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Effect of Phenobarbital and Naphthalene on Some of the Components of the Electron Transport System and the Hydroxylating Activity of House Fly Microsomes†

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ABSTRACT: (1) A house fly microsomal enzyme system that hydroxylates several type I substrates is inducible by naphthalene and phenobarbital administered in the food. Both inducers increase the levels of cytochrome P-450, NADPH–cytochrome *c* reductase, and cytochrome b_5. (2) Untreated house flies show a peak absorbance at 450 nm in the CO-differential spectrum, whereas the naphthalene- or phenobarbital-treated insects show a maximum absorbance at 448 nm. (3) Cessation of phenobarbital or naphthalene treatment is promptly followed by a progressive reduction in the amounts of cytochromes P-450 and b_5 and in NADPH–cytochrome *c* reductase. (4) During the induction period, the relationship between the level of cytochrome P-450 and the hydroxylating activity of microsomal preparations depends on the substrate hydroxylated and the inducer employed. Good correlation is obtained with naphthalene hydroxylation and aminopyrine N-demethylation, but not with 2-isopropoxyphenyl-*N*-methylcarbamate (Baygon) hydroxylation because of competition with the substrate for cytochrome P-450 binding site I. This effect is not evident with naphthalene because the latter apparently is metabolized at a faster rate than phenobarbital, thus making the cytochrome P-450 binding site I available for other substrates. (5) The CO and ethyl isocyanide differential spectra, as well as the metabolic activities of induced cytochrome P-450, suggest that contrary to what happens in mammals, both phenobarbital and naphthalene stimulate the synthesis of a new species of hemoprotein, cytochrome P-448.

A moderate to substantial increase in the activity of insect microsomal mixed function oxidases can be produced by the administration of several insecticides as well as by phenobarbital and 3-methylcholanthrene (Agosin *et al.*, 1969; Gil *et al.*, 1968; Morello, 1964; Perry *et al.*, 1971; Plapp and Casida, 1970; Walker and Terriere, 1970). The increase in enzyme activity is preceded by an increase in the synthesis of messenger-like RNA (Balazs and Agosin, 1968; Litvak and Agosin, 1968) and of DNA-dependent RNA polymerase levels (Agosin, 1971). These effects can be prevented by the administration of RNA and protein synthesis inhibitors such as actinomycin D (Balazs and Agosin, 1968) and cycloheximide (Walker and Terriere, 1970). These observations indicate that house fly treatment with the above chemicals results in an increase in the quantity of microsomal enzymes. The latter, as in the case of mammalian microsomes (Hayaishi, 1969), correspond to a NADPH[1]-linked enzyme system which has as its terminal oxidase the CO-binding hemoprotein P-450 (Ray, 1967; Perry, 1970; Morello *et al.*, 1971). The administration of dieldrin (Matthews and Casida, 1970) or phenobarbital (Perry *et al.*, 1971) increases the levels of cytochrome P-450 in house flies, but a corresponding increase in microsomal activity is not always proportional. This may probably be due to the fact that the chemically induced changes in the kinetics of microsomal enzymes are complex functions of the inducing agent used and the substrate investigated (Gram *et al.*, 1968). Another possibility which might explain the lack of correlation between levels of cytochrome P-450 and hydroxylating activity may be the existence of more than one microsomal enzyme system involving cytochrome P-450

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[1] Abbreviations used are: NAD^+ and NADPH, oxidized and reduced nicotinamide adenine dinucleotides, respectively.

(Alvares and Mannering, 1970) or the induction of new and distinct cytochrome P-450 species with different catalytic activities and depending on the inducer used (Kuntzman *et al.*, 1971).

Recently, cytochrome P-450 has been characterized in a new microsomal preparation obtained from whole house flies (Morello *et al.*, 1971). This preparation has been used in the present work to investigate further the correlation of microsomal mixed-function oxidase activity during induction with the levels of cytochrome P-450, cytochrome b_5, and NADPH-dependent cytochrome P-450 reductase. The enzyme activity includes aromatic hydroxylation, as measured by the formation of 1-naphthol from naphthalene, and N-demethylation, as determined by the formation of formaldehyde from aminopyrine. The *in vitro* metabolism of the insecticide, 2-isopropoxyphenyl *N*-methylcarbamate (Baygon) has also been investigated. This compound is mainly ring hydroxylated by insect microsomes, although some N-demethylation also occurs (Shrivastava *et al.*, 1969).

