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IS THERE A PLEIOTROPIC RESPONSE IN BARBITURATE INDUCTION?

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Abstract—C57BL/6J mice were treated with barbitone, phenobarbitone, thiopentone and hexobarbitone, respectively, for a period of 6 days, and the resulting induction effects compared. Among parameters measured were metabolic rates for ethylmorphine, biphenyl, ethoxycoumarin and pentoxyresorufin. Effects of inducers were easily distinguishable from each other. No fixed proportions were seen among increased metabolic rates, but increases of relative liver weight, cytochrome P450 per g liver and pentoxyresorufin metabolism were accompanied by a corresponding decrease of biphenyl-2-hydroxylation. Doubling the dose (NMRI mice) of hexobarbitone and thiopentone increased inductive responses, but did not render them more similar to each other. Pentoxyresorufin metabolism was increased 5- to 30-fold, calculated per nmol cytochrome P450. The results argue against the existence of one pleiotropic response—at least in a strict sense—in barbiturate induction.

When Poland *et al.* [1] presented 1,4-bis(2-(3,5-dichloropyridyl-oxy))benzene (TCPOBOP) as a potent phenobarbitone-like inducer, they noted: "For other foreign chemicals, which are considered as either phenobarbitone-like or 3-methylcholanthrene-like, usually on the basis of their induction of microsomal monooxygenase activity, it is assumed, but infrequently documented that these compounds are faithful mimetics of the entire pleiotropic response produced by the prototype compounds." If it is true that phenobarbitone-like inducers provoke the same concerted response as the prototype compound phenobarbitone, then in any case that same response should be produced by other barbiturates. In this paper we provide evidence that this is not the case. We investigated the catalytic activities of global cytochrome P450, induced comparatively by barbitone, phenobarbitone, hexobarbitone and thiopentone. The substrate metabolisms assayed included pentoxyresorufin, which has recently been shown to be very specific for phenobarbitone induction [2, 3]. Each of these barbiturate inducers proved to produce distinguishable catalytic activity patterns.

MATERIALS AND METHODS

Animals. Male C57BL/6J Han mice were purchased from the Zentralinstitut für Versuchstiere, Hannover, 4 weeks ± 2 days of age, weighing 18–20 g. They were kept in plastic cages (3 animals each) with sawdust bedding. Bedding was taken from the same charge as used by the breeder. Animals received commercial pellet diet (SSNiFFM) 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. Male Han:NMRI mice, grown in normalized litters (N = 4), were purchased from the Zentralinstitut für Versuchstiere, Hannover, 4 weeks ± 2 days of age. They were kept litterwise. Litters were not split off until sacrifice, one animal of each litter being allocated to one of four treatment groups. All other conditions were as described above.

Treatment. C57BL/6J mice were injected s.c. twice a day (8 a.m. and 8 p.m.) during a period of six days, the last dose being given 21 hr before sacrifice. Food was removed 16 hr before sacrifice. Doses consisted of 0.9% NaCl or 2 × 40 mg barbitone or 2 × 50 mg phenobarbitone or 2 × 80 mg hexobarbitone or 2 × 30 mg thiopentone per kg body weight, respectively. NMRI mice were injected s.c. twice a day (8 a.m., 8 p.m.) or four times a day (8 a.m., 1 p.m., 6 p.m., 12 p.m.), controls three times a day (8 a.m., 1 p.m., 6 p.m.) for a period of six days. Doses consisted of 0.9% NaCl or 2 × 80 mg hexobarbitone or 4 × 80 mg hexobarbitone or 2 × 30 mg thiopentone or 4 × 30 mg thiopentone per kg body weight. All other conditions were as described above. In most animals, hexobarbitone caused "sleeping times" of 20 min maximum, part of the thiopentone-injected animals were excited and/or sedated. Treated groups lost body weight as shown in Table 1.

Preparation. Animals were sacrificed on Mondays between 8.30 and 10 a.m. by cervical dislocation. Livers were perfused *in situ* with ice-cold 1.15% KCl for 2 min, 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 of 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 pentoxyresorufin and ethoxycoumarin metabolism were stored overnight at −20°.

