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DIFFERENCES BETWEEN INDUCTION EFFECTS OF 1,4-bis[2-(3,5-DICHLOROPYRIDYLOXY)]BENZENE AND PHENOBARBITONE

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Abstract—The inductive effects of phenobarbitone (PB) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) were compared in C57BL/6J mice. Induction parameters included six substrates: ethylmorphine (EM), benzphetamine (Bph), biphenyl, ethoxycoumarin (EtoC), pentoxyresorufin and dichloro-*p*-nitroanisole (DPNA). In order to validate this descriptive approach the comparison was extended to diazepam, rifampicin, warfarin, and pregnenolone-16α-carbonitrile (PCN). All inducers were clearly distinguishable from each other. Warfarin was similar to PB, rifampicin was similar to PCN. TCPOBOP differed significantly from PB in relative liver weight, cytochrome P-450 content of liver microsomes, EM-, Bph- and DPNA-demethylations, biphenyl-hydroxylations, EtoC de-ethylation and absorption maximum of reduced CO–cytochrome P-450. TCPOBOP, as an inducer, was less "specific" than PB: total metabolic rates were excessively increased due to microsomal protein (1.5 times) and cytochrome P-450 (4 times) augmentation, whereas cytochrome P-450-related metabolic rates were less increased than those after PB. Thus TCPOBOP does not seem to be as similar to PB as was suggested in the first description of its inducing potency.

TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene) was synthesized and described as inducing the hepatic microsomal monooxygenase system by Poland and coworkers [1]. They found the induction effects of TCPOBOP to be very similar to those of phenobarbitone (PB) and the inductive potency to be 650 times that of PB (on the basis of increased aminopyrine demethylation). The authors write: "If TCPOBOP is a phenobarbarbital-like agonist, then it should evoke all of the pleiotropic effects produced by phenobarbital." Similarity to PB as inducer and substantial potency would render TCPOBOP a tool for investigation of a possible receptor mechanism in PB induction.

In a previous paper [2] we have shown that the inductive effects of even true barbiturates are clearly distinguishable from each other when compared by a rather conventional set of induction parameters in a refined experimental design. This casts some doubt on the idea of a pleiotropic response and an underlying receptor mechanism. If this doubt applies even to true barbiturates, it should apply even more to the "phenobarbital-like agonist" TCPOBOP. Therefore, we now extend our descriptive approach to that inducing agent.

If our approach were able to differentiate even between barbiturates, it should differentiate also between barbiturates and non-barbiturate inducers, and even more so between inducers of, for example, the rifampicin-PCN type [3–7]. Thus, we complemented the comparison between phenobarbitone and TCPOBOP by including other inducers such as diazepam, rifampicin, PCN and warfarin in order to validate the approach and illustrate the amount of difference. Warfarin has been described to be an inducer similar to phenobarbitone [8]. Inductive effects of diazepam in humans and rats have been described earlier [9–11].

Induction parameters measured were body weight, relative liver weight, microsomal protein and cytochrome P-450 content of the liver, wavelength of reduced CO–cytochrome P-450, ethylmorphine and benzphetamin demethylations, 2- and 4-hydroxylations of biphenyl, ethoxycoumarin de-ethylation and pentoxyresorufin dealkylation. C57BL/6J mice were used as experimental animals.

MATERIALS AND METHODS

Animals. Male C57BL/6J/Han mice were purchased from Zentralinstitut für Versuchstierzucht, Hannover (F.R.G.), 5 weeks ± 3 days of age. They were kept in plastic cages (4 animals each) with sawdust bedding. Bedding was taken from the same charge as used by the breeder. Animals received commercial pellet diet (ssniffM from Intermast, Bockum-Hövel, F.R.G.) and water *ad libitum*. Room temperature was 22–25°. Darkness was timed from 6 p.m. to 6 a.m. Animals were allowed to acclimatize for one week before treatment.

Treatment. Diazepam (2 × 5 mg/kg body wt) and rifampicin (2 × 40 mg/kg body wt) were given in 1% methylcellulose p.o. over a period of 6 days. Warfarin (120 mg/kg) was given in 1% methylcellulose p.o. over a period of 3 days. PCN (2 × 50 mg/kg body wt) was given in aqueous suspension i.p. over a period of 3 days. Phenobarbitone (2 × 50 mg/kg body wt) was given in aqueous solution i.p. during a period of 6 days. TCPOBOP (3 mg/

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kg body wt) was given in Arachis oil as a single dose 3 days before sacrifice. Controls received 1% methylcellulose or 0.9% NaCl plus one dose of Arachis oil, respectively, over a period of 6 days. In all cases, the last dose was given 20 hr, and food removed 16 hr, prior to sacrifice.

Preparation. Animals were sacrificed on Tuesdays between 8.30 and 10.00 a.m. by cervical dislocation. Livers were perfused *in situ* with ice-cold 1.15% KCl for 2 min then excised, gall bladders removed, weighed and homogenized by a glass–Teflon homogenizer (8 strokes in 2 min) and microsomes prepared. 100,000 *g* pellets were equally resuspended in a 3 ml volume pH 7.4 buffer, containing 20 mM Tris and 0.25 M saccharose, and following protein determination, diluted to 5 mg microsomal protein per ml suspension. Microsomes for benzphetamine, ethoxycoumarin and pentoxyresorufin metabolism were stored overnight at −20°.

