9]. The microsomal pellets resuspended in 0.05 M Tris–HCl buffer, pH 7.5, were kept on ice and used for further assays within 1–1.5 hr after preparation.

Measurement of the microsomal protein content was performed as described by Albro [10]. The Hitachi-850 spectrofluorometer (Japan) was used in the enzyme activities measurement. The AHH activity was measured fluorometrically as described by Dehnen *et al.* [11]. The method described by Patil *et al.* [12] was used for the measurement of ECOD activity. Both reactions were performed at +25°.

SDS–PAGE and immunoblotting of microsomal proteins. SDS–PAGE of microsomal proteins was performed as described by Laemmli [13] using constant current of 19 mA. A total of 20 μg microsomal protein was applied to each well. The stacking gel of 1 mm thickness contained 4.5% and the separating gel 10% polyacrylamide. Five hundred micrograms of microsomal protein were loaded on to enable identification of hemoprotein containing areas [14].

Gels were either stained with Coomassie brilliant blue or subjected to the immunoblotting procedure as described [15]. Briefly, proteins were transferred to 0.45 μm nitrocellulose (Schleicher & Schuell, NH, U.S.A.) in a Transblot apparatus (Bio-Rad) at 0.6 A for 12 hr. The MoAbs 13-2e or 8-1d were diluted 100-fold in 5% (w/v) nonfat dry milk in PBS and incubated with the membrane for 3 hr at 37° on a rocking platform. After a number of incubation and washing steps [15] the blot was then stained with the 0.25 mM solution of 3,3-diaminobenzidine in PBS with 0.01% H_2O_2.

A densitometric analysis of the gels and immunoblots to quantify the cytochrome P450 forms profile were performed with LKB densitometer.

RESULTS

The data of kinetics of MFO induction by PB in IS and IR flies are summarized in Figs 1 and 2. In the Oregon R strain, ECOD activity increased only on the second day of induction and reached its maximum on the same day. This effect was dose dependent and exhibited a significant increase of up to 2.5 times when compared with the controls. When the inducer was omitted, ECOD activity dropped to the control level within 1 day, irrespective of the PB concentration in the former diet (Fig. 1).

The inducibility of ECOD activity in homogenates from 91R flies was lower than in Oregon R ones. The marginal but statistically significant increase in this activity (1.35 times control) was observed only

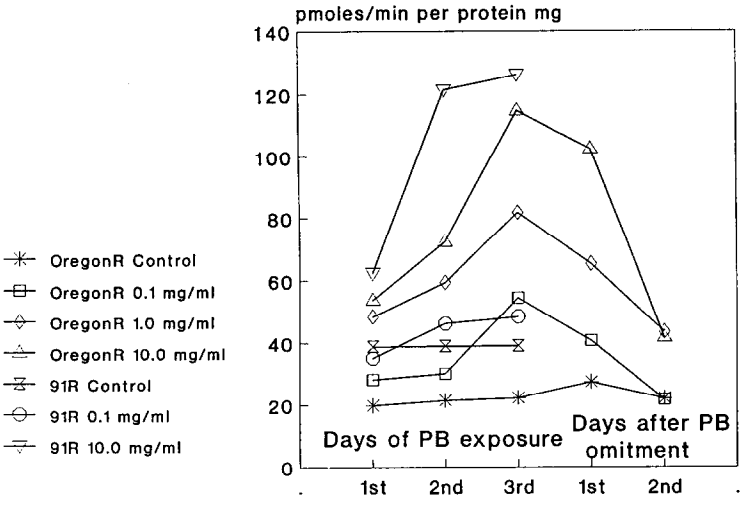

Fig. 2. The kinetics of AHH activity in homogenates from 91R and Oregon R flies treated with PB. Data were measured as pmol of 3-hydroxybenzo[a]pyrene formed/min per mg protein; each value represents mean of at least four separate experiments.