Assays. Cytochrome P450 was measured according to Omura and Sato [4], using an Aminco DW-2 spectrophotometer. Wavelengths of CO-cytochrome P450 peaks were measured but did not vary with

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Table 1. Loss of body weight after 6 days treatment with barbiturate inducers

C57BL/6J			NMRI					
Inducer	Body wt. (g)	Significance of difference to control	Inducer	Body wt. (g)	Significance of difference to control	Inducer	Body wt. (g)	Significance of difference to control
Con	20.5		Con	28.7		Con	28.6	
Bar 2 × 40	19.9	n.s.	Hex 2 × 80	26.1	**	Thi 2 × 30	28.5	n.s.
Phe 2 × 50	19.2	n.s.	Hex 4 × 80	24.5	**	Thi 4 × 30	26.3	*
Hex 2 × 80	20.0	n.s.						
Thi 2 × 30	19.3	n.s.						

Con, control; Bar, barbitone; Phe, phenobarbitone; Hex, hexobarbitone; Thi, thiopentone; n.s., not significant; *, $\alpha \leq 5\%$; **, $\alpha \leq 1\%$. Significances were calculated following two factor analysis of variance by Scheffé-test in C57BL/6J and by Duncan-test in NMRI mice (see Fig. 1).

treatment. Ethylmorphine demethylation was measured by a modified method according to Mazel [5]. One millilitre of diluted microsomal suspension was incubated with a NADPH regenerating system of glucose-6-phosphate, glucose-6-phosphate-dehydrogenase and NADP, $MgCl_2$ (5 μmol per sample), 0.1 M phosphate buffer pH 7.4 and 0.1 ml 1 mM ethylmorphine (without nicotinamide) for 10 min and 37°. Biphenyl 4- and 2-hydroxylation were measured according to Creaven *et al.* [6]. Fluorescence intensity was determined at 290/415 and 275/338 nm excitation/emission wavelengths by a Perkin-Elmer 204-A spectrofluorometer. Pentoxyresorufin depentylation (increase of resorufin fluorescence at 530/585 nm excitation/emission wavelengths) was followed in the cuvette containing a NADPH regenerating system of isocitric acid, isocitric acid dehydrogenase, NADP and $MgCl_2$ (0.64 μmol per sample) at 25° and calibrated by adding 1 nmol resorufin. Ethoxycoumarin-deethylation was measured according to Aitio [7]. All assays were finished within two days.

Chemicals. Barbitone, ethylmorphine and Folin-Ciocalteu's reagent were purchased from Merck, Darmstadt; phenobarbitone from Fluka, Buchs, Switzerland; hexobarbitone from Bayer, Leverkusen; thiopentone from Byk Gulden, Konstanz; bovine serum albumin, biphenyl, 2-OH-biphenyl, 4-OH-biphenyl and isocitrate from Serva, Heidelberg; glucose-6-phosphate, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and NADP from Boehringer, Mannheim; 7-hydroxycoumarin from Janssen, Düsseldorf. Pentoxyresorufin was synthesized and kindly donated by R. T. Mayer, then College Station, Texas. Ethoxycoumarin was synthesized in this laboratory by W. Legrum according to Ullrich and Weber [8].

Design and statistical evaluation. Since there are significant differences between parameters of the oxidative foreign compound metabolism in animals of different consignments, the experiment was repeated three times. The resulting design is given in Fig. 1a. One complete treatment group consists of 12 animals out of 4 dates; one experiment (one date) consists of 5 treatments with 3 animals per treatment. The results were subject to a two factor-analysis of variance, followed by Scheffé-tests for differences between levels. The design of experiments with NMRI mice is given in Fig. 1b. One complete treatment group consists of 12 animals out of 3 dates; one experiment (one date) consists of three treatments with four animals per treatment. In