Assays. Cytochrome P-450 was measured according to Omura and Sato [12], using a Kontron Uvikon 810 spectrophotometer. CO-cytochrome P-450 peaks were recorded as sections of the spectrum following determination of cytochrome P-450 content. All other assays were performed as described before [2]. They were finished within 2 days. Synthesis and determination of dichloro-*p*-nitroanisole (DPNA) will be published in a following paper.

Chemicals. Ethylmorphine and Folin-Ciocalteu's reagent were purchased from Merck (Darmstadt, F.R.G.), phenobarbitone, 2,3,5-trichlorpyridin and hydroquinone from Fluka (Buchs, Switzerland); bovine serum albumin, biphenyl, 2-OH-biphenyl, 4-OH-biphenyl and isocitrate from Serva (Heidelberg F.R.G.); glucose-6-phophate, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and NADP from Boehringer (Mannheim, F.R.G.); 7-hydroxycoumarin from Janssen (Düsseldorf, F.R.G.). Warfarin was kindly donated by Richardson-Merrell (Groß-Gerau, F.R.G.), rifampicin by Chemie Grünenthal (Stolberg, F.R.G.); PCN by R. Schulte-Hermann, the material being synthesized by Schering (Berlin, F.R.G.); pentoxyresorufin by R. T. Mayer, the material being synthesized by M. D. Burke; benzphetamine by Temmler, Marburg, the material being synthesized by B. Stick. Ethoxycoumarin was synthesized in this laboratory by W. Legrum according to Allen and Gates [13]. TCPOBOP was synthesized by E. G. according to Poland *et al.* [1]. Analytical data: melting point, 157°, CHN analysis, C, 47, 85% (theoretically 47.80%), H, 2.06% (theoretically 2.09%), N, 6.99% (theoretically 6.97%); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.10 (s, 4 H aromatic), δ = 7.70 (d, 2 H aromatic, J = 2.4 Hz), δ = 7.8% (d, 2 H aromatic, J = 2,4 Hz); mass spectrum: EI, m/e = 400 (M^+ for Cl^{35}).

Design and statistical evaluation. The design of the whole experiment is given in Fig. 1. In order to adjust the warfarin results (see Fig. 1) the second cell of date 3.7.84, was discarded and an artificial cell for date 5.6.84 formed by averaging the first, second, third and fourth members of the remaining cells. Twenty parameters were measured or calculated and statistically evaluated: 8 "non-metabolic" parameters (body weight, liver weight, relative liver weight (% of body wt), weight of 100,000 *g* pellet, relative pellet weight (% of liver wt), microsomal protein, cytochrome P-450 content of microsomes and wavelength of CO–cytochrome P-450 absorption maximum), 12 metabolic parameters (metabolism of ethylmorphine, benzphetamine, ethoxycourmarin, pentoxyresorufin, biphenyl 2- and 4-hydroxylation, calculated both per mg microsomal protein—not quoted in this paper – and per nmol cytochrome P-450; for DPNA metabolism see Results section). Three types of analysis of variance were used for statistical evaluation

(1) Simple analysis of variance exclusively with *controls* (six cells according to six days of experiment, see Fig. 1 and paragraph "time dependence" in the Results section); (2) Two-factor (4 × 3) analysis of variance, factors being "treatment" and "time", with control and three inducers (see Tables 2a and 2b); (3) Simple analysis of variance with six cells (= treatments). In this type, values were expressed as per cent of the control mean of the respective day and the 3 days of one treatment combined, in order to render series 1 and 2 (see Fig. 1) comparable (see Table 2c).

Homogeneity of variances was evaluated in simple analyses of variance by Bartlett tests, in two factor analyses of variance by Cochran tests. Where homogeneity of variances had to be rejected with $\alpha \leq 5\%$, values were subject to logarithmic (ln) transformation and tested once more (indicated in Tables 2a–c). Comparison of levels in the two factor analyses of variance was achieved by Scheffé tests, comparison of groups following simple analyses of variance by Duncan tests. Calculations were performed by the Rechenzentrum der Philipps-Universität Marburg.

RESULTS

Time dependence of induction parameters

Table 1 lists the respective findings in *controls* (exclusively). As is to be seen from the significances of simple analyses of variance (left hand side of Table 1), the six control groups must not be regarded as random samples drawn from the same population in almost all parameters (exceptions: body weight, cytochrome P-450 content, wavelength of CO–P-450 peak, biphenyl-4-hydroxylation and ethoxycoumarin de-ethylation, calculated per nmol cytochrome P-450). Additionally, the two *series* (see Fig. 1) of controls were compared by *t*-tests (right-hand side of Table 1). In almost all parameters (exceptions: wavelength of CO–P-450 peak and pentoxyresorufin depentylation) series 2 shows the lower means. In 8 parameters the difference is significant. A closer view reveals that the (unknown) influence seems to concern rather the composition of the endoplasmatic reticulum (pellet weights, microsomal protein) than the composition of cytochrome P-450 (exception: biphenyl-2-hydroxylation). If the whole body of data is considered (control and treatment groups), variances between dates of experiment (or animal consignments, respectively) should appear as significances of factor "time" in the two factor analyses of variance. This is the case in several parameters: if all analyses of variance are excluded in which the conditions of normality of samples and homogeneity