Table 1. The effect of various inducers on the microsomal MFO activities in *Drosophila melanogaster*

Strain	Treatment	No. of experiments	ECOD activity*	AHH activity†
Oregon R	Control	9	47 ± 1	144 ± 7
	PB 1 mg/mL‡	7	107 ± 4§	504 ± 27§
	TSO 10 mg/mL‡	4	105 ± 6§	348 ± 18§
	DBP 0.1 mg/mL‡	4	52 ± 6	180 ± 27
	TPD 0.1 mg/mL‡	4	104 ± 4§	529 ± 53§
	BP 0.2 mg/mL‖	4	56 ± 5	226 ± 18
91R	Control	7	105 ± 4	233 ± 15
	PB 1 mg/mL	8	142 ± 11¶	514 ± 13¶
	BP 0.2 mg/mL	6	232 ± 24¶	447 ± 19§

* Measured as pmol of 7-hydroxycoumarin formed/min per mg protein, each value represents mean ± SE.

† Measured as pmol of 3-hydroxybenzo[a]pyrene formed/min per mg protein, each value represents means ± SE.

‡ Treatment for 72 hr.

§ $P < 0.001$ to controls in the Student's *t*-test.

‖ Treatment for 20 hr.

¶ $P < 0.01$ to controls in the Student's *t*-test.

at the highest dose of PB by the second day of exposure (Fig. 1).

A different type of induction kinetics was observed in the case of AHH. In Oregon R flies, induction of this activity began earlier, at the first day of PB feeding and increased for the next 72 hr, until PB was removed. The inducibility of AHH was higher overall than ECOD and it reached a level of 5.2 times over the control after a 3-day exposure to the highest (10 mg/mL) concentration of PB. The residual enhanced level of this activity maintained for at least 2 days subsequent to the transfer of flies on the PB-free diet (Fig. 2). In 91R flies, AHH activity was significantly induced only by a high dose of PB (10 mg/mL) reaching a peak which was 4.35 times of the control by the second day of exposure (Fig. 2).

The effects of different mammalian cytochrome P450 2B subfamily inducers and BP as measured in microsomes MFO activities in *Drosophila* are shown in Table 1. Compared to the IS Oregon R strain, the DDT-resistant 91R strain was found to have 2.2 times and 1.6 times higher the constitutive levels of ECOD and AHH activities, respectively. This is in general agreement with a number of previous studies [1, 4, 6] and with the data obtained for MFO activities measured in homogenates (Figs 1 and 2). Treatment with 1 mg/mL PB caused 2.3-fold and 1.3-fold

