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THE RELATIVE ETHYLUMBELLIFERONE DEALKYLASE ACTIVITY CHARACTERIZING THE EFFECT OF DIFFERENT INDUCERS ON THE HYDROXYLASE SYSTEM IN THE LIVER MICROSOMES OF FEMALE MICE*

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Abstract—D,L-Camphor was detected as a new inducer of hydroxylase in the liver microsomes of female mice. After a 2-day inhalation of D,L-camphor, cyt. P-450 and the ethylumbelliferone dealkylase were increased by 250 per cent and the NADPH–cyt. P-450 reductase by 350 per cent. The product [NADPH–cyt. P-450 reductase activity × cyt. P450 concentration] was shown to be a suitable reference parameter for the ethylumbelliferone dealkylase activity in the liver microsomes during the treatment with four different inducers. The relative dealkylase activity Q was much decreased during inhalation of cyclohexane or D,L-camphor.

$$Q = \frac{\text{mU ethylumbelliferone dealkylase}}{\text{mU NADPH–cyt. P-450 reductase} \times \text{nmoles cyt. P-450/mg protein}}$$

Obviously these two inducers preferably enhanced cyt. P-450 species with a low dealkylase activity. The Q-values were reproducible. Q was increased by 100 per cent during induction of a MC-sensitive mouse strain with 3-methylcholanthrene, but it was only moderately decreased by induction with phenobarbital. Corresponding to this, methylcholanthrene is known to selectively induce a cyt. P-448 with high dealkylase activity whereas phenobarbital is known to change the hydroxylase specificity in the liver microsomes not very much.

The premise of this paper is the following working hypothesis: There are different cyt. P-450 species in the liver microsomes with different hydroxylase specificities [1–6]. However in the adult organism this cyt. P-450 population remains constant under normal and constant environmental conditions. An exogenous inducer may alter the P-450 population and with optimal doses a new population of cyt. P-450 species is induced again remaining constant as long as the treatment is continued.

To recognize a change in the microsomal hydroxylase population we need only a single hydroxylase substrate, if the apparent molar activity of the hydroxylase system is known. As the hydroxylases are bound to the microsmal membranes, not only the kinetic parameters of the enzymes, but also the solubility of the substrates in the membranes may decisively influence the velocity of hydroxylations [7–9]. Therefore we can get reference parameters only in an empirical way.

Some time ago we reported: Treating female albino mice (HOM) by cyclohexane inhalation, the product [NADPH–cyt. P-450 reductase activity × cyt. P-450 concentration] is a suitable reference parameter for the ethylumbelliferone dealkylase activity [8, 10]. The quotient Q is the apparent molar activity searched for, which means the relative dealkylase activity.

$$Q = \frac{\text{mU ethylumbelliferone dealkylase}}{\text{mU NADPH–cyt. P-450 reductase} \times \text{nmoles cyt. P-450/mg protein}}$$

This relation is valid because one reductase molecule is combined with an excess of cyt. P-450 molecules in the order of 10–20 [11]. The reductase cannot be 'saturated' with a single cyt. P-450 species.

Changing the cyt. P-450 population, an inducer will also change the relative ethylumbelliferone dealkylase activity. If the cyt. P-450 population becomes constant again, the relative dealkylase activity will also become constant despite a further increase in its specific and total activity.

In the paper presented here we show, that the reference parameter [P-450 reductase activity × P-450 concentration] for the ethylumbelliferone dealkylase activity is valid after induction with cyclohexane, D,L-camphor, phenobarbital or 3-methylcholanthrene. We prefer inducers without or with only one reactive group like cyclohexane and camphor, because these may show a more uniform effect than many drugs and xenobiotics. Phenobarbital and methylcholanthrene are well suited for testing the applicability of our reference parameter, because they are the most thoroughly investigated agents of two groups of inducers with varying effectiveness [12–16].

Abbreviations (used mainly in the figures): EU = ethylumbelliferone; CH = cyclohexane; CA = D,L-camphor; PB = phenobarbital; MC = 3-methylcholanthrene; K = controls fed only 2 hr/day; V = mice with freely accessible food; U = unit of the enzyme activity = 1 μmole/min.

* A summary of this paper was a part of a short lecture during the 558th Meeting of the Biochemical Society, Edinburgh 1975, see *Biochem. Soc. Transact.* **3**, 972.