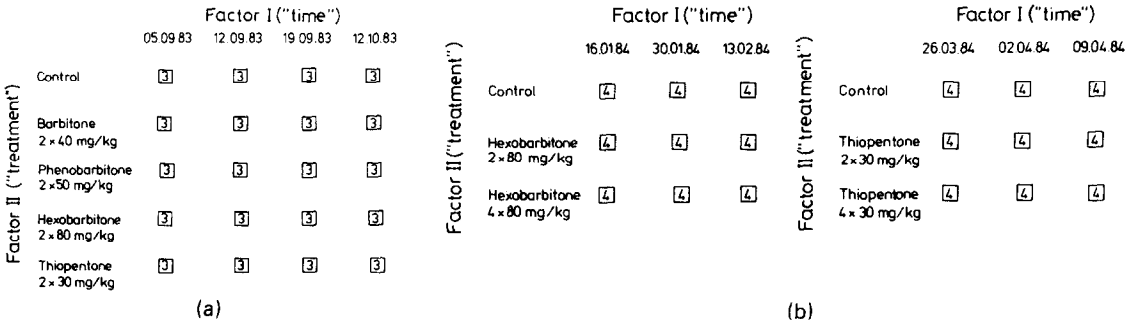

Fig. 1. Design of experiments. Each quad represents a cell in a two factor analysis of variance with its number of animals examined: (a) comparison of inducers (C57BL/6J mice); (b) comparison of doses (NMRI mice).

Table 2. Statistical significances of differences between effects of 4 different barbiturate inducers

	Relative liver weight					Relative pellet weight					mg Protein/g liver				
	Con	Bar	Phe	Hex	Thi	Con	Bar	Phe	Hex	Thi	Con	Bar	Phe	Hex	Thi
Con		**	**	•	**		•	**	*	**		•	**	•	**
Bar			**	*	**			*	•	**			•	•	•
Phe				**	**				•	•				•	•
Hex					**					•					•
Thi															

	nmol P-450/g liver					nmol P-450/mg prot.				
	Con	Bar	Phe	Hex	Thi	Con	Bar	Phe	Hex	Thi
Con		**	**	*	**		**	**	•	**
Bar			**	•	•			**	•	•
Phe				**	**				**	**
Hex					*					•
Thi										

	EM metab./nmol P-450					Biphenyl-4-hydr./P-450					Biphenyl-2-hydr./P-450				
	Con	Bar	Phe	Hex	Thi	Con	Bar	Phe	Hex	Thi	Con	Bar	Phe	Hex	Thi
Con		•	**	•	•		•	•	•	•		**	**	**	**
Bar			**	•	•			•	•	•			•	•	•
Phe				**	*				•	•				•	•
Hex					•					•					•
Thi															

	Pentoxyr. metab./P-450					Ethoxyc. metab./P-450				
	Con	Bar	Phe	Hex	Thi	Con	Bar	Phe	Hex	Thi
Con		**	**	**	**		•	**	•	**
Bar			**	*	•			**	•	**
Phe				**					**	•
Hex				**						**
Thi										

Each inducer is compared to each other one and to control in 10 matrices, corresponding to 10 induction parameters. •, no significant difference between 2 inducers compared; *, $\alpha \leq 5\%$; **, $\alpha \leq 1\%$; Scheffé-test following two factor analysis of variance (see Fig. 1a).

each of the three treatment groups of one date, litters were represented equally (the fourth treatment group, which was dosed four times a day with a small dose did not serve the purpose of this paper and is not shown here). The results were subject to a two factor-analysis of variance with repeated measures on the second factor (followed by Duncan-tests). Quotients between metabolic parameters were handled in the same manner. Calculations were performed by the Rechenzentrum der Philipps-Universität Marburg.

RESULTS

Induction patterns

Figure 2 and Table 2 summarize the results of our comparative experiments with C57BL/6J mice. Figure 2 gives the mean values of every induction parameter measured after treatment with the four inducers; Table 2 gives the significances of the differences between them. Metabolic rates of substrates are calculated per nmol cytochrome P450, to which we were led by the following considerations. Inducers may differ in how much they augment microsomal protein content and in how much they augment cytochrome P450 content of microsomal protein. Both kinds of augmentation may be specific (in the sense that the inducers are distinguishable from each other in these regards). But, as we are concerned with the oxidative drug metabolizing system, we are interested mainly in its catalytic activities. These reside in the cytochrome P450 and are specified by its (unknown) shares of different isoenzymes. Thus, the catalytic activities of the (global) cytochrome P450 content express the more important part of the pleiotropic response, and different catalytic activities of the (global) cytochrome P450 would express different pleiotropic responses. Therefore, in this paper, metabolic rates of substrates are calculated per nmol cytochrome P450.