The inducers used include phenobarbital and naphthalene, both type I substrates (Schenkman *et al.*, 1966; Orrenius *et al.*, 1971). The effect of these inducers on the *in vivo* tolerance to Baygon has also been explored. The results obtained indicate that induction of a new species of cytochrome P-450, the rate of metabolism of the inducer by the microsomal system, and the relative affinity of inducers and substrates for binding sites on the hemoprotein may be factors involved in the presence or lack of correlation between levels of hemoprotein and enzyme activity. In addition, the rate of degradation of the induced hemoprotein appears to be involved in the *in vivo* correlation between microsomal activity and tolerance to a foreign compound. A striking observation is that contrary to the situation in mammals, both naphthalene and phenobarbital seem to induce a new species of cytochrome P-450.

Materials and Methods

Chemicals. Naphthalene and 1-naphthol were obtained from Amersham-Searle, Des Plaines, Ill., and Baker Chemical Co., Phillipsburg, N. J., respectively. Aminopyrine was purchased from Merck and Co., Inc., Rahway, N. J. Phenobarbital was obtained from American Pharmaceutical Co., New York, N. Y. Baygon, 98% technical grade, was obtained from Chemagro Corp., Kansas City, Mo. 2,4-Pentanedione was purchased from Baker Chemical Co., Phillipsburg, N. J., and $NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and cytochrome *c* from Sigma Chemical Co., St. Louis, Mo. [^{14}C]Phenobarbital (specific activity, 3.15 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. Ethyl isocyanide was synthesized in the laboratory. Other chemicals used were of the highest purity commercially available.

Insect Materials. Nonsexed 4-day old flies of the Fc strain (Gil *et al.*, 1968) were used throughout. The insects were reared in CSMA larva media obtained from Ralston Purina Co., St. Louis, Mo., and the adults were fed *ad libitum* a mixture of nonfat powdered mild-sugar-powdered egg (6:6:1).

Treatment of Insects. Phenobarbital and naphthalene were administered in the food at a concentration of 0.5 and 5%, respectively, for periods ranging from 48 to 120 hr.

Preparation of Microsomes. Microsomes were obtained from whole house flies by the "mortar" procedure described by Morello *et al.* (1971). Microsomes derived from 10 g of flies were suspended in 5 ml of 0.1 M potassium phosphate buffer, pH 7.5, and used immediately. In some cases, microsomes were stored as a pellet at −80° for at least 10 days without loss of activity.

Differential Spectra of Microsomal Fractions. CO-differential spectra were measured according to Omura and Sato (1964) as described by Morello *et al.* (1971). The concentration of cytochrome P-450 was determined from the CO-differential spectrum using the extinction coefficient of 91 cm^{-1} mM^{-1} for the difference in absorbance between 450 and 490 nm (Omura and Sato, 1964). The ethyl isocyanide differential spectra were obtained as follows: microsomal suspensions in 0.1 M potassium phosphate buffer, pH 7.5, were pipetted into the sample and reference cuvets. Both samples were then reduced with solid $Na_2S_2O_4$, and ethyl isocyanide was added to the sample cuvet at concentrations varying from 1 to 100 mM. The differential spectrum was then recorded for each ethyl isocyanide concentration. Microsomes from control insects had a protein content of 4.0–4.5 mg/ml and 0.60–0.65 nmol of cytochrome P-450 per milliliter. Microsomes from phenobarbital and naphthalene-treated insects contained 4.8–5.5 mg of protein/ml and 1.1–1.3 nmol of cytochrome P-450/ml. To determine the effect of pH on the ethyl isocyanide spectra, the microsomes were suspended in 0.1 M potassium phosphate buffer at pH values varying from 6 to 9 and the difference spectra were then determined as above. Scanning was done over a wavelength from 390 to 500 nm. Cytochrome b_5 was determined from the difference spectra between the oxidized and the $Na_2S_2O_4$ reduced form, assuming an extinction coefficient of 163 cm^{-1} mM^{-1} for the difference between 426 and 409 nm (Garfinkel, 1958). Cytochrome P-420 was determined by first establishing a base line for the oxidized microsomes in both reference and sample cuvets. The experimental cuvet was then saturated with CO by bubbling the gas gently for 30 sec and both reference and sample suspensions were reduced with solid $Na_2S_2O_4$. An extinction coefficient of 110 cm^{-1} mM^{-1} for the change in absorbance between 422 and 490 nm was used for estimating the amount of cytochrome P-420. It should be pointed out that both cytochrome b_5 and 420 have different absorbance maxima in insects as compared with the corresponding mammalian cytochromes. Maximum absorbance for cytochrome b_5 in the oxidized *vs.* reduced differential spectrum is 424 nm in mammals (Omura and Sato, 1964; Garfinkel, 1958) *vs.* 426 in house flies; maximum absorbance for cytochrome P-420 in the CO-differential spectrum is 420 nm in mammals (Omura and Sato, 1964) and 422 in house flies.