MATERIALS AND METHODS

Chemicals. Inorganic chemicals (p.a.), sodium phenobarbital (puriss.) and solvents (p.a.) were obtained from Merck, Darmstadt, D,L-camphor (m.p. 175–177°) from Aldrich-Europe (Janssen, Düsseldorf), 3-methylcholanthrene (puriss.) from Fluka, Buchs and biochemicals from Boehringer Mannheim. The silicon Nr. 0860 from Roth, Karlsruhe was taken as antifoam agent.

Animals. Female albino mice of 22–25 g were taken from our own inbred strain WHW/HOM, which came originally from the Deutsche Gesellschaft für Versuchstierzucht, Hannover-Linden. It was inbred in our institute during 10 years by Dr. Gertrud Werth, Homburg, Saar. As the strain WHW/HOM is MC-insensitive [17], for the experiments with 3-methylcholanthrene also female black mice (18–21 g) were taken from the well known MC-sensitive strain C57BL/6N kindly supplied by Dr. D. W. Nebert, Institutes of Health, Bethesda, MD.

Feeding. In all experiments drinking water was freely accessible. Inhalating cyclohexane or camphor, the mice slept often for many hours especially at the 1st day of induction. Therefore these mice and their controls K received Altromin food (Juchem, Eppelborn) each day from 8–10 o'clock in the morning, outside of the cages in fresh air.

After application of methylcholanthrene and even after phenobarbital the mice did not sleep. It was not necessary to fix and limit their feeding time.

Induction. For inhalation of cyclohexane or camphor, four mice were set together into a round plexiglass cage with wire bottom and cover. This was set into an outer cage with a cone-shaped metal gauze on a large funnel. A urine flask with 25 ml 2 N H_2SO_4 was attached to the funnel tube. Four flat disks with sodium peroxide were placed into the outer cage, which was closed air-tightly as soon as the volatile inducer had been brought into it. Fresh inducer was given each day. Table 1 shows inducer dosage and duration of induction.

Cyclohexane and camphor treated animals were set into fresh air 1 and 2 hr respectively before death by cerebrospinal stretching. Phenobarbital or methylcholanthrene treated mice were killed 24 hr after the last injection. All animals were killed at 8 o'clock in the morning. With each of the four inducers the short-term experiments from 0–24 hr were done simultaneously for the purpose of comparison. The room temperature was 23–26°.

Concentration of cyclohexane and camphor in the induction cages. 1 ml cyclohexane (0.778 g) was given upon a cotton plug in the outer cage of 40 l. The initial CH-concentration was 2.3×10^{-4} M each day. The daily consumption of CH by the four mice may be in the order of 20 per cent of the dosage. 4 ml of a solution of 40 g D,L-camphor in 100 ml acetone were equally distributed on both sides of a 2-times folded paper handkerchief of 3.2 g. This was impregnated with 1.2 g camphor after evaporating the solvent. At 24° such a preparation was placed in each of four induction cages containing sodium peroxide and 2 N H_2SO_4, but no mice. The handkerchief lost 182 mg camphor after 2 hr and 246 mg (S.D. = ±5 mg) after 4, 6 and 12 hr. According to this the concentration of camphor should be about or less than 4×10^{-5} M in the air of the cage 3–4 hr after closing it (see also Ref. 18). On average four mice consumed 300 mg camphor/day leaving 650 mg in the handkerchief. Therefore we can assume: Between 3–4 hr after closing and the next opening the camphor concentration in the air of the cages remains constant, being easily filled up by the excess in the handkerchief. In further experiments the exact values of CH- and CA-concentrations in air and mouse and particularly the metabolism of CH and CA in treated and untreated mice will be investigated with the aid of a gas–liquid-chromatograph.

Controls. Originally we had four different control groups according to the different experimental conditions (Table 2). Investigating the time-course of inductions, we needed the untreated animals K_1 especially for all short-term, but also for all other experiments, K_1, K_2 and K_3 could be joint to a single group V, because their liver microsomes showed identical results.

Analytical methods. The liver microsomes were isolated as described by Mohn and Philipp [10], and their protein was estimated according to Gornall *et al.* [19]. The cytochromes P-450 and b_5, the activity of the ethylumbelliferone dealkylase and the NADPH–cyt. P-450 reductase activity were estimated according to Omura and Sato [20, 21], Ullrich and Weber [17] and Diehl *et al.* [22] respectively.