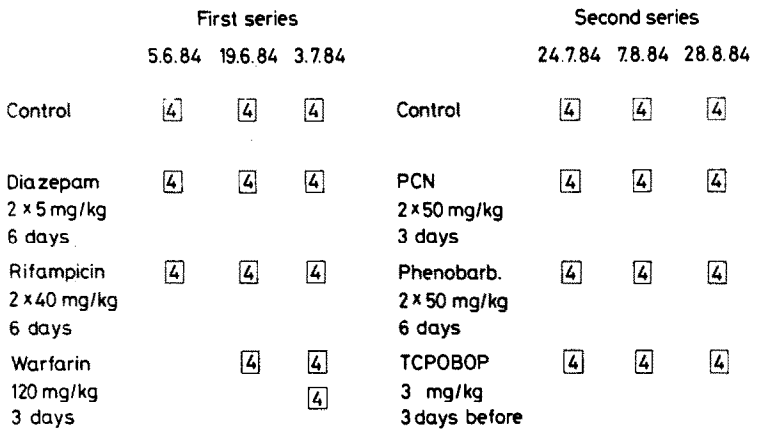

Fig. 1. Design of experiments. Each cell contains four animals.

Table 1. Dependence of controls upon date of experiment

Parameter	Significance α (analysis of variance)	Means Ser.1	Means Ser.2	Significance α (t-tests)
Body weight (g)	NS	19.44	15.15	NS
Liver weight (g)	<2.5%	0.97	0.90	NS
Relative liver weight (% of body weight)	<0.1%	4.86	4.69	NS
Pellet weight (g)	<0.1%	0.22	0.18	<1%
Relative pellet weight (% of liver weight)	<0.1%	22.5	19.8	<1%
Microsomal protein (mg/g liver)	<0.5%	18.8	15.8	<0.1%
Cytochrome P-450 (nmol/mg micr. prot.)	NS	0.733	0.665	NS
Wavelength CO–P-450 max. (nm)	NS	449.8	449.8	—
Metabolism/mg micr. prot.				
Ethylmorphine (μg formaldehyde/10 min)	<0.5%	6.81	5.79	NS
Benzphetamine (μg formaldehyde/10 min)	<5%	2.81	2.18	<5%
Biphenyl-4-hydroxylation (nmol 4-OH-biphenyl/min)	<0.1%	3.72	3.16	<1%
Biphenyl-2-hydroxylation (nmol 2-OH-biphenyl/min)	<0.1%	1.10	0.82	<0.1%
Ethoxycoumarine (nmol 7-OH-coumarine/10 min)	<0.1%	45.73	38.67	<1%
Pentoxyresorufin (pmol resorufin/min)	<1%	12.54	14.99	NS
Metabolism/nmol cytochrome P-450 (units as above)				
Ethylmorphine	<1%	9.72	8.75	NS
Benzphetamine	<5%	4.06	3.30	NS
Biphenyl-4-hydroxylation	NS	5.29	4.78	NS
Biphenyl-2-hydroxylation	<5%	1.55	1.24	<2%
Ethoxycoumarine	NS	64.7	58.3	NS
Pentoxyresorufin	<5%	17.7	22.9	NS

With the indicated significances α have to be rejected the zero hypotheses that all control groups are drawn from one population (left) or that the controls of series 1 and of series 2 (see Fig. 1) are drawn from one population (right).

of variances are not adequately met, then in series 1 (Fig. 1) eight parameters show significances on factor "time" of 1% or less (liver weight, relative liver weight, microsomal protein, relative pellet weight, ethoxycoumarin de-ethylation per protein and per P-450, 4-hydroxylation of biphenyl per P-450). In series 2 (Fig. 1) seven parameters show significances of 2% or less: (body weight, liver weight, pellet weight, cytochrome P-450 content, ethylmorphine demethylation per protein, biphenyl-4-hydroxylation per protein and per P-450).

Interactions between factor "time" and factor "treatment" were significant for 2 induction parameters in series 1 and for 10 induction parameters in series 2 ($\alpha \leq 5\%$, analyses of variance calculated either with untransformed or with transformed values — see Methods section — excluding all having positive Cochran tests and including cytochrome P-450 — as well as protein-related metabolisms).

