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STUDIES ON METABOLIC ACTIVATION OF VINYL CHLORIDE IN
DROSOPHILA MELANOGASTER AFTER PRÉ-TREATMENT WITH
PHENOBARBITAL AND POLYCHLORINATED BIPHENYLS

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SUMMARY

It is known that vinyl chloride is metabolized by the mixed function oxygenase system in the liver to reactive mutagenic and carcinogenic metabolites. This metabolic activation was studied in *Drosophila melanogaster* by measuring the uptake of ^{14}C from labelled vinyl chloride in different strains and with different pretreatments with phenobarbital and polychlorinated biphenyl (PCB) (Clophen A50), well known inducers of cytochrome P-450. In accordance with previously obtained data on vinyl chloride induced sex linked recessive lethals, it was shown that pretreatment with inducers increased the uptake of labelled compound up to ten times. There was, however, a marked difference in response between the five strains used. In particular, the strain Hikone, known to be resistant to insecticides, had a comparatively high initial radioactivity from vinyl chloride without any pretreatment, but it was not or insignificantly inducible with phenobarbital or PCB. Crosses between Hikone and an inducible strain indicated essentially a dominance for the Hikone genotype. Tests on inducible strains showed the same response to phenobarbital by 2 h old larvae and adult males and females. Dimethylsulphoxide (DMSO) used as a solvent decreased both the initial uptake of ^{14}C and particularly the induction by PCB. The use of Tween 80 as an emulsifier did not have such an effect.

It is emphasized that the interstrain variation in metabolic activation and inducibility has to be taken into consideration in order to optimize the use of *Drosophila* for mutagenicity testing. This variation also opens up new possibilities of analyzing the mixed function oxygenase system biochemically and genetically.

Abbreviations: DMSO, dimethylsulphoxide; PCB, polychlorinated biphenyl.

INTRODUCTION

The screening for mutagenic effects by chemicals, involves two principal problems which have to be considered when developing test protocols. The first concerns the necessity of covering various kinds of mutagenic effects at the chromosome and gene level, and the second relates to the metabolic conversion which occurs in living organisms. The fact that investigations of mutagenicity usually aim at an evaluation of possible risks to humans, the metabolism occurring in the mammalian body and especially the human body with necessity gets into the focus of attention. This aspect of mutagenicity testing is all the more important as most known carcinogenic chemicals are inactive by themselves and require a metabolic activation before becoming carcinogenic. The wide use of short term mutagenicity tests for the screening also of carcinogenic properties, which is based on the close correlation between induction of mutations and cancer, requires a system which at least imitates the mammalian metabolism. Unfortunately the genetic test systems best suited for genetic screening are seldom appropriate from the point of view of metabolism and therefore a number of combined test methods have been developed, in which mutations are measured in one organism but the metabolic conversion of a mammalian system is introduced in some way. The best known systems in this respect have been the host mediated assay, developed by Garbridge and Legator [1] and Ames' test system on *Salmonella* with the addition of mammalian liver microsomes [2].

Although particularly the bacterial tests developed by Ames have turned out to be a powerful tool to discover also indirectly acting mutagens and carcinogens, a serious drawback is the fact that the genetic system used does not represent the eukaryotic level of chromosomal organization. Important genetic effects like chromosome aberrations and alterations of the normal chromosome number are not accessible for investigation with such a bacterial system. Also at the gene level there are differences between prokaryotes and eukaryotes in the organization of DNA, although we may at this point and time not be able to fully evaluate the consequence of such a difference when considering screening for mutagenic and carcinogenic properties.

Being the classical material for genetic research *Drosophila melanogaster* appears as a particularly prominent test organism, which combines an eukaryotic organization with a unique range of test systems. The suitability of *Drosophila* from a genetic point of view for mutagenicity screening is well known and has been emphasized by many authors, e.g. Sobels, Vogel, Würzler and Zimmering [3-8]. But also concerning the other aspect of mutagenicity screening, pointed out above that is, the metabolic conversion of chemicals, *Drosophila* has turned out to be remarkably useful. Carcinogenic compounds which are known to require a metabolic activation in mammals by the microsomal enzyme system, have been shown to be active in mutagenicity tests in *Drosophila* [9,10]. This is in accordance with the demonstration that insects have the ability of oxidatively metabolizing

chemicals by means of membrane bound microsomal enzymes in a similar way as mammals (for reviews see Casida [11] and Wilkinson and Brattsten [12]).