RESULTS

Induction by cyclohexane. During the inhalation of 2×10^{-4} M cyclohexane the values of the NADPH–cyt. P-450 reductase and the cyt. P-450 concentration rose from the beginning, but the activity of the ethyl-

Table 1. Treatment of female mice with different inducers. Dosage and duration of treatment

Inducer	Daily dosage	Kind of application	Duration of treatment (days)
Cyclohexane	~2×10^{-4} M in the air	Inhalation	0.25–3
D,L-Camphor	~4×10^{-5} M in the air	Inhalation	0.25–4
Phenobarbital	100 mg/kg	i.p.	0.25–3
3-Methylcholanthrene	20 mg/kg	i.p.	0.25–2

Table 2. The controls for the experiments with the mice WHW/HOM

Group		Treatment	Feeding	Corresponding experimental animals
K		Housed in closed induction cages for 0.25–4 days	8–10 a.m. outside the cages	CH- and CA-treated mice
V	K_1	Untreated	Not limited	Especially short-term treated mice HOM
	K_2	0.2 ml 0.9% NaCl i.p. at each of 3 days	Not limited	PB-treated mice
	K_3	0.2 ml wheat germ oil i.p. at each of 2 days	Not limited	MC-treated mice HOM

$K_1 + K_2 + K_3$ represent the control group *V*.
CH = Cyclohexane; CA = D,L-camphor; PB = phenobarbital; MC = 3-methylcholanthrene.

umbelliferone dealkylase did not increase before the first 24 hr [10]. This behavior of the enzymes was proved to be reproducible and this is to be best seen in the time dependence of the relative dealkylase activity (Fig. 1).

After 1 day of cyclohexane inhalation the *Q*-value became constant and was equal to 0.04 mU dealkylase/[mU reductase × nmoles cyt. P-450/mg protein]. In former experiments we found 0.05. The *Q*-values of the treated animals were significantly lower than those of the controls as early as 12 hr after the beginning of the experiment. The difference rose with induction time. Obviously the controls got accustomed to the feeding limited to 2 hr/day.

D,L-*Camphor as a new inducer for microsomal hydroxylation.* Other saturated hydrocarbons being related to cyclohexane were tested for their inducing properties. *Cis-* and *trans*-decaline were too toxic in that concentration range, which would be necessary for induction [23]. Then D,L-camphor was chosen, although its molecule is inflexible and under high strain and therefore little related to cyclohexane. Camphor is known to be hydroxylated in the mammalian organism *in vivo* and *in vitro* [24, 25]. Moreover it is inducer and substrate of the cyt. P-450 system in *Pseudomonas putida* [26, 27]. The present study demonstrates that camphor is also an inducer of the hydroxylase system in the mouse liver microsomes (Figs. 2 and 3, see also Kessler and Mohn, in preparation).

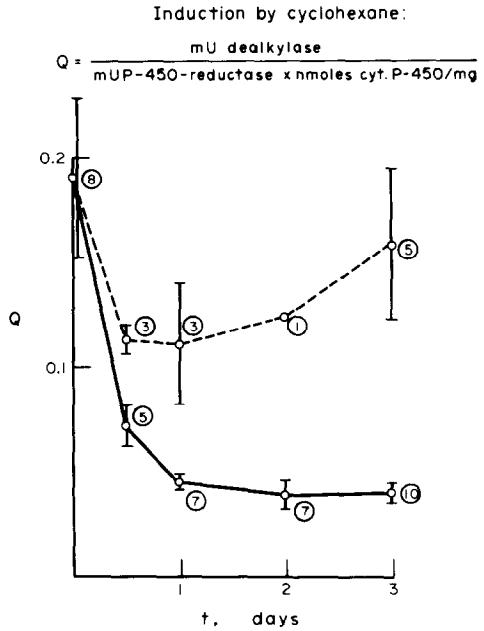

Fig. 1. Induction by cyclohexane. The relative ethylumbelliferone dealkylase activity *Q* in the liver microsomes of albino mice WHW/HOM as a function of the induction time. *Q* = mU dealkylase/[NADPH–cyt. P-450 reductase × nmoles cyt. P-450/mg protein]. O——O Induced mice; O- - -O controls. Bars represent ±S.E.M. The numbers in circles are the numbers of investigated groups at each point of time. 8 livers were pooled per group.