NADPH-Dependent Cytochrome c Reductase. The enzyme activity of microsomal fractions was determined by following the reduction of cytochrome *c* at 550 nm. It is assumed that this enzyme is the same that reduces cytochrome P-450 (Estabrook and Cohen, 1969). The reaction mixtures contained, in a final volume of 1.0 ml, 0.1 μmol of $NADP^+$, 0.2 μmol of glucose 6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, 0.5 mg of cytochrome *c*, and 100 μmol of potassium phosphate buffer, pH 7.5. The reaction was started by the addition of the microsomal suspension (62–68 μg of protein). The reference cuvet contained the same components but microsomes. The enzyme activity was linear for at least 4 min. Under these conditions, a change in absorbance of 1.0 corresponds to the reduction of 0.0467 μmol of cytochrome *c* (Masters *et al.*, 1967).

Microsomal Hydroxylating Activity. The mixed function oxidase activity of microsomes toward naphthalene, aminopyrine, and Baygon was measured in a reaction mixture containing in a final volume of 3.5 ml, the following: potassium

phosphate buffer, pH 7.5, 100 μmol; $NADP^+$, 3.5 μmol; glucose 6-phosphate, 20 μmol; $MgCl_2$, 20 μmol; glucose-6-phosphate dehydrogenase, 2.5 units; and microsomes equivalent to 3.6 g of flies. The substrates were added as follows: aminopyrine, dissolved in 0.1 M potassium phosphate buffer, pH 7.5, 1.87 μmol; naphthalene, dissolved in 20 μl of ethanol, 6.5 μmol; Baygon, dissolved as naphthalene, 1.9 μmol. The reaction was started by the addition of the microsomes and incubation was carried out at 30°. At zero time and after 15 and 30 min of incubation, 1.0-ml aliquots were pipetted into test tubes containing 0.1 ml of concentrated HCl for naphthalene, 0.1 ml of 100% trichloroacetic acid for aminopyrine, and a mixture of benzene and acetone (5:1, v/v) for Baygon. The formation of 1,2-dihydro-1,2-dihydroxynaphthalene and 1-naphthol was determined by the procedure of Booth and Boyland (1958). The formation of formaldehyde from aminopyrine was determined by the procedure of Nash (1953). The yellow color produced by the addition of 2,4-pentanedione and 4 M NH_4Cl was allowed to develop for 2 hr at room temperature. Baygon hydroxylation was followed by measuring the disappearance of substrate by gas chromatography as described by Perry *et al.* (1971). Under the above conditions, the $NADP^+$, reducing system is in excess (Morello *et al.*, 1971).

In Vitro Uptake of [^{14}C]Phenobarbital by Microsomes. The labeled compound was dissolved in 0.1 M potassium phosphate buffer, pH 8.0, to give a specific activity of 3×10^4 dpm/μg and mixed with microsomal preparations derived from control insects at a proportion of 340 μg of phenobarbital/10 mg of microsomal protein. Incubation was carried out for 30 min at 30°. The microsomes were then isolated by differential centrifugation and the bound radioactivity determined as indicated below. The effect of substrates on the amount of bound [^{14}C]phenobarbital was determined by adding the substrates to 2.0-ml suspensions of labeled microsomes (2.24 mg of protein) at concentrations of 160 μg each. The microsomes were incubated for 15 min at 30° and then isolated by centrifuging the reaction mixtures at 204,000*g* × 60 min. The microsomal pellets were resuspended in a suitable amount of 0.1 M potassium phosphate buffer, pH 7.5, and aliquots containing 0.5 mg of protein were pipetted into test tubes containing trichloroacetic acid at the final concentration of 5%. After standing for 30 min at 2–4°, the material was filtered through Millipore filters and washed five times with 15-ml portions of 5% trichloroacetic acid. The Millipore filters were dried under infrared lamp and placed into scintillation vials containing 10 ml of scintillation mixture (Litvak and Agosin, 1968).

Measurement of Radioactivity. Radioactivity was measured in a Nuclear-Chicago Mark I scintillation counter at an efficiency of 85%.

Effect of Induction on Resistance to Baygon. To determine if changes in the levels of cytochrome P-450 are paralleled by changes in house fly susceptibility to an insecticide metabolized primarily by microsomes, female house flies were induced with phenobarbital and naphthalene as indicated above and, after 48 hr, Baygon dissolved in acetone was applied topically at doses varying from 1 to 10 μg/fly. The insects were maintained for an additional 24-hr period with food containing no inducers at which time the mortality was determined. From the mortality data obtained with the different concentrations of Baygon, an LD_{50} value was calculated.

To correlate the mortality data with the *in vivo* metabolism of Baygon, female flies were induced as above and then treated topically with 2.5 μg of Baygon per insect. Two hours after application, the insects were rinsed with acetone to remove unabsorbed Baygon; then they were ground with anhydrous Na_2SO_4 and extracted with benzene in a Soxhlet extractor for 4 hr. The extracts were cleaned by silica gel chromatography and analyzed by gas chromatography as described by Perry *et al.* (1971).