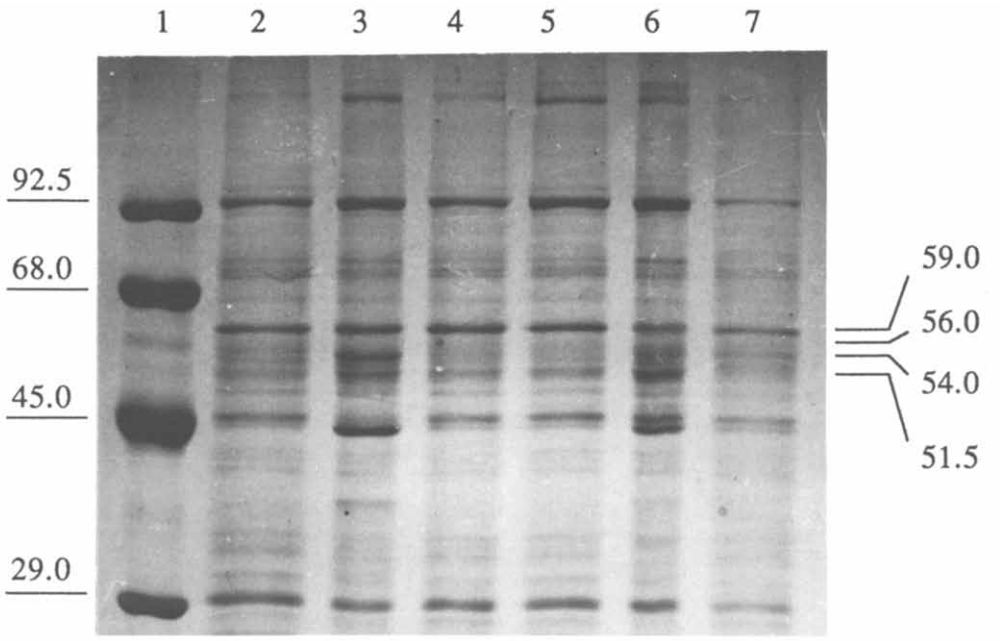

Fig. 3. SDS–PAGE analysis of microsomal proteins from flies of two *Drosophila* strains. Each well was loaded with 20 μg of microsomal protein. Lane 1, molecular mass standards; lane 2, Oregon R, control; lane 3, Oregon R, PB (1 mg/mL); lane 4, Oregon R, TSO (10 mg/mL); lane 5, Oregon R, DPB (0.1 mg/mL); lane 6, Oregon R, TPD (0.1 mg/mL); lane 7, 91R, control. The proteins were stained with Coomassie brilliant blue.

respectively. Other cytochrome P450 2B subfamily inducers TPD [16] and, to a lesser degree, TSO [17] induced MFO activities in IS flies in a similar manner as PB. On the other hand, TCPOBOP [18] failed to increase these enzyme activities (Table 1). A reversed responsiveness to induction by PB and BP was observed in susceptible and resistant strains. PB caused a more effective increase in activity in the IS Oregon R strain, compared with BP which was more potent in IR 91R one, especially in ECOD induction (Table 1).

Proteins with apparent molecular masses of 59,000, 56,000, 54,000 and 51,500 Da were found in the hemoprotein-containing area of gels after SDS–PAGE analysis of *D. melanogaster* microsomes (data not shown). A marked increase in the intensity of proteins having molecular masses of 56,000 and 54,000 Da was noted in microsomes from untreated 91R compared with those of Oregon R control (compare lanes 2 and 7, Figs 3 and 4). The treatment of IS flies with PB essentially increased the protein content of three bands with apparent molecular masses of 56,000, 54,000 and 51,500 Da. A similar effect by TPD was also observed. TSO treatment caused a slight but reproducible increase in the 56,000- and 54,000-protein content. No alteration in the electrophoretic profile of microsomal proteins was observed in IS flies treated with TCPOBOP.

In order to determine the expression profile of *Drosophila* cytochrome P450, immunoblotting

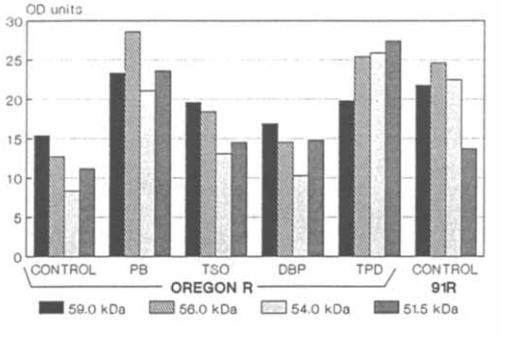

Fig. 4. The average densitometric data obtained by duplicate tracing at 550 nm of at least two gels with SDS–PAGE-resolved microsomal proteins from *Drosophila* (see gel in Fig. 1).

experiments were performed (Figs 5 and 6). Immunoblots of SDS–PAGE-resolved microsomal proteins of Oregon R and 91R strains with MoAbs 13-2e or 8-1d recognizing P450A or P450B subsets, respectively [7], revealed cytochrome P450 forms comigrating with 59,000-, 56,000-, 54,000- and 51,500-protein bands (Figs 5 and 6). In some experiments, the P450 form with molecular mass of 52,000 Da was also observed (Fig. 6). MoAbs 13-2e were specific for 59,000-protein band (Fig. 5). These observations are in accordance with those obtained