Induction patterns

Figure 2 and Tables 2a–c and 3 summarize the results of our comparative induction experiments. Figure 2 gives the mean values of ten selected induction parameters measured after treatment with the six inducers, calculated as percentages of controls. Metabolic rates, before, had been calculated per nmol cytochrome P-450. From Fig. 2 and Tables 2a–c may be seen: (1) The six induction patterns are clearly *distinguishable*. The most prominent distinc-

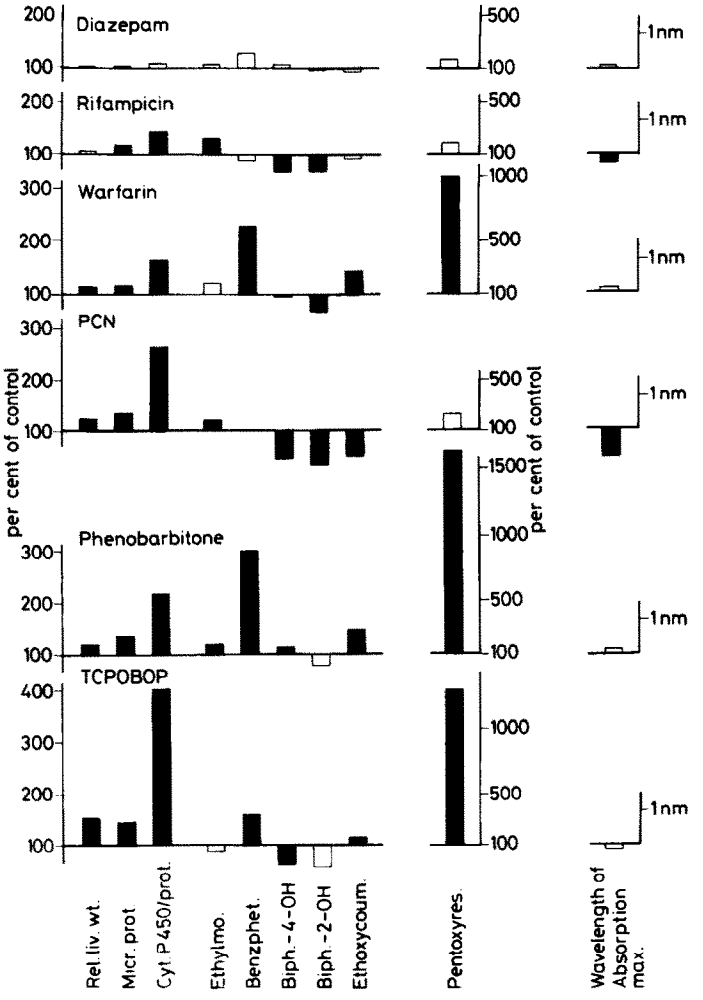

Fig. 2. Ten selected induction parameters in mouse liver microsomes after pretreatment with six non-Ah inducers. Inducers diazepam, rifampicin, warfarin, above (= Series 1, Fig. 1), inducers PCN, phenobarbitone, TCPOBOP, below (= Series 2, Fig. 1). Design of experiments, see Fig. 1. Black columns indicate statistically significant difference from control, $\alpha \leq 5\%$; for significances of differences between inducers see Tables 2 a–c. Absolute means of controls are given in Table 1. Wavelength findings: positive deviation means red shift, negative deviation means blue shift.

tive features of the individual inducers are: diazepam shows no significant alteration at all; rifampicin, although a rather weak inducer (no increase of relative liver weight) shifts the CO–P-450 absorption maximum to shorter wavelengths (blue shift); PCN is the only inducer to decrease three metabolic rates; phenobarbitone produces the maximum increase in pentoxyresorufin metabolism and TCPOBOP the maximum increase in cytochrome P-450. Only warfarin seems to have no distinctive single feature, although it is different from all other inducers; (2) Obviously, there are two pairs of inducers: if the induction parameters are ranked according to the magnitude of deviation from control, the rank order is similar between rifampicin and PCN on the one hand and between phenobarbitone and warfarin on the other (although, in each case, the partner is related to different controls, see Fig. 1). Additionally, rifampicin and PCN have in common the shift of CO–cytochrome P-450 absorption maximum to shorter wavelengths and the failure to increase pentoxyresorufin and benzphetamine metabolisms; (3) The inductive effect of TCPOBOP, which, originally [1], had been introduced as a phenobarbitone-like inducer, is, nevertheless, clearly distinguishable from that of phenobarbitone. TCPOBOP increases liver weight and cytochrome P-450 content excessively and the microsomal protein content is the highest among all inducers. It does not increase ethylmorphine and only slightly increases benzphetamine metabolism, which would be typical for phenobarbitone induction. Biphenyl-4-hydroxylation is even decreased. There is a significant difference between the wavelengths of reduced CO–cytochrome P-450 peaks (see Fig. 2, Tables 2b and c) after PB and TCPOBOP. Additionally, there are differences between PB and TCPOBOP with respect to their effects on the metabolism of dichloro-*p*-nitroanisole, a substrate which was introduced and found to be phenobarbitone-specific in rats by

Table 2. Significance of differences between six non-Ah inducers in 10 selected induction parameters

(a)

Rel. liv. wt.	Dia	Rif	War
Con	•	•	**
Dia		•	**
Rif			**

Micr. prot.	Dia	Rif	War
Con	•	**	**
Dia		**	**
Rif			•

Cyt. P-450§	Dia	Rif	War
Con	•	**	**
Dia		**	**
Rif			•

Ethylm./P-450§	Dia	Rif	War
Con	•	*	•
Dia		•	•
Rif			•

Benzph./P-450§	Dia	Rif	War
Con	•	•	**
Dia		•	**
Rif			**

Biph.-4-OH/P-450	Dia	Rif	War
Con	•	**	•
Dia		**	•
Rif			**

Biph.-2-OH/P-450	Dia	Rif	War
Con	•	**	**
Dia		**	**
Rif			•

Ethoc./P-450	Dia	Rif	War
Con	•	•	**
Dia		•	**
Rif			**

Pentoxy./P-450‡	Dia	Rif	War
Con	•	•	**
Dia		•	**
Rif			**

Peak wavelength‡	Dia	Rif	War
Con	•	**	•
Dia		**	•
Rif			**

(b)