At the beginning the reductase activity was increasing more markedly than cyt. P-450 whereas the main increase of the dealkylase activity was not seen before 24 hr of induction. Within 2 days the nmoles cyt. P-450 and mU dealkylase per mg protein increased by 250 per cent and the mU reductase by 350 per cent (Fig. 2). The first decrease of the specific activities or concentrations respectively was seen at 2.5 days of induction. Obviously this was caused in part by dilution of the hydroxylase proteins by other microsomal proteins, because the total values per liver were increasing until 2.5 days (Fig. 3).

During induction by camphor the behaviour of the relative ethylumbelliferone dealkylase activity *Q* was

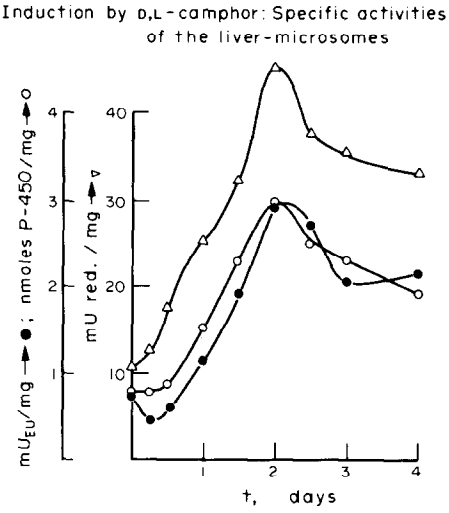

Fig. 2. Induction by D,L-camphor. The specific activity of NADPH–cyt. P-450 reductase △ and ethylumbelliferone dealkylase ● and the nmoles cyt. P-450/mg protein ○ as a function of the induction time.

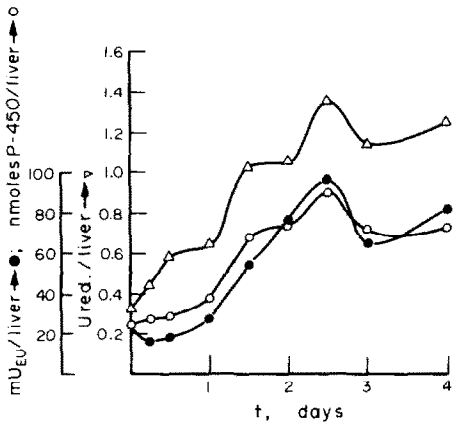

Fig. 3. Induction by D,L-camphor. The total activity of NADPH–cyt. P-450 reductase △ and ethylumbelliferone dealkylase ● and the total quantity cyt. P-450 ○ per liver as a function of the induction time.

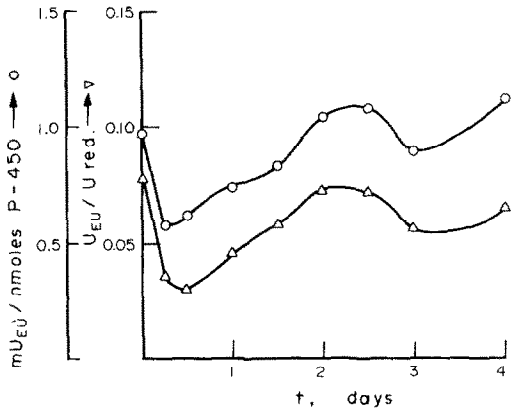

Fig. 5. Induction by D,L-camphor. The quotients mU ethylumbelliferone dealkylase/mU NADPH–cyt. P-450 reductase △ and mU ethylumbelliferone dealkylase/nmoles cyt. P-450 ○ as a function of the induction time.

quite similar to that during induction with cyclohexane (Figs. 4 and 1). Q was quickly decreasing somewhat longer than 24 hr. Then it remained constant for at least 3 days. This Q-value was significantly lower than that during permanent induction with cyclohexane ($P < 0.05$). The controls were the same as in Fig. 1. If we stopped the camphor inhalation after 3 days, the relative dealkylase activity increased within 2 days to the value of untreated animals.

The curve of the relative dealkylase activity is not only more continuous than the curves of the specific and total activities (Figs. 2 and 3), but especially more continuous than that of the dealkylase activity related to the reductase activity or to the cyt. P-450 concentration alone (Fig. 5). From the two latter curves we obtain little informations about the alteration of the hydroxylase system by camphor, and that is also true for analogous curves during induction with cyclohexane [10]. However, we see that reductase and cyt. P-450 are induced simultaneously after the 1st day of camphor inhalation (see also Fig. 3). This was not seen during cyclohexane inhalation.