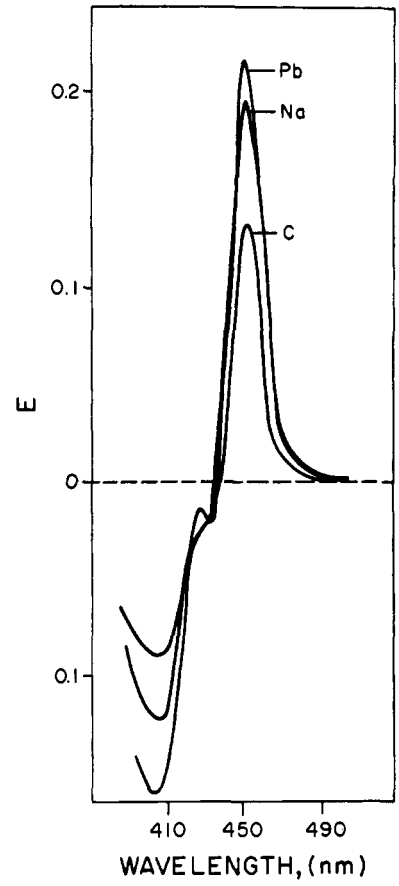

FIGURE 1: CO-differential spectra of microsomal suspensions derived from control, phenobarbital-, and naphthalene-treated insects. Inducers administered in the feed for 48 hr. Results are expressed per 10 g of flies. See the Materials and Methods section for further details.

Analytical Procedures. RNA was determined according to Mejbaum's procedure (1939) and protein according to Lowry *et al.* (1951). All spectrophotometric determinations were done at room temperature (22°) using cuvets of 1.0 ml capacity and 1.0-cm light path in a Unicam SP-1800 spectrophotometer. The results reported correspond to the average of four or more experiments.

Results

CO and Oxidized vs. Reduced Differential Spectra of House Fly Microsomes. Figure 1 represents the CO-differential spectra of cytochrome P-450 from microsomes derived from control, naphthalene-, and phenobarbital-treated insects. The absorbance maxima of the hemoprotein from induced insects is at 448 nm, while in control insects it is at 450 nm. This could be clearly evident when expanded spectra were recorded. Figure 2 shows the oxidized *vs.* the reduced differential spectra of microsomal suspensions from control and induced insects. In all three instances, the absorbance maxima was at 426 nm.

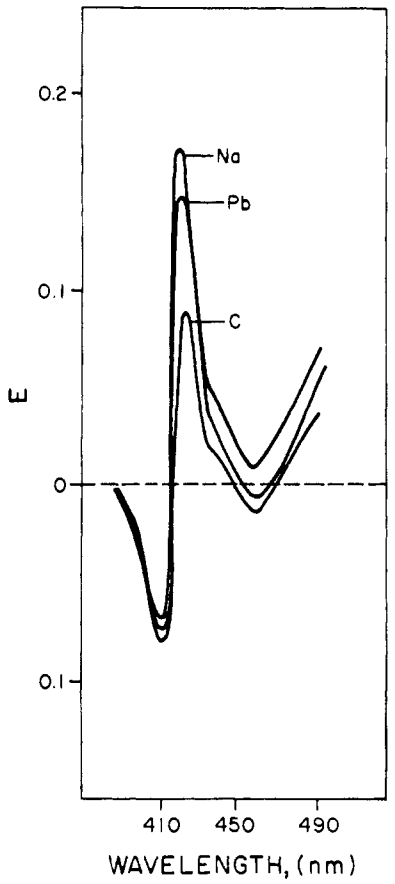

FIGURE 2: Oxidized *vs.* reduced differential spectra of microsomes derived from control, phenobarbital-, and naphthalene-treated insects. Results expressed per 10 g of flies.