Fig. 5. Immunoblot of SDS–PAGE-resolved microsomal proteins from flies of two *Drosophila* strains. Each well contained 20 μg of microsomal protein. Lane 1, Oregon R, control; lane 2, Oregon R, PB (1 mg/mL); lane 3, Oregon R, TSO (10 mg/mL); lane 4, Oregon R, DPB (0.1 mg/mL); lane 5, Oregon R, TPD (0.1 mg/mL); lane 6, 91R, control; lane 7, 91R, PB (1 mg/mL). The entire blot was probed with 1:100 diluted MoAbs 13-2e.

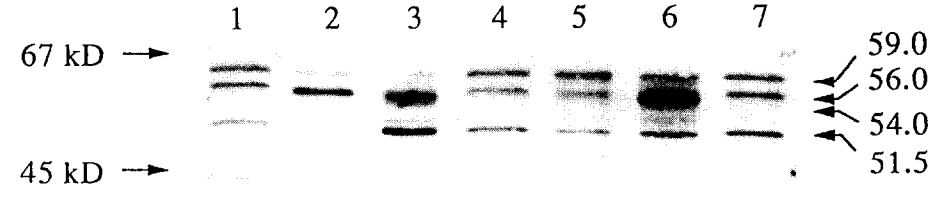

Fig. 6. Immunoblot of SDS–PAGE-resolved microsomal proteins from flies of two *Drosophila* strains. Each well contained 20 μg of microsomal protein. Lane 1, 91R, PB (1 mg/mL); lane 2, 91R, control; lane 3, Oregon R, TPD (0.1 mg/mL; lane 4, Oregon R, DPB (0.1 mg/mL); lane 5, Oregon R, TSO (10 mg/mL); lane 6, Oregon R, PB (1 mg/mL); lane 7, Oregon R, control. The entire blot was probed with 1:100 diluted MoAbs 8-1d.

by Sundseth *et al.* [7]. As to MoAbs 8-1d, they recognized epitopes on the hemoproteins with molecular masses of 56,000, 54,000, 52,000, 51,500, and also with 59,000 Da in microsomes from both strains (Fig. 6).

The effects of various inducers on the cytochrome P450 isozymes profile observed in the immunoblots were similar to those in SDS–PAGE analysis (Figs 3 and 4). Comparing the basal level of microsomal cytochrome P450 forms in 91R with those in Oregon R, a consistent difference was found between the two strains. The content of isozymes of 56,000 and 54,000 was essentially higher in IR strain. Treatment of flies with PB, TPD and, to a lesser extent, with TSO, but not with TCPOBOP, resulted in a significant induction of these forms in IS strain. PB or TPD treatment also caused an increase in 51,500-protein content (Fig. 6). Comparison of the cytochrome P450 profiles in the untreated 91R flies with those in the PB treated ones revealed that PB did not induce P450 isozymes of 56,000 and 54,000 in the IR strain in contrast to IS one. Treatment with this agent resulted in the induction of hemoproteins with molecular masses of 59,000 and 51,500 Da in the latter strain (compare lanes 1 and 2, Fig. 6). However, when the blots were treated with MoAbs 13-2e, 59,000-protein content was not enhanced (compare lanes 6 and 7, Fig. 5).

DISCUSSION

It is believed that various forms of cytochrome P450 differ in their activities toward various substrates [19]. In human, the main (but not specific) sources for AHH and ECOD activity are 1A1 and 2A6 P450 forms, respectively [20]. Different types of induction kinetics observed for AHH and ECOD activities in the IS strain (Oregon R) treated with PB may be evidence for the suggestion that different inducible cytochrome P450 isozymes by PB are mostly responsible for AHH and ECOD measured activities. This is supported by the lack of correlation between AHH and ECOD constitutive activities in 11 strains of *D. melanogaster* reported by Hällström and Blanck [21].