From Fig. 2 and Table 2 may be seen the following:

(1) Barbiturate inducers may be distinguished almost completely merely by their individual pattern of *non-metabolic* parameters: Phenobarbitone shows the highest increases, hexobarbitone the lowest ones, both are obviously different from barbitone as well as

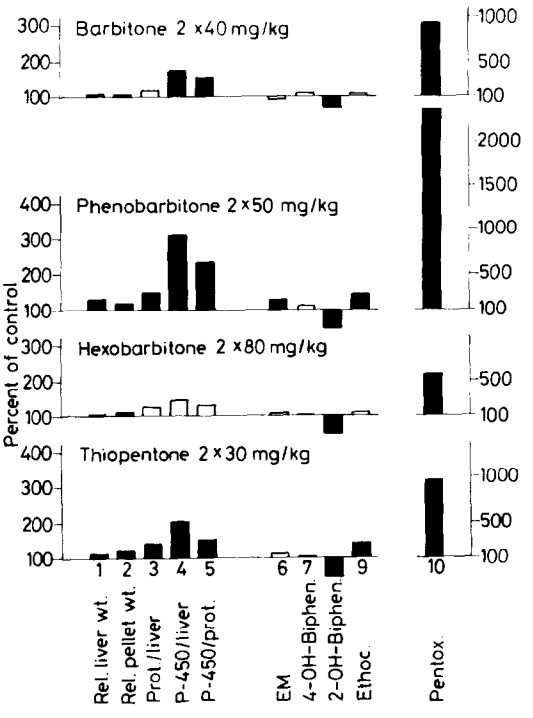

Fig. 2. Average effects of 4 barbiturate inducers in 10 induction parameters. Metabolic parameters (6–10) are calculated per nmol cytochrome P450. EM, ethylmorphine-demethylation; Ethoc, ethoxycoumarin-deethylation; Pentox, pentoxyresorufin-depentylation. Black columns indicate statistically significant differences ($\alpha \leq 5\%$, Scheffé test following two factor analysis of variance see Fig. 1) from *control*.

from thiopentone. The difference between barbitone and thiopentone is less obvious, but significant in parameters 1 and 2, barbitone does, in parameter 3, not differ from control.

(2) Barbiturate inducers may be distinguished as well by their individual pattern of *metabolic* parameters: Phenobarbitone produces significant alterations in 4 out of 5 parameters, thiopentone in 3 out of 5, barbitone and hexobarbitone, which alter only two out of 5 parameters, differ significantly in parameter 10.

(3) Barbiturate inducers may be distinguished in any case, if non-metabolic and metabolic parameters are combined: Hexobarbitone now yields only 3 significant alterations, barbitone 6, thiopentone 8 and phenobarbitone 9 ones.

(4) The only substrate, which is specific for one inducer (in the sense that only that inducer enhances its P450-related metabolism) is ethylmorphine: Phenobarbitone induction increases that parameter significantly different from control and all competitors, whereas other inducers cause no difference, either to control or to each other.

(5) The only P450-related reaction which is not enhanced at all (significant differences neither to control nor to competitors) is 4-hydroxylation of biphenyl. That means that no inducer has shifted the relative share(s) of isoenzyme(s) which catalyze(s) this hydroxylation away from the share(s) in constitutive P450 (although 3 inducers did increase global cytochrome P450).

(6) There are two parameters in which 9 out of 10 possible comparisons between inducers are significant (see Table 2), relative liver weight and pentoxyresorufin metabolism (hexobarbitone did not differ from control with respect to relative liver weight, and hexobarbitone did not differ from thiopentone with respect to pentoxyresorufin metabolism). If it were defendable to rely only on two induction parameters, this pair of parameters would be sufficient to prove that inductive effects of all barbiturate inducers differ from each other.

(7) If a "relative inducing power" of a compound were to be determined, these two parameters would indicate the same order: phenobarbitone-thiopentone-barbitone-hexobarbitone from maximum to minimum effect. The same order would be indicated by parameter 4, cytochrome P450 per g liver, although, on this ladder, only eight of the ten possible comparisons between the five rungs are significant.