The presence in *Drosophila* of cytochrome P-450, the terminal electron acceptor of the mixed function oxidase system in the microsomes has been demonstrated by Hodgson [13,14] and Baars et al. [15]. Baars et al. furthermore demonstrated the occurrence of aryl hydrocarbon hydroxylase and studied the activity of this enzyme in three strains of *Drosophila*.

In a previous study [16] we have shown that the mixed function oxidase system can be induced by phenobarbital in the same way as in mammals. This was done by measuring sex linked recessive lethals produced by vinyl chloride, which is known to be metabolized by this enzyme system to mutagenic and carcinogenic compounds [17-20]. The inducibility of the detoxification enzymes does not only indicate an interesting parallel to the mammalian metabolism, but it also opens possibilities to analyze the enzymatic drug metabolism genetically and biochemically in *Drosophila*.

In this paper we report further investigations of the metabolic conversion of vinyl chloride in *Drosophila* with special reference to the induction of the metabolizing enzymes. The experimental system is based on the occurrence of non-volatile ^{14}C -labelled metabolites in the flies from the distribution of labelled vinyl chloride.

MATERIALS AND METHODS

Three wild type strains were used, Karsnäs 60, Berlin and Hikone. The last strain was obtained from Dr. E. Vogel, which we gratefully acknowledge. Tests were also made with one strain carrying a white mutation and one carrying white-apricot. All the strains used were kept in mass cultures on ordinary corn agar substrate.

^{14}C -labelled vinyl chloride dissolved in orthoxylene was supplied from New England Nuclear, Boston, Mass. It had a specific activity of 0.48 mCi/mM and 99% of the labelling was on vinyl chloride. Not labelled vinyl chloride (99.5% purity) of the same batch as used in previous experiments [16] was used for the present experiments. Clophen A50 (Bayer, G.F.R.) is a mixture of PCBs. The types of PCB molecules present have been analyzed by Jensen and Sundström [21]. Phenobarbital (sodium barbiturate) was obtained from ACO, Stockholm, Sweden, Tween 80 (polyoxyethylene sorbitan monooleate) from Hopkin and Williams, England and DMSO from Merck, Darmstadt, G.F.R. (spectrographic purity 99.7%). Pretreatment of flies for induction with phenobarbital and PCB was performed in 13 ml glass vials. 0.5 ml of the solution were given to the flies on a piece of tissue paper (Kleenex) in the bottom of the vials.

Phenobarbital was dissolved in a 1% solution of sucrose, while Clophen was dissolved in DMSO or emulgated in Tween 80 before being mixed in a similar sucrose solution. The controls were handled in the same way but only given 1% sucrose. Pretreatment with the inducers and the sucrose solution

was given to the flies for 24 h before the exposure to vinyl chloride. Before this pretreatment the flies were kept in empty vials for about 4 h in order to increase their consumption of liquid. The treatment with vinyl chloride was performed for 3 h in a 2-l three necked round-bottom flask (Fig. 1). The concentration of vinyl chloride in the air was 1%, consisting of 0.1% ^{14}C -labelled compound and the rest cold vinyl chloride. The procedure of treatment with the vinyl chloride was as follows (the method principally according to Osterman-Golkar et al. [22]). A test tube containing 1 ml ^{14}C -labelled vinyl chloride dissolved in xylene was connected to a tube containing a stop-cock (Fig. 1). At the other end this tube was connected to one of the necks of the flask. The air of the flask was evacuated to about 10 mm Hg. When the stop-cock between the test tube and the flask was opened the low pressure in the flask caused the vinyl chloride to boil and pass into the flask as a gas. Air was let into the flask and through a rubber membrane on another of the necks of the flask cold vinyl chloride was injected to a final concentration of vinyl chloride in the flask of about 1%. The flies were kept in glass tubes with gauze at both ends. These tubes were then inserted into the flask through the third neck.

In the experiment with larvae these were collected at an age of 2 ± 2 h. They were treated with phenobarbital in a 1% sucrose solution or only the sucrose solution for 48 h. The treatment procedure both with phenobarbital and vinyl chloride was the same for the larvae as the adult flies.

After the treatment with vinyl chloride the flies or the larvae were killed and dried in room temperature for 24 h. They were thereafter mashed with a glass pistil in scintillation vials and 2 ml of ethanol was added. Before counting in the scintillation counter 2 ml of scintillation fluid (instagel) was added. In most cases 20 flies were put in each scintillation vial and counted in the scintillator for 40 min. The results in the tables are presented as counts/min/fly or larva. The values have been corrected for quenching efficiency by means of an external standard.