A number of studies, including the present data, indicate a higher inducibility of the cytochrome P450 system by PB in susceptible insects compared to IR ones [1, 4, 22–24]. Because the PB molecule by itself, but not by its derivatives induces MFO enzymes in mammals [25], it is possible that PB fails to induce this system in IR strains due to its enhanced degradation by the high basal level of MFO activities, as it was assumed previously [22–24]. A higher PB dose required to induce AHH in 91R flies (Fig. 2) may support this suggestion. Further evidence comes from a finding that, in Oregon R flies, the content of cytochrome P450B (56,000- and 54,000-proteins) and ECOD activity can be induced to, but not above, the level in 91R ones (Figs 1 and 4, Table 1).

In contrast to PB, BP has been shown to be a more effective inducer of this system in IR 91R strain then in the wild-type IS Oregon R one (Table

1). We have recently found that the insecticide resistance-related P450 isozyme with molecular mass of 56,000 Da, not induced by PB (Fig. 6), was essentially increased after treatment of 91R but not Oregon R flies with BP*. This may be attributed to initial steps of MFO induction which are different with PB and BP. In contrast to PB [25], polycyclic aromatic hydrocarbons induce this system by their metabolites formed by the basal MFO activity [26]. Thus, if the same is true for *Drosophila*, the high level of MFO activity in IR flies will result, at least partially, in decrease of the PB level and in increase of the level of BP derivatives with inducing activity.

Very intriguing were the different effects of TPD and TCPOBOP on the Oregon R cytochrome P450 system. TCPOBOP is known to be a potent inducer of the cytochrome P450 2B subfamily (CYP2B) forms in mice, but not in rats [18]. TPD, in contrast, has been demonstrated to be a strong inducer of these isozymes in rats, but not in mice [16]. In the present study, TCPOBOP failed to affect the cytochrome P450 system in Oregon R (Table 1, Figs 3, 4 and 6), leading us to assume that the MFO system inducibility in *D. melanogaster* is more similar to that in rats than in mice. This finding might be taken into consideration in connection with the screening of genotoxic procarcinogens (including drugs) in *Drosophila* assays, because there are a lot of examples demonstrating differences in carcinogenic activities of chemicals in these two rodent species [27].

The SDS–PAGE and immunoblotting analyses of microsomes from IS flies have shown that PB, TPD and, to a lesser degree, TSO induced the P450 isozymes of 56,000 and 54,000 Da which are constitutively enhanced in IR ones. The relationship between the elevated expression of 56,000- and (or) 54,000-hemoproteins and resistance to pesticides has been previously reported by Sundseth *et al.* [28] and Hällström and co-workers [4, 21], respectively. Thus, PB-like inducers increased the content of insecticide resistance-related cytochrome P450 forms in susceptible strain. The present data are in line with those obtained in houseflies by Wheelock and Scott [29], who reported that PB induced the pyrethroid resistance-related cytochrome $P450_{ipr}$ in the IS strain of *Musca domestica*.

It is also likely that these forms are involved mainly in the level of measured ECOD activity. This activity has been shown to increase in malathion resistant strain of *D. melanogaster* [12]. Our suggestion may also be indirectly supported by the data showing the lack or failure of induction of 56,000- and 54,000-hemoprotein content and ECOD activity by PB in 91R (Table 1, Fig. 6). Waters *et al.* [30] have found that mRNA transcript of resistance-related P450B gene (CYP6A2) contains regions determining the instability of mRNA in the wild-type strain of *D. melanogaster* 91C, but not in 91R. This probably might explain the rapid drop of ECOD activity after omission of PB in IS flies (Fig. 1).

In 91R flies, PB treatment caused an increase in the content of 59,000-protein recognized by MoAbs 8-1d (Fig. 6), but not by MoAbs 13-2e (Fig. 5). Thus, this band may represent different proteins. Further studies of cytochrome P450 isozymes multiplicity in this species, as well as mechanisms of their inducibility and the role in metabolism of different xenobiotics and endogenous substrates seems to be expedient.

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