Induction of the ethylumbelliferone dealkylase by phenobarbital and 3-methylcholanthrene. The experiments with phenobarbital (Fig. 6) were carried out with the strain WHW/HOM whereas those with 3-methylcholanthrene were carried out with the MC-sensitive black strain C57BL/6N giving similar high dealkylase activities after treatment with methylcholanthrene as our mice HOM after induction by the other three substances.

In contrast to cyclohexane and camphor, phenobarbital and methylcholanthrene made the dealkylase increase from the beginning of the induction and that considerably more rapidly than the other two parameters. After 24 hr of treatment with phenobarbital the reductase was obviously rising selectively. With both of the inducers the time course of the total activities and concentrations of the enzymes (not shown here) was quite similar to that of the specific values.

Despite the similar increase in the specific dealkylase activity during the first 24 hr (Figs. 6 and 7). Figure 8 shows us the difference expected in the relative dealkylase activities. During phenobarbital treatment Q is decreasing somewhat, but not so much as during treatment with cyclohexane or camphor. In contrast, Q is rising 2-fold during the short-term induction by methylcholanthrene.

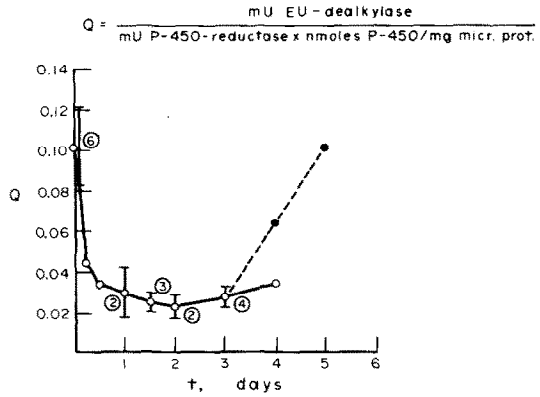

Fig. 4. Induction by D,L-camphor. The relative dealkylase activity Q as a function of the induction time. ○——○ CA-treated mice; ●– – –● CA-treated mice after stopping the camphor inhalation.

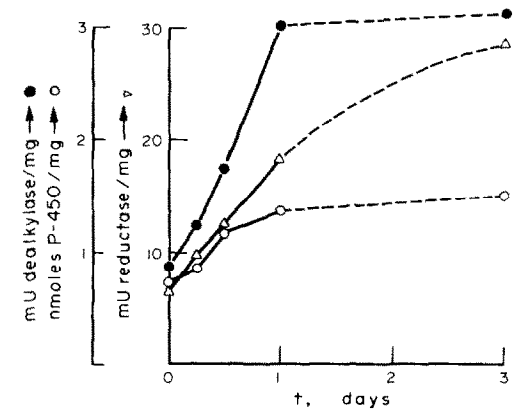

Fig. 6. Induction by phenobarbital. The specific activity of NADPH–cyt. P-450 reductase △ and ethylumbelliferone dealkylase ● and the nmoles cyt. P-450/mg protein ○ in the liver microsomes of mice HOM as a function of the induction time.

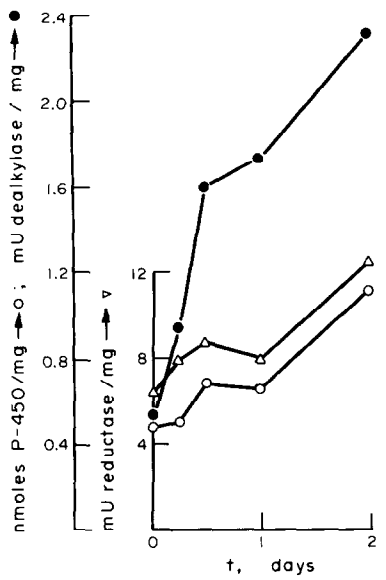

Fig. 7. Induction by 3-methylcholanthrene. The specific activity of NADPH–cyt. P-450 reductase △ and ethylumbelliferone dealkylase ● and the nmoles cyt. P-450/mg protein ○ in the liver microsomes of female black mice C57BL/6N as a function of the induction time.

Comparison of the effects of the four inducers after maximal induction in the mouse strain WHW/HOM. All of the experiments in Figs. 9–11 were done with our MC-insensitive strain WHW/HOM. PB- and MC-treated animals are to be compared with the controls V and CH- or CA-treated animals with the controls K (see under Methods).