(8) There is only one parameter to be *decreased*: biphenyl-2-hydroxylation. Here, the effects of all inducers are significantly different from control, but not from each other. However, the order of ranks between inducers (see above) in relative liver weight, pentoxyresorufin metabolism and cytochrome P450 content per g liver is reproduced in a reversed fashion: not increase but decrease of 2-hydroxylation of biphenyl follows the order phenobarbitone-thiopentone-barbitone-hexobarbitone from maximum to minimum degree. This is visualized in Fig. 3.

Dose dependence

The inductive response may depend not only upon the kind of inducer but also upon the dosage. This raises the question whether different doses of the same inducer provoke the same pattern of catalytic

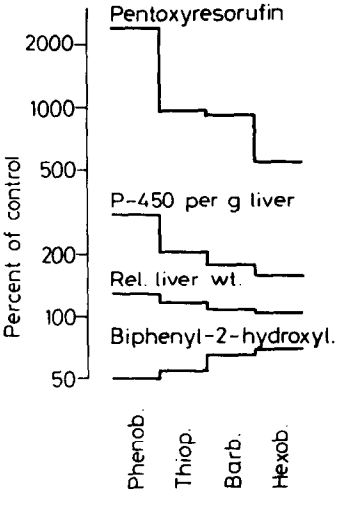

Fig. 3. Action of 4 barbiturate inducers upon 4 selected induction parameters. Metabolic parameters calculated per nmol cytochrome P450. Logarithmic scale. Biphenyl-2-hydroxylation exhibits an inverse pattern.

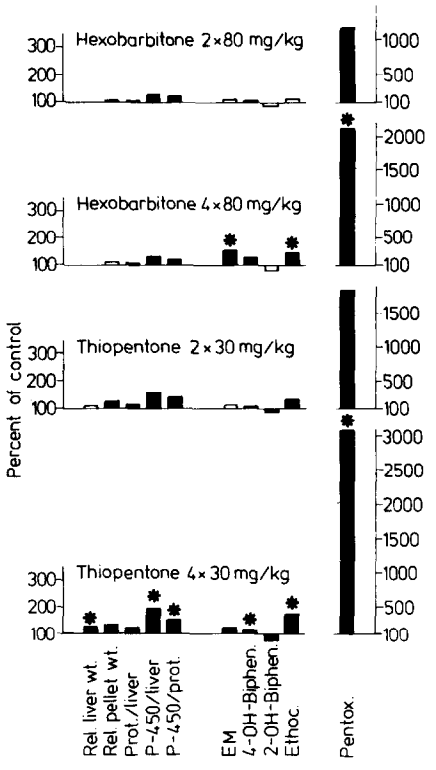

Fig. 4. Average inductive effects of 2 different doses of hexobarbitone and of thiopentone. Metabolic parameters are calculated per nmol cytochrome P450. For abbreviations see Fig. 2. Black columns indicate statistically significant difference from *control* ($\alpha \leq 5\%$). Asterisk indicates statistically significant difference between *doses* ($\alpha \leq 5\%$). Duncan-tests following two factor analysis of variance with repeated measures on the second factor (see Fig. 1b).

activities (in only different amounts) or a qualitatively different pattern. Therefore, we examined the inductive response after two different doses of hexobarbitone and of thiopentone (in NMRI mice). Figure 4 sums up our findings in respect to this problem:

(1) The pattern of inductive effects produced by hexobarbitone and thiopentone in NMRI mice is roughly similar to that produced in C57BL/6J mice (compare Fig. 2). Pentoxyresorufin metabolism, however, is more increased and 2-hydroxylation of biphenyl less decreased than in C57BL/6J mice (our NMRI strain lacks the Ah complex).

(2) Doubling the dose enhances inductive effects but leaves the inducer-specific patterns roughly unaltered. In this case, "roughly" means that, if increases (or a decrease) over control of metabolic parameters are ranked in an order according to their magnitude, parameters obtain the same places.