RESULTS

The exposure of the flies to labelled vinyl chloride was performed in four sets of experiments and the data pertaining to the different experiments are indicated in the tables and figures. In all the experiments it is clear that phenobarbital treatment causes an increase of the uptake of vinyl chloride. There is, however, an important exception for the strain Hikone (Tables I, II and Fig. 1). This strain has initially a higher uptake of vinyl chloride than any of the other strains tested, as indicated by the controls using only sucrose. The uptake of vinyl chloride in Hikone, however, is unaffected by phenobarbital even with a 10-fold higher dose of phenobarbital than the highest dose tested with the other strains (Fig. 1). The other strains are all inducible, but the response varies between strains. Thus in both experiments Berlin gives a higher response than Karsnäs 60, and it is also higher than the white and white-apricot strains in Expt. 2, shown in Table II.

TABLE I

UPTAKE OF ¹⁴C-LABELLED VINYL CHLORIDE IN MALES OF THREE WILD TYPE STRAINS, KARSNÄS 60, BERLIN AND HIKONE (Expt. 1)

Pretreatment on 1% sucrose, phenobarbital dissolved in 1% sucrose or PCB dissolved and emulgated in DMSO or Tween 80

No.	Treatment				Counts/min/fly in strains		
	Clophen (%)	DMSO (%)	Tween 80 (%)	Phenobarb. (%)	Karsnäs 60	Berlin	Hikone
1	0.2	0.8			5.09	2.85	5.82
2	0.05	0.2			3.39	2.93	11.64
3		0.8			0.92	1.25	6.38
4	0.2		0.4		27.22	16.15	9.50
5	0.05		0.1		17.30	16.75	9.34
6			0.4		2.48	3.96	9.76
7				1.0	13.17	16.04	6.43
8				0.5	15.50	14.04	5.68
9				0.1	15.90	21.25	6.60
10					2.32	3.43	10.06

To study the dose effect of phenobarbital the data from both experiments are combined in Fig. 1. Although the treatment conditions may not have been quite identical for a number of reasons, the fact that the overlapping data between the two experiments on controls with only sucrose and on 0.1% phenobarbital give rather similar counts, may justify that both sets of results are dealt with in the same context, as in Fig. 1. The results indicate that an optimal induction is obtained around 0.1% phenobarbital, whereafter there seems to be a decrease at 0.5%.

TABLE II

UPTAKE OF ¹⁴C-LABELLED VINYL CHLORIDE IN MALES OF FIVE STRAINS, KARSNÄS 60, BERLIN, HIKONE, WHITE AND WHITE-APRICOT (Expt. 2)

Pretreatment on 1% sucrose, phenobarbital dissolved in 1% sucrose. Clophen emulgated in Tween 80.

No.	Treatment		Counts/min/fly in strains				
	Clophen	Phenobarb.	Karsnäs 60	Berlin	Hikone	Sw-a	Sw-a
1	0.2		22.23	23.51	22.88	33.83	29.49
2	0.02		4.94	6.10	14.16	4.84	4.15
3		10.0			7.54		
4		3.0			11.90		
5		0.1	16.57	26.96		7.39	15.47
6		0.02	8.48	18.57		5.40	8.45
7		0.005	2.95	4.14			
8			2.07	1.78	9.55	1.85	2.60

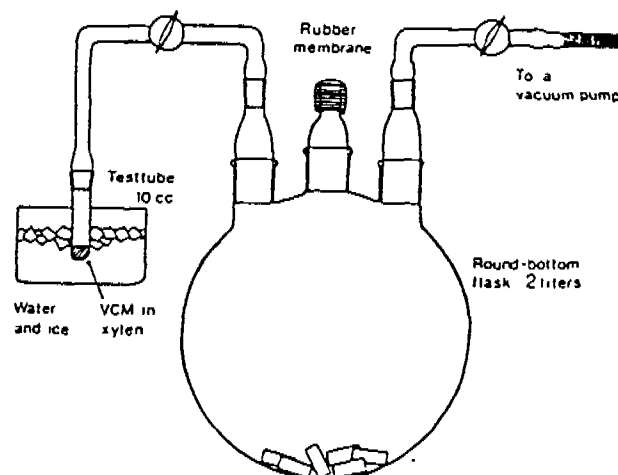


Fig. 1. Experimental set up for treatment of *Drosophila* with ^{14}C -labelled vinyl chloride. In the bottom of the flask the tubes with flies are shown. See further in the text.