On the left side of Fig. 9 the sequence of the relative dealkylase activities is $Q_{MC} \geq Q_K \approx Q_V \geq Q_{PB} > Q_{CH} > Q_{CA}$. In the hydroxylase population the percentage of cyt. P-450 molecules with high dealkylase activity should decrease in the same sequence. On the right side the relative values of the total dealkylase activities Q' are shown, calculated from the total activities of reductase and dealkylase and the total amount of cyt. P-450 in the liver. Here the Q' value of the controls K is quite out of place. In contrast to the cyclohexane or camphor treated mice, the K-mice lost not only 20 per cent of body weight, but also 40 per cent of liver weight. This severe loss of liver weight, caused by the limited feeding, was abolished by the induction with cyclohexane or camphor. Beyond this both of these inducers enhanced the mg microsomal protein per g liver by 50–100 per cent.

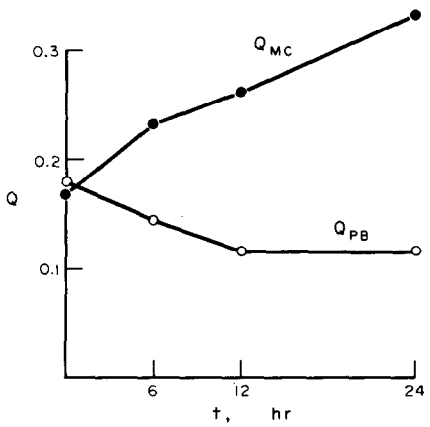

Fig. 8. The relative ethylumbelliferone dealkylase activity Q dependent on the induction time. ○——○ Phenobarbital treated mice HOM; ●——● 3-methylcholanthrene treated mice C57BL/6N.

The comparison of the specific and the total activities of reductase and dealkylase and of the specific and the total amount of cyt. P-450 shows us some interesting details in the different effectiveness of the four inducers. Phenobarbital enhanced the dealkylase more than the other 3 substances did (Fig. 10, left side). Methylcholanthrene induced a highly significant increase in the dealkylase activity even in our MC-insensitive albino strain ($P < 0.001$). Cyclohexane and camphor treated mice had a total dealkylase activity being 6-fold higher than that of the controls K. Cytochrome P-450 and the P-450 reductase were most highly induced by camphor (Fig. 10, right side; Fig. 11, left side). The total reductase activity was 10-fold higher than in the liver of K-mice. After induction by methylcholanthrene even the total values of cyt. P-450 and P-450 reductase were not significantly different from those of the controls V ($P < 0.1$; $P = 0.3$).

On the right side of Fig. 11 we find the specific and the total content of cyt. b_5 in the liver microsomes. The latter particularly should be a measure for the portion of endoplasmatic reticulum in the liver cell. Each of the four inducers increased cyt. b_5 significantly, but cyclohexane did so to the greatest degree.

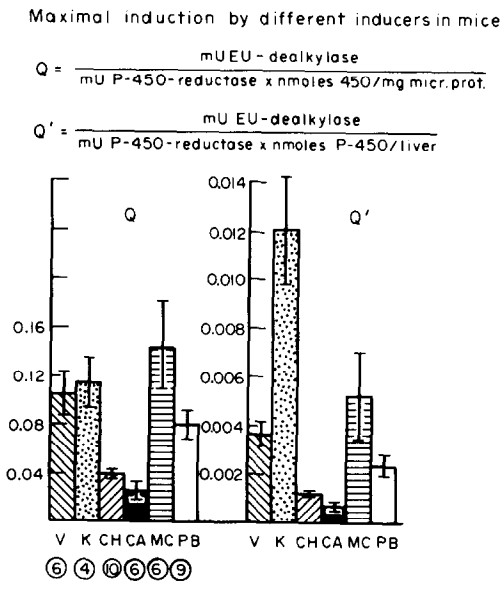

Fig. 9. Treatment of mice HOM by different inducers. Comparison of the relative ethylumbelliferone dealkylase activity Q and Q'. Q = mU dealkylase/[mU reductase × nmoles P-450 per mg protein]. Q' = mU dealkylase/[mU reductase × nmoles P-450 per liver]. V = untreated mice; K = controls fed only during 2 hr/day; CH, CA or PB = mice treated for 3 days by cyclohexane, D,L-camphor or phenobarbital; MC = mice treated for 2 days by methylcholanthrene. Bars represent ±S.E.M. The numbers in circles are the numbers of groups investigated. 4–8 livers were pooled per group.