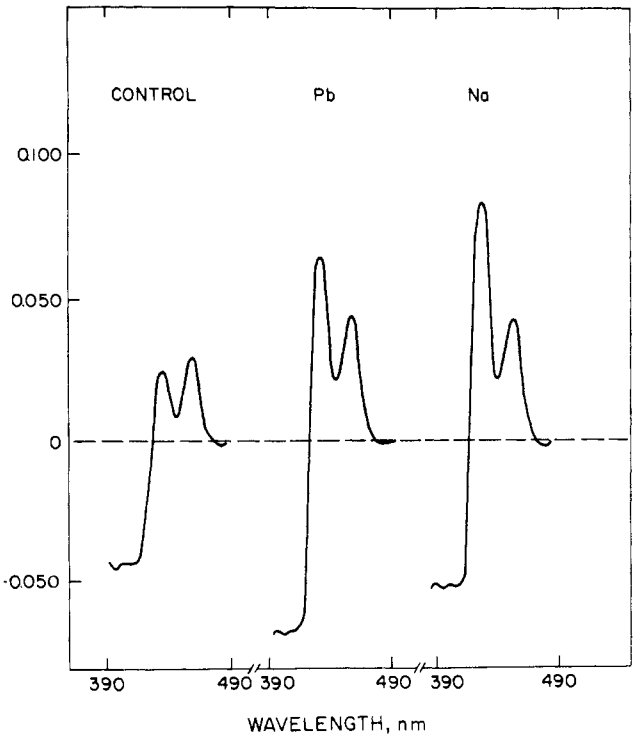

FIGURE 3: Ethyl isocyanide differential spectra of chemically reduced microsomes from control, naphthalene- and phenobarbital-treated insects. Ethyl isocyanide was added at a concentration of 0.1 M. Spectra were obtained at pH 8.0.

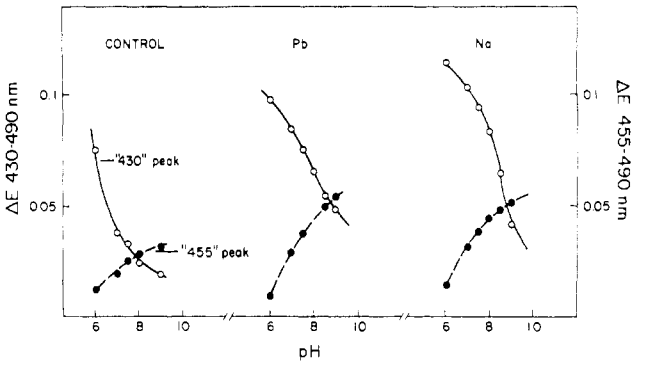

FIGURE 4: Plot of ΔE "430"–490 nm and ΔE "455"–490 nm *vs.* pH. Ethyl isocyanide concentration is as in Figure 3.

Ethyl Isocyanide Differential Spectrum. The addition of ethyl isocyanide to chemically reduced house fly microsomes resulted in the appearance of two peaks in the Soret region at 430 and 455 nm, similar to the peaks of mammalian cytochrome P-450 (Omura and Sato, 1964). It has been shown that the height of either peak is affected by prior treatment of experimental animals. For example, at a given pH, the ratio of the 455 peak height relative to that of the 430 peak is increased by pretreatment with 3-methylcholanthrene (Sladek and Mannering, 1969a,b). The Soret peaks of the induced house fly cytochrome P-450 also occur at 430 and 455 nm, and at pH 8.0 there was a greater increase in the amount of the "455" peak relative to the controls (Figure 3). When the heights of the Soret peaks were plotted as a function of pH, it was found that the peaks had the same height in the cytochrome P-450 of the controls at pH 7.85, while a similar relationship was obtained at pH 8.8 for naphthalene- and 8.85 for phenobarbital-induced hemoprotein. In Figure 4, the ΔE 430–490 nm and the ΔE 455–490 in the ordinate axis are plotted against pH, showing that the pH at which the height of both peaks is of the same magnitude is different for the control and the induced cytochrome P-450.

Kinetics of Induction of the Microsomal Hemoproteins and of the Reductase. The levels of cytochrome P-450 tend to change with age in control insects, an observation previously reported by Perry (1970). The levels tend to increase up to the seventh day of age and then decrease afterward (Figure 5). The reductase shows a similar pattern as cytochrome P-450, but cytochrome b_5 increases during the entire experimental

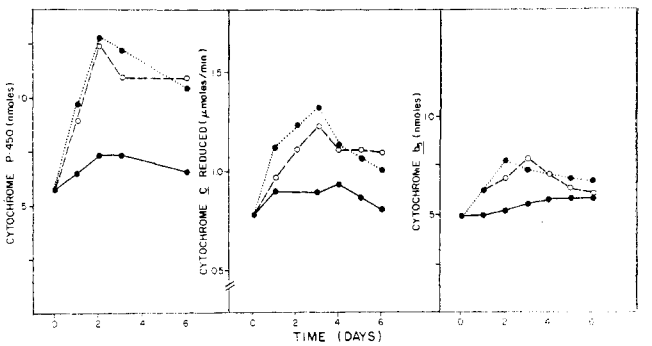

FIGURE 5: Changes in cytochrome P-450 and b_5 and NADPH-cytochrome c reductase in house fly microsomes during induction by phenobarbital and naphthalene. Values expressed per 10 g of flies. Inducers are administered in the feed for the indicated time: (●—●), control; (● · · · ●), naphthalene; (○— —○) phenobarbital.