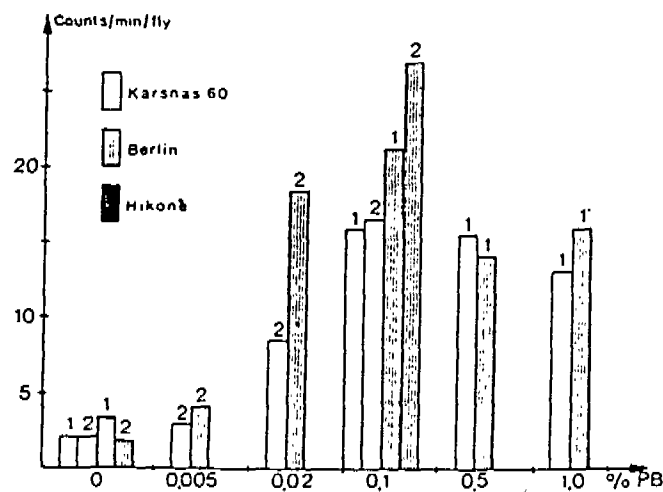
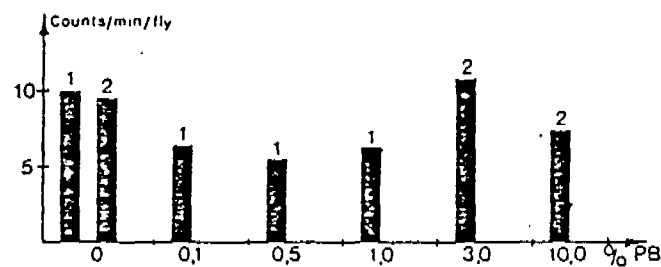


Fig. 2. The effect of pretreatment with phenobarbital on the uptake of ^{14}C -labelled vinyl chloride. Data pertaining to Expts. 1 and 2 are indicated in the columns in the figure.

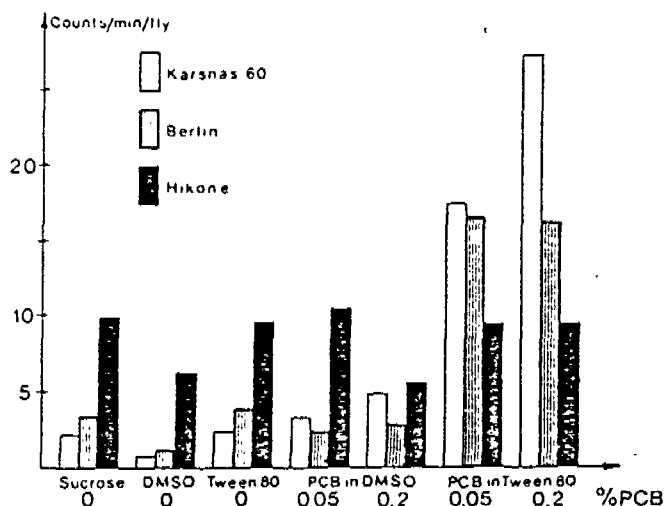


Fig. 3. The effect of pretreatment with PCB (Clophen A50) dissolved in DMSO or emulgated in Tween 80, on the uptake of ^{14}C -labelled vinyl chloride. Data from Expt. 1.

The situation with PCB is similar. In Expt. 1 (Table I and Fig. 3) an increase of the uptake of vinyl chloride is induced by PCB for Karsnäs 60 and Berlin, but again Hikone seems unaffected. In Expt. 2, however, some increase in counts at least with the highest dose of PCB can be discerned for Hikone, which may indicate that Hikone is not totally uninducible. Among the other four strains tested in Expt. 2 it may be pointed out that a particularly high level of induction is indicated for the white strain at the highest dose level of PCB.

The use of solvents with PCB revealed some unexpected effects. As shown in Table I and Fig. 3, DMSO itself suppresses the uptake of vinyl chloride in all the strains and evidently also eliminates the inducing action of PCB. The emulgator Tween 80 apparently did not have such an effect.