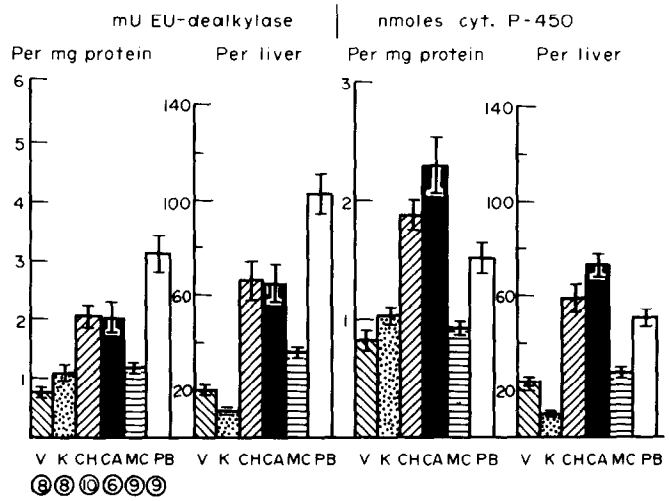

Fig. 10. Treatment of mice HOM by different inducers. Left side: Comparison of the specific and the total ethylumbelliferone dealkylase activity. Right side: Comparison of the nmoles cyt. P-450 per mg protein and per liver. Further details see Fig. 9.

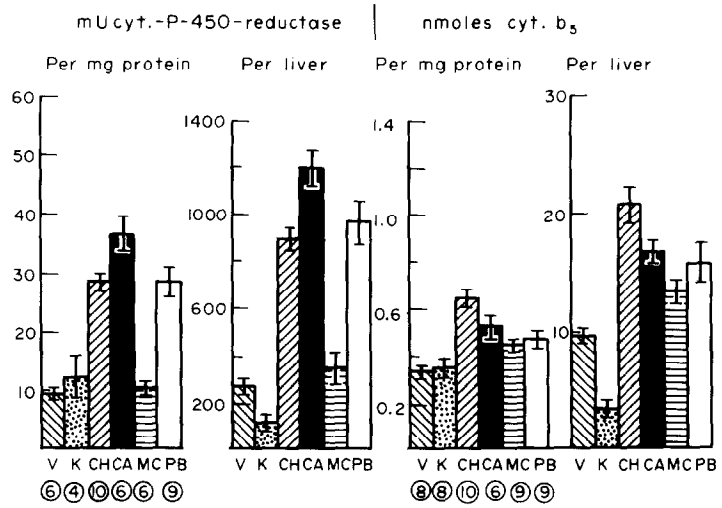

Fig. 11. Treatment of mice HOM by different inducers. Left side: Comparison of the specific and the total activities of the NADPH–cyt. P-450 reductase. Right side: Comparison of the nmoles cyt. b_5 per mg protein and per liver. Further details see Fig. 9.

DISCUSSION

The product [NADPH–cyt. P-450 reductase activity × cyt. P-450 concentration] is a suitable reference parameter for the ethylumbellferone dealkylase activity especially during induction with cyclohexane or camphor (Figs. 1 and 4). Plotting the relative dealkylase activity Q against the induction time, we receive continuous curves indicative of a permanent induction. This is not the case, if we refer the dealkylase activity to the reductase activity or to the cyt. P-450 concentration alone (compare Figs. 4 and 5; see also [10]).

With the aid of the relative ethylumbelliferone dealkylase activity the main difference between induction by phenobarbiyal and 3-methylcholanthrene can be shown. The induction by phenobarbital is comprehensive. Gillette and co-workers write: "Phenobarbital increases the metabolism of most of the drug substrates of the microsomal enzymes" (see Ref. 28, p. 72). Many of the hydroxylase activities are enhanced by a factor of 2–4 [12, 29, 30], and this is also true for the EU-dealkylation (Fig. 10). Therefore Q is not altered very much (Fig. 8). Methylcholanthrene induces cyt. P-448 selectively [14, 15]. According to Nebert such a hydroxylase system has a specificity range much in favor of the ethylumbelliferone dealkylation and the N-demethylation of azo-dyes (see Ref. 16, p. 153). Accordingly, Q increases markedly (Fig. 8). Obviously cyclohexane and camphor also induce selectively, but contrary to methylcholanthrene, one or more cyt. P-450 species with a low dealkylase activity. There may be other hydroxylase activities, which are preferably induced, for instance the hexobarbital hydroxylation (Weber and Mohn, in preparation) and the N-Demethylation of aminopyrine (Mohn and Philipp, in preparation).