Rel. liv. wt.	PCN	PBa	TCP
Con	**	**	**
PCN		•	**
PBa			**

Micro. prot.‡	PCN	PBa	TCP
Con	**	**	**
PCN		•	*
PBa			•

Cyt. P-450	PCN	PBa	TCP
Con	**	**	**
PCN		**	**
PBa			**

Ethylm./P-450§	PCN	PBa	TCP
Con	**	**	•
PCN		•	**
PBa			**

Benzph./P-450	PCN	PBa	TCP
Con	•	**	**
PCN		**	**
PBa			**

Biph.-4-OH/P-450	PCN	PBa	TCP
Con	**	**	**
PCN		**	**
PBa			**

Biph.-2-OH/P-450	PCN	PBa	TCP
Con	**	**	**
PCN		**	•
PBa			**

Ethoc./P-450†‡	PCN	PBa	TCP
Con	**	**	**
PCN		**	**
PBa			**

Pentoxy./P-450§	PCN	PBa	TCP
Con	•	**	**
PCN		**	**
PBa			**

Peak wavelength	PCN	PBa	TCP
Con	**	•	•
PCN		**	**
PBa			**

(c)

rel. liver wt§	Dia	Rif	War	PCN	PBa	TCP
Dia		•	**	**	**	**
Rif			**	**	**	**
War				**	**	**
PCN					•	**
PBa						**

micr. prot./g liver§	Dia	Rif	War	PCN	PBa	TCP
Dia		**	*	**	**	**
Rif			•	**	**	**
War				**	**	**
PCN					•	**
PBa						•

P-450/mg micr. prot.‡	Dia	Rif	War	PCN	PBa	TCP
Dia		**	**	**	**	**
Rif			*	**	**	**
War				**	**	**
PCN					**	**
PBa						**

EM/nmol P-450	Dia	Rif	War	PCN	PBa	TCP
Dia		*	•	•	*	•
Rif			•	•	•	**
War				•	•	*
PCN					•	*
PBa						**

Benzph./nmol P-450‡	Dia	Rif	War	PCN	PBa	TCP
Dia		**	**	*	**	*
Rif			**	•	**	**
War				**	*	**
PCN					**	**
PBa						**

Biph.-4-OH/nmol P-450§	Dia	Rif	War	PCN	PBa	TCP
Dia		**	•	**	**	**
Rif			**	**	**	•
War				**	**	**
PCN					**	**
PBa						**

Biph-2-OH/nmol P-450‡	Dia	Rif	War	PCN	PBa	TCP
Dia		**	**	**	**	**
Rif			•	**	•	**
War				**	•	**
PCN					**	•
PBa						**

Ethoxyc./nmol P-450‡	Dia	Rif	War	PCN	PBa	TCP
Dia		**	**	**	**	•
Rif			**	**	**	**
War				**	•	**
PCN					**	**
PBa						**

Pentoxy./nmol P-450‡	Dia	Rif	War	PCN	PBa	TCP
Dia		•	**	**	**	**
Rif			**	**	**	**
War				**	**	*
PCN					**	**
PBa						•

Peak wavelength	Dia	Rif	War	PCN	PBa	TCP
Dia		**	•	**	•	*
Rif			**	**	**	*
War				**	•	*
PCN					**	**
PBa						**

* $\alpha \leq 5\%$; ** $\alpha \leq 1\%$.

‡ After logarithmic transformation (see Materials and Methods section); § homogeneity of variances to be rejected with $\alpha \leq 5\%$. (a) Series 1 (see Fig. 1), inducers diazepam, rifampicin, warfarin; Scheffé tests. (b) Series 2 (see Fig. 1), inducers PCN, phenobarbitone, TCPOBOP; Scheffé tests. (c) Both series, all inducers. Series have been made comparable by expressing values as percentages of the control mean of the respective day; Duncan tests.