The use of the uptake of vinyl chloride as an indication of a metabolic conversion of vinyl chloride to one or more metabolites is based on the low reactivity of vinyl chloride itself as compared to these metabolites. In order to establish that the labelling of the flies depends on metabolites either covalently bound to proteins or nucleic acids or as conjugates or simple non-volatile metabolites and not vinyl chloride as such, the retention of labelling in the flies and the excretion was followed in one experiment. The excretion of non-volatile metabolites will result in a gradual shift of labelling from the flies to the substrate, but the total amount will be roughly constant. If vinyl chloride, which is volatile, is involved there will be a net decrease of the total labelling of the flies and the substrate. The result of this experiment is presented in Table III. During 24 h between one half and two thirds of the labelling disappeared from males of Karsnäs 60 and Berlin. At the same time, however, there was a corresponding increase of labelling of the substrate. The total amount of labelling remained constant, indicating that the para-

TABLE III

UPTAKE AND EXCRETION OF ¹⁴C-LABELLED VINYL CHLORIDE IN MALES OF THE WILD TYPE STRAINS KARSNÄS 60 AND BERLIN WITH AND WITHOUT PRETREATMENT WITH 1% PHENOBARBITAL, DISSOLVED IN 1% SUCROSE (Expt. 3).

Time after treatment	Phenobarb.	Counts min/fly in strains Karsnäs 60			Berlin		
		Excreted	Residue in flies	Total	Excreted	Residue in flies	Total
0	+	—	10.95	10.95	—	12.05	12.05
4	+	9.59	6.30	15.89	5.38	6.36	11.74
8	+	10.85	4.48	15.33	5.26	5.08	10.34
12	+	12.19	3.05	15.24	6.77	4.57	11.34
24	+	14.19	3.43	17.62	8.64	5.06	13.70
0	—	—	2.57	2.57	—	2.58	2.58
4	—	0	1.90	1.90	0.26	1.98	2.24
8	—	0	1.40	1.40	0.13	2.44	2.57
12	—	0.70	1.12	1.82	0.95	2.34	3.29
24	—	—	2.05	2.05	0.81	1.80	2.61

TABLE IV

UPTAKE OF ¹⁴C-LABELLED VINYL CHLORIDE IN FEMALES OF THE WILD TYPE STRAINS KARSNÄS 60 AND BERLIN WITH AND WITHOUT PRETREATMENT WITH 1% PHENOBARBITAL DISSOLVED IN 1% SUCROSE (Expt. 4).

Phenobarb.	Counts/min/fly in strains	
	Karsnäs 60	Berlin
+	6.20	13.72
—	1.01	1.96

TABLE V

UPTAKE OF ¹⁴C-LABELLED VINYL CHLORIDE OF LARVAE OF THE STRAINS KARSNÄS 60, BERLIN AND HIKONE WITH AND WITHOUT PRETREATMENT WITH PHENOBARBITAL IN 1% SUCROSE SOLUTION FOR 48 H (Expt. 3).

Phenobarb. (%)	Counts/min/larvae in strains		
	Karsnäs 60	Berlin	Hikone
0.2	34.00	40.09	45.15
0.02	20.56	14.07	42.34
0	15.36	8.67	37.75

TABLE VI

UPTAKE OF ¹⁴C-LABELLED VINYL CHLORIDE IN MALES FROM RECIPROCAL CROSSES OF THE WILD TYPE STRAINS BERLIN AND HIKONE (Expt. 3).

Cross	Counts/min/fly	
	Treated with phenobarb.	Not treated with phenobarb.
Berlin x Berlin	87.69	8.75
Berlin x Hikone	29.78	21.66
Hikone x Berlin	53.12	29.96
Hikone x Hikone	35.15	26.81

meter used in fact reflects non-volatile metabolites of vinyl chloride and not vinyl chloride as such.

So far all the experiments have dealt with male flies. In order to establish whether the induction of the metabolizing enzymes is a general property and not limited to one sex an experiment with phenobarbital induction was performed on females of the strains Karsnäs 60 and Berlin. As shown in Table IV the results are in good agreement with those on males, excluding the possibility of a sex limitation. In one experiment larvae (12 h old) were investigated for induction by phenobarbital. The results in Table V show that both the strains Karsnäs 60 and Berlin which were inducible in adult flies also behaved in a similar way when larvae. The strongest induction was exhibited by Berlin. In agreement with the results on adult males Hikone differed distinctly from the other two strains. The initial uptake of vinyl chloride was considerably higher and no, or possibly a very weak response to phenobarbital occurred. The variation between the *Drosophila* strains in their reaction to the inducing agents opens the possibility of analyzing the genetical determination of this process. As yet no attempts have been made to carry out such analyses, but one experiment was performed to study the heterozygotes between one of the inducible wild type strains, Berlin, and the non-inducible Hikone strain. The heterozygotes are closest to Hikone, but they seem to exhibit a weak inducibility with phenobarbital (Table VI).