(3) There is a marked difference between pentoxyresorufin metabolism on the one hand and all other parameters on the other. Pentoxyresorufin is multiplied at least 10 times, whereas all other parameters are increased less than twice. A thirtyfold increase (calculated per nmol cytochrome P450), as following the high dose of thiopentone, is the highest we saw in this laboratory in phenobarbitone-like induction.

(4) Doubling the dose does not render the hexobarbitone and thiopentone patterns more similar to each other.

Proportions between metabolic rates

If a pair of substrate metabolisms is regulated in common, the quotients between these metabolic rates should be constant. This would indicate a part of a pleiotropic response. Table 3 summarizes all possible six quotients between our four substrate reactions (biphenyl-2-hydroxylation must be excluded because it is *de*creased). Within each of the six quotients, the five treatments are compared to each other (C57BL/6J mice). Table 4 contains the same quotients, within which the two doses of hexobarbitone and of thiopentone are compared to each other (NMRI mice). The comparisons are based on the individual quotients of twelve mice which received one treatment. From Table 3 may be seen:

(1) If all inducers were to increase the metabolic rates in the pre-existing proportions, no significant differences at all should be seen. If all inducers would increase the metabolic rates in the same proportions which but are different from the proportions in controls, significances should be seen only in the first row. But these two cases are realized only once each (biphenyl - 4 - hydroxylation/ethylmorphine-demethylation and ethoxycoumarine-deethylation/pentoxyresorufin-depentylation), and there are quite numerous significant differences between inducers.

(2) In the first case (4-hydroxylation of biphenyl/ethylmorphine-demethylation) the mean quotients are: control, 0.38; barbitone, 0.40; phenobarbitone 0.32; hexobarbitone 0.37; and thiopentone, 0.34. Although no statistically significant differences between these mean quotients exist, phenobarbitone obtains an extreme position. Phenobarbitone, simultaneously, as seen in Table 2, is the only inducer to cause an increase of ethylmorphine demethylation significantly different from all other inducers and control. Thus, the conclusion that ethylmorphine demethylation and 4-hydroxylation of biphenyl are regulated in common, should be drawn with caution.

(3) In the second case (ethoxycoumarin-deethylation/pentoxy-resorufin-depentylation) the mean quotients are: control, 312.0; barbitone, 139.5; phenobarbitone, 103.4; hexobarbitone, 240.7; and thiopentone, 186.2. Although these means are significantly different only from control, they are ranked in almost the same order as mentioned above. Thus, also, the conclusion that ethoxycoumarin and pentoxyresorufin metabolism are regulated in common, should not be drawn.

From Table 4 may be seen:

(1) If both doses of one inducer were to increase the metabolic rates in the same proportions, no significant differences should appear. But there are significant differences, two in hexobarbitone, four in thiopentone induction. In these instances doubling the dose has shifted the proportion between metabolic rates.

(2) Two quotients (biphenyl-4-hydroxylation/ethylmorphine-demethylation and ethoxycoumarin-

Table 3. Proportions of metabolic rates after treatment with 4 different barbiturate inducers

	Con	Bar	4-Biph/EM Phe	Hex	Thi	Con	Bar	Pento/EM Phe	Hex	Thi	Con	Bar	Ethoc/EM Phe	Hex	Thi
Con		●	●	●	●		**	**	**	**		●	●	●	*
Bar			●	●	●			**	*	●			●	●	●
Phe				●	●				**	**				●	●
Hex					●					*					*
Thi															

		Con	Bar	Pento/4-Biph Phe	Hex	Thi	Con	Bar	Ethoc/4-Biph Phe	Hex	Thi
Con			**	**	●	**		●	*	●	**
Bar				**	●	●			*	●	**
Phe					**	**				●	●
Hex						●					*
Thi											

					Con	Bar	Ethoc/Pento Phe	Hex	Thi
Con						**	**	**	**
Bar							●	●	●
Phe								●	●
Hex									●
Thi									

Each inducer is compared to each other one and to control in 6 matrices, corresponding to 6 quotients. ●, no significant difference between two inducers compared; *; $\alpha \leq 5\%$; **, $\alpha \leq 1\%$; Scheffé-test following two factor analysis of variance (see Fig. 1a).

deethylation/pentoxyresorufin-depentylation) show no significant differences between doses in hexobarbitone as well as in thiopentone induction. This finding would be compatible with the hypothesis that, at least in thiopentone induction, both these pairs are "sibling increases", not connected to each other. On the other hand, Table 4 contains twelve comparisons: in eleven of them (exception: ethoxycoumarin - deethylation/biphenyl - 4 - hydroxylation after hexobarbitone) the higher dose is connected with a greater deviation from control than is the smaller one. Therefore, as above, the conclusion of common regulations should only be drawn with caution.