TABLE I: *in Vitro* Metabolism of Naphthalene, Aminopyrine, and Baygon.[a]

Conditions	Na[b]			Aminopyrine[b]			Baygon[b]		
	Total	Per mg of Protein	Per nmol of Cytochrome P-450	Total	Per mg of Protein	Per nmol of Cytochrome P-450	Total	Per mg of Protein	Per nmol of Cytochrome P-450
Control	370	11.4	50	395	12.2	53.2	612	15.8	97.2
Napthalene treated	505	16.2	40.5	780	25.0	62.5	2715	67.6	246
Phenobarbital treated	490	14.3	38.5	770	22.5	60.0	1382	31.9	141.7

[a] By microsomes obtained from house flies treated with naphthalene and phenolbarbital. The latter were administered for 48 hr as in Figure 1. Results expressed as nanomoles of substrate metabolized in 15 min at 30° for 10 g of flies. For experimental details see text under Materials and Methods. [b] Substrate metabolized nanomoles.

period. The significance of these normal changes is not clear at present, but it is possible that hormonal and metabolic changes associated with aging may be involved in thier production (Perry, 1970). Maximum increases in the levels of cytochrome P-450 were obtained after 48 hr of treatment with either phenobarbital or naphthalene (65–70% of the control values). The levels of the reductase increased approximately 30–35% at 48 hr of induction but the maximum levels (50%) were attained after 72 hr (Figure 5). Curiously, naphthalene treatment produced a maximum increase in cytochrome b_5 after 48 hr (about 50%) while phenobarbital treatment resulted in higher levels at 72 hr (50%). Whether this observation indicates the induction of different cytochrome b_5 species by phenobarbital or naphthalene is difficult to ascertain at present, although no differences in the oxidized *vs.* reduced spectra are apparent (Figure 2). Neither naphthalene nor phenobarbital had a significant effect on the levels of protein. Similarly, the levels of RNA that were about 19.7 mg/10 g of flies were not changed by treatment with the inducers up to 120 hr.

Kinetics of Degradation of the Microsomal Cytochromes and of the Reductase after Removal of the Inducers. Following the removal of the inducer, previously administered for 48 hr in the case of cytochrome P-450, 72 hr in the case of the reductase, 48 hr for naphthalene-induced, and 72 hr for phenobarbital-induced cytochrome b_5, the levels of the above microsomal components decreased rapidly (Figure 6). Naphthalene- and phenobarbital-induced cytochrome b_5 decreased at similar rates, but naphthalene-induced cytochrome P-450 and reductase decreased faster than those induced by phenobarbital. Four days after removal of the inducers, the levels of reductase and cytochrome b_5 approached normal values, but cytochrome P-450 was slightly higher in induced house flies than in the controls. No determinations were made at longer intervals than 4 days after removal of the inducers.

Hydroxylating Activity. The data of Table I indicate that there is a correlation between the levels of cytochrome P-450 and the hydroxylation of naphthalene and N-demethylation of aminopyrine, the activity of the induced hemoprotein in terms of nanomoles of substrate metabolized per nanomole of cytochrome P-450 being close to the control values. However, such correlation is not evident when Baygon is the substrate. Naphthalene-induced microsomes have a 2.6-fold increase in catalytic activity, while phenobarbital-induced microsomes are 1.6 times more active than the controls.

Effect of Inducers on the in Vivo Susceptibility of House Flies to Baygon. Since naphthalene and phenobarbital treatment increased the catalytic activity of microsomes toward Baygon, it was expected that administration of these inducers would increase the tolerance of the insects to this insecticide. Table II shows the results of such an experiment. The LD_{50} for Baygon in control insects was 2.7 μg per insect. When the

TABLE II: Susceptibility of House Flies Treated with Naphthalene and Phenolbarbital to Baygon.[a]

Baygon (μg/fly)	% Mortality in 24 hr		
	Control	Phenobarbital Treated	Naphthalene Treated
1.0	15.3 ± 12.4	20 ± 6.9	6.4 ± 5.6
2.5	43.7 ± 6.5	68 ± 15.7	26 ± 7.4
5.0	80.7 ± 13.7	85.6 ± 12.2	4.56 ± 8.0
10.0	100	93 ± 6.0	54 ± 4.6
LD_{50}	2.7 ± 0.37	1.75 ± 0.18	6.15 ± 0.98

[a] The figures after the ± sign correspond to the standard error of the mean. For experimental details, see text under Materials and Methods.