Table 3. "Total" and "specific" activity of inducers

A				B			
Rank	Inducer	Substrate	Multiple of control (times)	Rank	Inducer	Substrate	Multiple of control (times)
1	TCPOBOP	Pentoxyres.	137.26	1	Phenobarbitone	Pentoxyres.	16.62
2	Phenobarbitone	Pentoxyres.	65.30	2	TCPOBOP	Pentoxyres.	13.18
3	Warfarin	Pentoxyres.	21.87	3	Warfarin	Pentoxyres.	10.13
4	TCPOBOP	Benzphetamine	17.13	4	Phenobarbitone	Benzphetamine	3.01
5	TCPOBOP	Ethoxycoum.	12.94	5	Warfarin	Benzphetamine	2.33
6	Phenobarbitone	Benzphetamine	11.87	6	PCN	Pentoxyres.	2.09
7	PCN	Pentoxyres.	9.93	7	TCPOBOP	Benzphetamine	1.64
8	TCPOBOP	Ethylmorphine	9.40	8	Phenobarbitone	Ethoxycoum.	1.49
9	TCPOBOP	Biphenyl (4-OH)	6.67	9	Warfarin	Ethoxycoum.	1.43
10	Phenobarbitone	Ethoxycoum.	5.85	10	Rifampicin	Ethylmorphine	1.28
11	Warfarin	Benzphetamine	5.46	11	Diazepam	Benzphetamine	1.27
11	PCN	Ethylmorphine	5.46	12	Phenobarbitone	Ethylmorphine	1.22
12	Phenobarbitone	Ethylmorphine	4.82	13	PCN	Ethylmorphine	1.21
13	PCN	Benzphetamine	4.70	14	Warfarin	Ethylmorphine	1.20
14	Phenobarbitone	Biphenyl (4-OH)	4.55	15	Rifampicin	Pentoxyres.	1.18
15	TCPOBOP	Biphenyl (2-OH)	3.43	16	Phenobarbitone	Biphenyl (4-OH)	1.16
16	Warfarin	Ethoxycoum.	3.17	17	TCPOBOP	Ethoxycoum.	1.15
17	Warfarin	Ethylmorphine	2.69	17	Diazepam	Pentoxyres.	1.15
18	PCN	Ethoxycoum.	2.45	18	Diazepam	Ethoxycoum.	1.05
19	Phenobarbitone	Biphenyl (2-OH)	2.44	19	Diazepam	Ethylmorphine	1.03
20	Rifampicin	Ethylmorphine	2.34	20	PCN	Benzphetamine	1.00
21	Warfarin	Biphenyl (4-OH)	2.15	21	Warfarin	Biphenyl (4-OH)	0.99
22	Rifampicin	Pentoxyres.	2.13	22	Diazepam	Biphenyl (4-OH)	0.98
23	PCN	Biphenyl (4-OH)	2.12	23	Diazepam	Biphenyl (2-OH)	0.95
24	Rifampicin	Benzphetamine	1.69	24	Rifampicin	Ethoxycoum.	0.91
25	Rifampicin	Ethoxycoum.	1.64	25	Rifampicin	Benzphetamine	0.90
26	PCN	Biphenyl (2-OH)	1.61	25	TCPOBOP	Ethylmorphine	0.90
27	Warfarin	Biphenyl (2-OH)	1.47	26	Rifampicin	Biphenyl (2-OH)	0.70
28	Diazepam	Benzphetamine	1.44	27	Warfarin	Biphenyl (2-OH)	0.69
29	Rifampicin	Biphenyl (2-OH)	1.29	28	Rifampicin	Biphenyl (4-OH)	0.68
29	Diazepam	Pentoxyres.	1.29	29	TCPOBOP	Biphenyl (4-OH)	0.65
30	Rifampicin	Biphenyl (4-OH)	1.26	30	Phenobarbitone	Biphenyl (2-OH)	0.62
31	Diazepam	Ethoxycoum.	1.17	31	TCPOBOP	Biphenyl (2-OH)	0.58
32	Diazepam	Ethylmorphine	1.14	32	PCN	Ethoxycoum.	0.53
33	Diazepam	Biphenyl (4-OH)	1.09	33	PCN	Biphenyl (4-OH)	0.46
34	Diazepam	Biphenyl (2-OH)	1.05	34	PCN	Biphenyl (2-OH)	0.37

Rank order of inducer-specific metabolic increases (or decreases) calculated (list A) as total activity (increase per animal, irrespective of effects outside the liver = metabolic rate per nmol P-450 × P-450 per mg microsomal protein × mg microsomal protein per g liver × liver weight) and calculated (list B) as "specific" activity per nmol cytochrome P-450.

Hultmark *et al.* [14, 15]. Dichloro-*p*-nitrophenol formation was unchanged in C57BL/6J mice after PB treatment, but decreased after TCPOBOP; in rats, it was increased after PB but unchanged after TCPOBOP (to be published elsewhere); (4) Another prominent feature of TCPOBOP induction is elucidated in Table 3: TCPOBOP holds the leading position in total increase of catalytic activity (Table 3, part A; ranks 1, 4, 5, 8, 9, 15), and is followed by phenobarbitone (ranks 2, 6, 10, 12, 14, 19), where "total" means increase per nmol cytochrome P-450 × cytochrome P-450 content × protein content × liver weight, i.e. the increase per whole animal irrespective of increases outside the liver. Phenobarbitone, on the other hand, holds the leading position in "specific" increase, i.e. increase of catalytic activity exclusively by changing the shares of cytochrome P-450 isoenzymes within global cytochrome P-450 (Table 3, part B; ranks 1, 4, 8, 12, 16, 30), and is followed by warfarin (ranks 3, 5, 9, 14, 21) and TCPOBOP (ranks 2, 7, 17, 25, 29, 31).

Pentoxyresorufin (POR) as substrate of the foreign compound metabolizing system

As may be seen from Table 3, POR metabolism shows the highest increases whatsoever (Table 3, part A: ranks 1, 2, 3, 7, 22, 29; part B: ranks 1, 2, 3, 6, 15, 17; Fig. 2). POR distinguishes very well between phenobarbitone, warfarin and TCPOBOP on the one hand and rifampicin and PCN on the other but to a lesser degree between phenobarbitone, TCPOBOP and warfarin themselves (see Fig. 2 Tables 2a–c). Its metabolism was never decreased, control values being rather low (mean 20.2 pmol/nmol P-450, range 9.8–39.3, in 24 control mice).