DISCUSSION

From various investigations of the action of vinyl chloride in experimental systems it can be concluded that its carcinogenic and mutagenic properties are due to reactive metabolites formed by the mixed function oxygenase system [19,20]. The enzymes involved in this metabolism evidently can be induced by phenobarbital and PCB in the mammalian body, particularly by the liver microsomal enzymes [17]. The experimental system used here is based on the formation of non-volatile metabolites by the microsomal enzymes. An induction of the microsomal enzymes involved will lead to an increased formation of such metabolites which will be retained in the flies. The increased uptake of labelled vinyl chloride after enzyme induction has

been used in order to study the induction process and the variation in this respect between *Drosophila* strains.

That the uptake of labelled vinyl chloride does reflect the metabolic conversion of vinyl chloride to non-volatile metabolites, which presumably form stable complexes, was indicated by the retention and excretion of labelled material. During 24 h after treatment with labelled vinyl chloride there was a gradual decrease in the flies but a simultaneous increase of labelling of the food from excretion products. There was no sign of a loss of the total labelling.

The results show that the uptake of labelled vinyl chloride is enhanced several times by phenobarbital and PCB (Clophen), both of which are widely used experimentally in mammals for the induction particularly of the mixed function oxidase system. Phenobarbital acts specifically on cytochrome P-450 while PCB apparently have a somewhat wider spectrum of action also inducing cytochrome P-448 [23]. Results with phenobarbital show that both sexes of flies are inducible. The studies on larvae are in agreement with the data on adults. This information is of some importance from the point of view of mutagenicity testing.

Both theoretically and practically the variation in the inducibility between different *Drosophila* strains is of interest. Four of the strains studied could be induced by phenobarbital and PCB although there obviously are quantitative differences in their response. The fifth strain, Hikone, which has been used quite extensively in mutagenicity testing, cannot be induced or at least only to a very small degree. In fact if induction investigations had been performed only on Hikone, *Drosophila* might well have been classified as a non-inducible species. Hikone also deviates from the other strains in another respect. It has a clearly elevated uptake of vinyl chloride and therefore presumably also an enhanced metabolic activation initially without any induction. The exceptional behaviour of Hikone concerning the mixed function oxygenase system is in accordance with a report by Vogel [24] on the induction of sex linked recessive lethals by the indirect mutagen 2,4,6-trichlorophenyldimethyltriazene. The yield of lethals in Hikone was distinctly smaller than in the Berlin strain.

Which enzyme or enzymes are affected by this difference between Hikone and the other strains is not known. The fact that vinyl chloride is metabolized by cytochrome P-450, and phenobarbital specifically induces this enzyme, suggests that the differences depends on this enzyme. In this context, it should be mentioned that Hikone is resistant to insecticides like DDT and parathion [25]. The involvement of cytochrome P-450 in such resistance is known from the house fly, *Musca domestica* [11,13,26]. The genetic determination of the difference between Hikone and the other strains has only been studied by investigation of the behaviour of hybrids from reciprocal crosses between Hikone and Berlin. The results indicated no, or only a small inducibility in the hybrids, suggesting essentially a dominance for the Hikone genotype. Vogel [10] reported an intermediary behaviour of the same hybrid between Hikone and Berlin for mutation

induction. The present data on the induction of the enzyme(s) involved in the metabolic activation of vinyl chloride is in accordance with the mutagenicity data reported previously [16]. It was shown that pretreatment with 1% phenobarbital increased the frequency of vinyl chloride induced recessive lethals in the strain Karsnäs 60 3—8-fold, which is in good agreement with the corresponding effect of phenobarbital in the present report. It should, however, be emphasized that a quantitative agreement with the induction of recessive lethals and the uptake of vinyl chloride as revealed in the system used here, can hardly be expected generally. Different metabolites may be involved in the two experimental systems. Furthermore alkylation is measured only at the DNA level when dealing with mutations, but in the present system binding is of course measured in a variety of tissues and molecules. Preliminary data on recessive lethals induced by vinyl chloride in other strains after phenobarbital induction shows less of a quantitative agreement between the two experimental systems.

Some practical conclusions for mutagenicity screening with *Drosophila* may finally be drawn from the present experiments. The data point to the fact that the induction of microsomal enzymes is not restricted to one sex or limited to larvae or adults. The use of DMSO and Tween 80 to dissolve and emulgate PCB revealed that DMSO interferes with the microsomal detoxification system and strongly diminishes the activation of vinyl chloride. When dealing with indirect mutagens it is therefore recommended not to use DMSO as a solvent.

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