Besides of this, there are other possibilities to explain a decrease of Q during treatment of mice with cyclohexane and camphor, for instance an enhanced

uncoupling in the presence of substrate or an alteration of the microenvironment of the enzymes (see also Ref. 31, p. 5855). Such possibilities will be discussed in our forthcoming papers. The selectivity of the induction is the most probable and the most interesting explanation.

We estimated the NADPH–cyt. P-450 reductase by the plunger method [10, 22, 32]. We obtained the best results measuring the reductase rate exactly 1 sec after the start (see also Ref. 32). In all experiments NADPH reduced at least 90 per cent of the cyt. P-450 being available with sodium dithionite.

After treatment of the albino mice HOM by cyclohexane, camphor or phenobarbital, the reductase activity was not significantly enhanced in the presence of ethylumbelliferone, aminopyrine or cyclohexane, in contrast to the behaviour of the P-450 reductase in the liver microsomes of male rats (Mohn, Kessler and Hoffmann, in preparation; [33]). Using the stopped-flow method [34] and adding ethylmorphine, cyclohexane or other substrates, Matsubara and coworkers [35] found reductase rates in the first 100 msec being 3–5 times higher than our values. However neither our values nor those from Matsubara are rate limiting in the aerobic hydroxylase reactions, during which the cyt. P-450 substrate complex can be reduced only as the nascent reduced complex is captured with high velocity by the following reaction steps. Obviously our reductase values are closely related to the reductase concentrations in the microsomes, and for this reason only is this reductase activity a factor in our reference parameter. It is questionable, whether the same would be valid in microsomal preparations, the reductase of which is highly substrate dependent [22, 28, 32, 35–37]. Matsubara and coworkers represent the idea, that the enzyme may not be rate limiting at the first reduction step of the hydroxylation. This is based on a former assumption of Estabrook [38], namely that a ternary complex of reduced cyt. P-450, substrate and O_2 was enriched during hydroxylation of hexobarbital. Recently Guengerich and co-workers found a similar complex with benzphetamine, O_2 and isolated reduced cyt. $P\text{-}450_{LM}$ [39]. However, the nature and function of these complexes are not clear. According to Guengerich, the NADPH–cyt. P-450 reductase may be rate limiting in the second reduction step of the hydroxylation.

Apart from the O_2-sensitivity of the P-450 reductase reaction there are two other arguments for the rate limiting role of this enzyme in hydroxylase reactions: the unfavourable relation reductase to P-450 molecules of 1:10–20 [11] and the heterogenity of cyt. P-450 [1–6]. Despite the apparent excess of P-450 molecules, the P-450 concentration is the 2nd factor in our reference parameter. Provided that under aerobical condition there is actually a considerable affinity between reductase and the P-450 substrate complex, this may not be effective, possibly because of a spatial separation of the active centers or of the active and binding centers and a relatively low mobility of the two hydroxylase components.

During treatment of female mice with cyclohexane and DL-camphor two different induction phases can be distinguished with the aid of the relative dealkylase activity Q: (1) the alteration of Q, that means a change of the specificity of the hydroxylase system by a change of the P-450 population (Figs. 1, 2 and 8); (2) a period, during which Q is constant, which may be explained by a similar increase of all components of the hydroxylase system being preferably present in the microsomes not earlier than 24 hr after beginning of the treatment (Figs. 1 and 4).

During the second phase also cyt. b_5 is enhanced, and this indicates an increase in the endoplasmatic reticulum [29] (Fig. 11). Besides, there may also be an inhibition of the degradation of the hydroxylases, as it is known from phenobarbital induction [40, 41]. Possibly the whole system can be activated by a selective enhancement of the NADPH–cyt. P-450 reductase. The treatment of our MC-insensitive albino mice by methyl-cholanthrene is particularly interesting. We saw no second induction phase. The change in the P-450 population took place as Q was rising and λ_{max} of the CO-complex of the hydroxylating cytochromes shifted from 450 to 449 nm. However the increase in reductase activity and P-'450'-concentration was lacking (Figs. 10 and 11).

Our results include the possibility, that each of the two hydroxylase components may appear to be rate limiting alone, if the other does not alter significantly. We assume that our reference parameter [NADPH–cyt. P-450 reductase activity × cyt. P-450 concentration] may be valid not only for the ethylumbelliferone dealkylation, but also for other hydroxylase reactions. We intend to investigate this in further experiments.

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