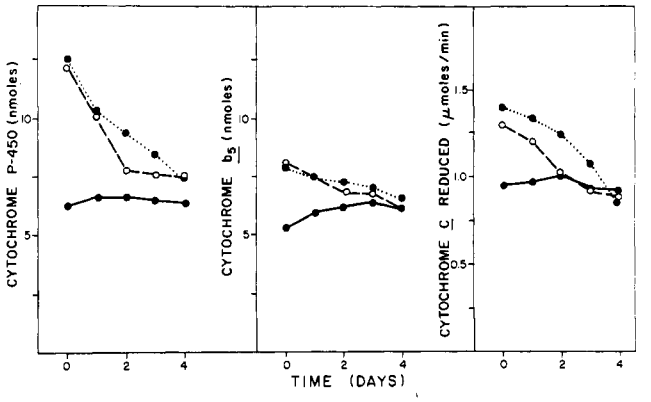

FIGURE 6: Changes in cytochrome P-450 and b_5 and NADPH–cytochrome *c* reductase in house fly microsomes after cessation of phenobarbital and naphthalene treatment. The insects were fed a diet containing the inducers and, after 48 hr, the diet was changed to a control one. Results are expressed per 10 g of flies: (●——●), control; (●···●), naphthalene; (○— —○), phenobarbital.

TABLE III: Effect of Added Phenobarbital on the *in Vitro* Hydroxylation of Baygon by House Fly Microsomes.[a]

[Phenobarbital] (mM)	Baygon Metabolized (nmol)		
	Total	Per mg of Protein	Per nmol of Cytochrome P-450
None	600	12.09	11.25
1	383	7.64	7.14
2	226	4.57	4.39
4	53.5	1.06	0.98
6	0		

[a] Results expressed as in Table II. For experimental details, see text under Materials and Methods.

TABLE IV: Effect of Substrates on the Retention of Phenobarbital Bound to House Fly Microsomes by *in Vitro* Pretreatment with [^{14}C]Phenobarbital.[a]

Conditions	cpm/mg of Protein	nmol of Phenobarbital Bound/mg of Protein	nmol of Phenobarbital Bound/nmol of Cytochrome P-450
[^{14}C]Phenolbarbital microsomes	2730	0.49	3.5
[^{14}C]Phenobarbital microsomes, control	750	0.27	1.9
[^{14}C]Phenobarbital microsomes + naphthalene	27	0.015	0.107
[^{14}C]Phenobarbital microsomes + aminopyrine	240	0.040	0.28
[^{14}C]Phenobarbital microsomes + Baygon	402	0.070	0.50

[a] Microsomes (10 mg of protein) were labeled with 1.59 nmol of phenobarbital (9.4×10^6 cpm) as indicated under Materials and Methods. Labeled microsomes (2.24 mg of protein) were then incubated with 160 μg of each of the indicated substrates for 15 min at 30°. A microsomal suspension containing no substrate was incubated for a similar period as control.

insects were previously treated with naphthalene for 48 hr, the LD_{50} increased to 6.15 μg, a value that correlates very well with the catalytic activity of naphthalene-induced cytochrome P-450 (Table I). However, treatment with phenobarbital decreased the LD_{50} to 1.75 μg/insect, a value 35% lower than that of the control. Since the penetration rate of Baygon is similar in susceptible and resistant strains (Shrivastava *et al.*, 1969), these results indicate that Baygon is metabolized faster by naphthalene-treated insects and slower in phenobarbital-treated ones, a fact which is supported by the following data. When the amount of internal Baygon remaining at 2 hr after application of 2.5 μg of Baygon/insect was determined as indicated under Materials and Methods, it was found that control insects contained 0.19 μg/specimen, while the naphthalene-treated ones contained only 0.08 μg, and the phenobarbital-treated ones contained an amount slightly higher than the controls, *i.e.*, 0.21 μg.

In Vitro Effect of Phenobarbital on the Hydroxylation of Baygon. The data of Figures 1, 3, and 4 suggested that naphthalene and phenobarbital may probably induce a new species of cytochrome P-450 but with catalytic activities that differ when Baygon is the substrate (Table I). The possibility that these differences in catalytic activity could be due to residual phenobarbital present in the microsomes was considered. Residual phenobarbital could compete with Baygon for the availability of site I in the hemoprotein, since both compounds are type I substrates. This would explain the enhancement of the toxicity of Baygon in phenobarbital-treated insects. The results of Table III indicate that under the hydroxylating conditions described in Table I, phenobarbital acts as a competitive inhibitor of Baygon hydroxylation, a concentration of 2 mM producing 62% inhibition.

Competition between Phenobarbital and Other Substrates for Binding Site I. The results presented in Tables I, II, and III may be interpreted as competition between phenobarbital and other substrates for binding site I of cytochrome P-450. Baygon metabolism would be inhibited in phenobarbital-treated house flies because it cannot displace the residual phenobarbital from binding site I. On the other hand, naphthalene hydroxylation and aminopyrine N-demethylation would not be inhibited because these substrates are capable of displacing phenobarbital from binding site I. When site I was occupied by [^{14}C]phenobarbital as described under Materials and Methods, 88% of the radioactivity was displaced by incubating the microsomes with naphthalene (Table IV). Aminopyrine displaced 68% and Baygon only 46%. In view of the results of Table III, it is even possible that under *in vivo* conditions, Baygon is less capable of displacing phenobarbital than is shown in Table IV. Furthermore, these results indicate that the catalytic activity toward Baygon of phenobarbital-induced cytochrome P-450 may be higher than that shown in Table I.