DISCUSSION

As the intent of this report is mainly descriptive, the question of representativity deserves special attention. It has been reported [16–18] that induction parameters of control mice of different dates (or

different consignments, respectively) vary more than they would do at random. Our results confirm this fact: control mice, in most parameters, must not be regarded as random samples drawn from one population (Table 1); in many parameters the controls of the second series of experiments (see Fig. 1) are significantly different from those of the first series (Table 1), which may be interpreted as a seasonal influence; and a factor "time" is retained in several parameters when both control and treated animals are included in the statistical calculations. It follows that this factor must be considered in the experimental design and evaluation.

We tried to meet this condition in two ways: first, when evaluating each series alone (see Fig. 1), the data were subject to two-factor analyses of variance with a factor "time" (three levels according to three days of experiment); second, when comparing the six inducers independently from the series, the data were expressed as per cent of the control mean of the respective day of experiment. Now, there may be made the following objection: we do not know the meaning of the different control values on different days; if these differences express different reagibilities (of animals to inducers), which means not only an additive but also a multiplicative [19] effect of the factor "time", the first way may be adequate in that interaction significances would emerge in two-factor analyses of variance and the second way might be adequate, although we cannot be sure about it. If the differences express no different reagibilities but only certain amounts to be added (or subtracted, respectively), the first way would still be adequate (no interaction effects would appear), but the second way would be inadequate because percentages, in this case, would be misleading.

No definitive decision concerning that objection is possible at the moment. In some of our parameters (see Results section, para. "Time dependence"), however, interaction effects as well as heterogeneity of variances were present, so that a simple additive model does not seem very likely. Thus, we think, our percentages are defendable (percentages in Fig. 2 are based on means of three days of experiment).

Pentoxyresorufin metabolism has already been shown to be increased excessively by phenobarbitone pretreatment in C57B1/10J- [20], C57B1/6J- [21] and NMRI [21] mice as well as in rats [22], whereas increase by 3-methylcholanthrene is minimal [20, 22]. In our experiments the excessive increase was confirmed and pentoxyresorufin metabolism shown to be a powerful discriminator of the phenobarbitone–warfarin against the rifampicin–PCN type of induction: rifampicin and PCN did not produce significant increases. Thus pentoxyresorufin obtains a prominent place among the model substrates in descriptive investigations on the induction phenomenon.

Diazepam had no significant effect on the induction parameters listed in Fig. 2 and Tables 2a–c. But there was a statistically significant increase in benzphetamine metabolism, calculated per mg microsomal protein (not quoted in the Results section). It has been shown earlier that diazepam pretreatment decreases pentobarbitone serum levels in humans and rats [9], shortens hexobarbitone sleeping times in rats [10] and decreases bilirubin serum levels in newborn infants [11]. Thus, its failure to influence our main parameters may not be taken as proof that diazepam has no inductive potency at all, although, in any case, it would be the least one among the inducers investigated here.

Among the remaining five inducers our results discriminate between 3 classes: phenobarbitone with warfarin, rifampicin with PCN, and TCPOBOP. Similarity between phenobarbitone and warfarin has been shown in this laboratory by means of metabolic [8, 22] and gel electrophoretic [23] methods and similarity between rifampicin and PCN by Tredger *et al.* [5]. Broad evidence exists concerning the difference between the glucocorticoid–PCN type and the PB type of induction even on the chromosomal level [6, 24–28]. Our findings show that this classification is reproducible by rather simple means. Differences between PB and TCPOBOP uncovered by these means should therefore be considered relevant.

The difference between PB and TCPOBOP—besides different catalytic activities, a remarkable superiority of TCPOBOP with respect to the inductive effect when calculated per whole animal—suggests, additionally, a classification on the scale "unspecific–specific". This reminds us of the still unsolved question of the "physiologic role" of the induction response. "Classically, the induction of cytochrome P-450 and other drug-metabolizing enzymes by foreign lipophilic chemicals is viewed as an adaptive physiologic response, a workload hypertrophy, the 'purpose' of which is to increase the rate of metabolism of the foreign compound and hasten its elimination from the body" [29]. If this is true, calculation per whole animal would be more adequate than per mg microsomal protein or per nmol cytochrome P-450. TCPOBOP would be the most effective non-Ah inducer and its toxicity needs to be scrutinized.