Table 4. Proportions of metabolic rates after treatment with 2 different doses of hexobarbitone and of thiopentone

	Hexobarbitone				Thiopentone			
	contr	2 × 80	4 × 80	sign	contr	2 × 30	4 × 30	sign
4-Biph/EM	0.413	0.402	0.335	n.s.	0.583	0.523	0.516	n.s.
Pento/EM	0.039	0.458	0.572	**	0.036	0.586	0.913	**
Ethoc/EM	3.77	3.69	3.45	n.s.	3.01	3.45	4.23	*
Pento/4-Biph	0.096	1.17	1.75	**	0.064	1.13	1.79	**
Ethoc/4-Biph	9.54	9.47	10.55	n.s.	5.28	6.61	8.23	**
Ethoc/Pento	118.6	9.75	6.33	n.s.	139.9	6.12	4.68	n.s.

The quotient of the lower dose is compared to the quotient of the higher dose. n.s., no statistically significant difference between doses; *, $\alpha \leq 5\%$; **, $\alpha \leq 1\%$; Duncan-tests following two factor analysis of variance with repeated measures on the second factor (see Fig. 1b).

DISCUSSION

"Pleiotropic response" denominates a certain type of coordination on the transcriptional level: a group of structural genes is simultaneously derepressed by a single derepressor. In the case of 3-methylcholanthrene-like induction, as Nebert and his coworkers have shown in a series of reports, the derepressor is an inducer–receptor-complex moving from cytosol to nucleus and giving rise to production of cytochrome P450 isoenzymes, resulting in a simultaneous increase of several catalytic activities. However, as cited in the Introduction of this paper, it is "infrequently documented that these compounds are faithful mimetics of the entire pleiotropic response produced by the prototype compounds" [1]. In phenobarbitone-like induction, in contrast, no evidence is available indicating a cytosolic receptor control mechanism. In this paper, we have tried to approach this problem with indirect means. Our results show clearly that, even among barbiturates themselves, there is no uniform response. This seems to exclude the existence of a common receptor regulating the expression of cytochrome P450 isoenzymes in one and the same way for all barbiturates. Perhaps we have to think of "weaker" forms of coordination.

To this conclusion might be objected that we did not completely exclude a uniform response. For it may be argued that different catalytic activities might require different doses for maximal induction, so that very high doses might transform different inducer-specific responses into a uniform one. Our results with hexobarbitone and thiopentone, however, speak against that consideration, for doubling the dose did not alter inducer-specific patterns into a direction of greater similarity. Besides that, it is doubtful whether higher doses would yield valid results. Already our doses produced weight losses, losing weight indicates starvation, and starvation interferes with induction [9, 10]. Thus, it will be impossible to answer the question, whether sufficiently high doses eventually produce a uniform response.

That phenobarbitone-like induction decreases the amount of cytochrome P450-isoenzymes characteristic for 3-methyl-cholanthrene-like induction, and vice versa, has already been shown by Thomas *et al.* [11], Ryan *et al.* [12] and Phillips *et al.* [13] by immunological methods. Accordingly, we found a decrease in biphenyl-2-hydroxylation after all barbiturate inducers examined. The decrease is more prominent in C57BL/6J than in NMRI mice. This is easily explained by the fact that C57BL/6J mice possess the Ah complex. But it is remarkable, then, that the "stronger" a barbiturate inducer is, the greater is the decrease of a counterpart catalytic activity (biphenyl-2-hydroxylation, see Fig. 3). This suggests an interaction of barbiturate inducers with the cytosolic 3-methylcholanthrene receptor site leading to a correspondingly graded reduction of its effectiveness.

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