Discussion

The NADPH-linked oxidative system involving cytochrome P-450 in insect microsomes is in many respects similar to the corresponding mammalian system. Contrary to what occurs with 3-methylcholanthrene (Sladek and Mannering, 1966), naphthalene behaves as phenobarbital in insects. Thus, treatment of mammals with phenobarbital (Alvares *et al.*, 1967) results in the formation of a hemoprotein similar to the uninduced one, having an absorbance maximum in the CO-differential spectrum at 450 nm. On the other hand, 3-methylcholanthrene treatment of mammals induces a pigment with a shift of about 2 nm to lower wavelength in the CO-differential spectrum. The latter type of hemoprotein appears to be induced in house flies by both phenobarbital and naphthalene, with an absorbance maximum at 448 nm. The spectral shift does not seem to be due to the presence of residual phenobarbital or naphthalene bound to the microsomes since addition of these compounds to microsomal suspensions derived from control insects at concentrations higher than 2 mM does not result in a modification of the absorbance maximum at 450 nm. However, neither phenobarbital nor naphthalene increases the levels of protein or RNA in insects as it has been shown for rat liver microsomal preparations obtained from phenobarbital or polycyclic hydrocarbons treated animals

(Conney, 1967). A strong evidence in favor of the induction of a new species of cytochrome P-450 by either naphthalene or phenobarbital is given by the ethyl isocyanide differential spectra, which indicates that a higher pH is required for equalization of the two Soret peaks in the new species (Figures 3 and 4). It should be recalled that the opposite occurs in mammalian cytochrome P-450 induced by 3-methylcholanthrene, *i.e.*, the pH at which both peaks have the same magnitude is lower than that of the noninduced hemoprotein (Jefcoate *et al.*, 1970).

The data presented here indicate that in certain instances a correlation exists between the levels of hydroxylating activity and cytochrome P-450, and that it depends on the inducer and substrate used. A first factor involves the binding capacity of the ligands, inducer and substrate. Kutt *et al.* (1971) have classified inducers and substrates as ligands with a strong capacity to bind to cytochrome P-450, a moderate capacity, and a weak capacity. The more strongly binding ligand will overcome the binding by weaker ligands, regardless if the latter are added *in vitro* (Tables III and IV) or if present because of previous treatment of the insects (Table II). Baygon may be classified as a ligand binding more weakly than phenobarbital (Table III) while aminopyrine is intermediate between Baygon and phenobarbital. On the other hand, naphthalene is a stronger binder than phenobarbital since it displaces phenobarbital from binding site I almost completely. The results of Table III indicate that about 0.05 μg of phenobarbital/mg of protein binds to the microsomes in 15 min at 30°. This value for mammalian microsomes is about 0.9 μg/mg of protein (Kutt *et al.*, 1971). Both represents a minimum value since *in vivo* pretreatment with [^{14}C]phenobarbital results in about 20 times more phenobarbital bound in rat liver microsomes (Kutt *et al.*, 1971). The latter remain bound to the microsomes even 48 hr after removal of the inducer, and a similar situation seems to occur in house flies. Microsomes isolated from phenobarbital-treated flies must contain residual phenobarbital because there is evidence of a competitive effect with Baygon in Table I. Furthermore, pretreatment with phenobarbital does not result in a protective effect against Baygon, but rather results in a synergistic effect (Table II).

A second factor is the rate at which a ligand is metabolized by the microsomes. It may be argued that naphthalene treatment should enhance Baygon metabolism to a greater extent than treatment with phenobarbital, since naphthalene is metabolized by microsomes of the Fc strain at very high rates (Morello *et al.*, 1971). As a result, binding site I of the hemoprotein is made available for the formation of the enzyme–substrate complex with a weaker ligand such as Baygon or aminopyrine. This may be reflected in the observation that naphthalene is very well tolerated by the flies while phenobarbital at concentrations higher than 0.5% becomes rapidly toxic.

A third factor that may play a role in correlating enzyme activity with levels of hemoprotein is the possibility that an inducer may give rise to a new species of hemoprotein with catalytic activities unlike those of the uninduced hemoprotein. The new hemoprotein may have a different extinction coefficient. Therefore, if its level is calculated on the basis of the extinction coefficient of the uninduced cytochrome, it may result in erroneous values (Nebert, 1970).

A fourth factor which is involved mainly with *in vivo* studies is the rate of degradation of the hemoprotein after removal of the inducer. A hemoprotein that is degraded slowly after cessation of induction may have a better protective effect against another substrate.

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