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REFERENCES

1. Poland A and Knutson JC, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22**: 515–554, 1982.
2. Heubel F, Kehl F and von Maxen A, Is there a pleiotropic response in barbiturate induction? *Biochem Pharmacol* **35**: 1889, 1986.
3. Lu AYH, Somogy A, West S, Kuntzman R and Conney AH, Pregnenolone-16α-carbonitrile: a new type of inducer of drug-metabolizing enzymes. *Arch Biochem Biophys* **152**: 457–462, 1972.
4. Heubel F and Netter KJ, Atypical inductive properties of rifampicin. *Biochem Pharmacol* **28**: 3373–3378, 1979.
5. Tredger M, Smith HM, Powell-Jackson PR, Davis M and Williams R, Effect of rifampicin on the mouse hepatic mixed-function oxidase system. *Biochem Pharmacol* **30**: 1043–1051, 1981.
6. Wrighton SA, Schutz EG, Watkins PB, Maurel P,

Barwick J, Bailey BS, Hartle HT, Young B and Guzelian PS, Demonstration in multiple species of inducible hepatic cytochromes P-450 and their mRNAs related to the glucocorticoid-inducible cytochrome P-450 of the rat. *Molec Pharmacol* **28**: 312–321, 1985.
7. Simmons DL, Lalley PA and Kasper CB, Chromosomal assignments of genes coding for components of the mixed-function oxidase system in mice. *J Biol Chem* **260**: 515–521, 1985.
8. Kling L, Influence of warfarin on cytochrome P-450 *in vitro* and *in vivo*. *Naunyn-Schmiedeberg's Arch Pharmacol (Suppl.)* **319**: R9, 1982.
9. Heubel F and Frank R, Zur induktiven Wirkung von Diazepam. *Arzneim-Forsch* **20**: 1706–1708, 1970.
10. Heubel F and Patutschnick H, Vergleichende Untersuchungen über die induktive Wirkung verschiedener Benzodiazepine an Hand der durch Pentobarbital bedingten Schlafzeit der Ratte. *Arzneim-Forsch* **22**: 1196–1199, 1972.
11. Heubel F and Mühlberger F, Senkung des Neugeborenen-Bilirubin-spiegels durch Diazepam. *Int J Clin Pharmacol* **6**: 332–341, 1972.
12. Omura R and Sato R, The carbon-monoxide binding pigment of the liver. *J Biol Chem* **239**: 2370–2378, 1964.
13. Allen CFH and Gates JW, o-*n*-Butoxynitrobenzene (ether, butyl-o-nitrophenyl). *Org Syntheses Coll* **III**: 140–141, 1955.
14. Hultmark D, Sundh K, Wachtmeister CA and Arrhenius E, Dichloro-*p*-nitroanisole o-demethylase—a convenient assay for microsomal mixed-function oxidase in isolated rat hepatocytes. *Biochem Pharmacol* **27**: 1129–1134, 1978.
15. Hultmark D, Sundh K, Johannson L and Arrhenius E, Dichloro-*p*-nitroanisole o-demethylase–II. Evidence for separate ethanol inhibited and phenobarbital-inducible enzymes. *Biochem Pharmacol* **28**: 1587–1590, 1979.
16. Robinson JR, Considine N and Nebert DW, Genetic expression of aryl hydrocarbon hydroxylase induction. Evidence for the involvement of other genetic loci. *J Biol Chem* **249**: 5851–5859, 1974.
17. Heubel F, Variation of control values in NMRI mice. *Naunyn-Schmiedeberg's Arch Pharmacol (Suppl)* **311**: R11, 1980.
18. Heubel F, Hedrich HJ and Kuhn-Nentwig L, Cytochrome P-450: Sources of variability in mice. *Naunyn-Schmiedeberg's Arch Pharmacol (Suppl)* **316**: R6, 1981.
19. Winer BJ, *Statistical Principles in Experimental Design*, p. 318. McGraw-Hill, New York 1971.
20. Mayer RT, personal communication.
21. Heubel F and Kehl F, Pentoxyresorufin—a very specific substrate in phenobarbitone-like induction. *Naunyn Schmiedeberg's Arch Pharmacol (Suppl)* **325**: R13, 1984.
22. Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T and Guengerich P, Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. *Arch Biochem Biophys* **238**: 43–48, 1985.
23. Kling L, Legrum W and Netter KJ, Induction of liver cytochrome P-450 in mice by warfarin. Comparison of warfarin-, phenobarbitone-, and cobalt-induced hepatic microsomal protein patterns by PAGE after partial purification on octyl-Sepharose Cl–4B. *Biochem Pharmacol* **34**: 85–91, 1985.
24. Schuetz E, Wrighton SA, Barwick JL and Guzelian PS, Induction of cytochrome P-450 by glucocorticoids in rat liver. *J Biol Chem* **259**: 1999–2006, 1984.
25. Heuman DM, Gallagher EJ, Barwick JL, Elshourbagy N and Guzelian PS, Immunochemical evidence for induction of a common form of hepatic cytochrome P-450 in rats treated with pregnenolone-16α-carbonitrile or other steroidal or non-steroidal agents. *Mol Pharmacol* **21**: 753–760, 1982.
26. Elshourbagy NA and Guzelian PS, Separation, purification and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolon-16α-carbonitrile. *J Biol Chem* **255**: 1279–1295, 1980.
27. Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B and Guzelian PS, Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450. *Biochem* **24**: 2171–2178, 1985.
28. Gonzales FJ, Nebert DW, Hardwick JP and Kasper CB, Complete cDNA and protein sequence of a pregnenolone 16α-carbonitrile-induced cytochrome P-450. *J Biol Chem* **260**: 7435–7441, 1985.
29. Poland A and Knutson JC, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Ann Rev Pharmacol Toxicol* **22**: 517, 1982.