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EVIDENCE FOR ENDOGENOUS TRIGGERING OF PERINATAL INDUCIBILITY OF HEPATIC MONOOXYGENASE

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Abstract—Perinatal activity of oxidative metabolism of xenobiotics was measured in rat foetuses during prolonged gestation and in early neonatal life in order to study the onset of inducibility. Cytochrome P-450 content, NADPH–cytochrome *c*-reductase activity, *p*-nitroanisole demethylation, and benzphetamine demethylation were determined in controls, phenobarbital-, and alpha-hexachlorocyclohexane (alpha HCH)-induced rats. Controls show low activities that rise during day 22–25 of gestation. After induction with phenobarbital the activities rise after day 22, and after alpha-hexachlorocyclohexane-induction there is a smaller response compared to phenobarbital. When molecular activities (activity per nmole spectrally measured cytochrome P-450) of the demethylation reaction are considered, there are marked fluctuations over the time period investigated. The finding of intrauterine inducibility is interpreted as an indication that the onset of inducibility in early life is independent of parturition itself but largely determined by the time passed since conception. The possibility of an independent internal time signal for triggering inducibility is contemplated.

The transition from prenatal to postnatal life constitutes a remarkable adaptational change in many parameters [1–4]. The postnatal adaptation to extrauterine life is—among other factors—characterised by an increase in microsomal drug oxidases and their inducibility by various enzyme inducers [5]. In rats an abrupt onset of inducibility is observed during the first twelve hours after birth [4, 6]. Phenobarbital and 3-methylcholanthrene (3-MC)† have been found by Guenthner and Mannering [2] to differentially induce foetal monooxygenase activity. Thus, there is no increase in aminopyrine and ethylmorphine demethylations after phenobarbital, while 3-MC increases the prenatal benzo(a)pyrene hydroxylation. After parturition, however, the inducibility also by phenobarbital became apparent within one day, pointing to the development of different induction mechanisms. Cresteil *et al.* [4] arrive at basically the same conclusions, namely non-inducibility by phenobarbital before birth and inducibility by 3-MC. They, furthermore, show the presence of cytochrome P-450 in foetal livers of non-induced animals at the gestational age of 20 days. In their review Gillette and Stripp [1] conclude that the formation of cytochrome P-450 begins at birth and requires several weeks to reach adult levels; this development is paralleled by a morphological change of the hepatic endoplasmic reticulum from the rough to the smooth form.

Since, obviously, the transition from intra- to extra-uterine life seems to trigger the onset of inducibility, the question was raised, whether parturition as such is essential or whether there is an endogenously operated mechanism that 'switches on' the inducibility. This could possibly occur by endogenous inducers such as postulated for the biosynthesis of epoxide hydratase by Oesch [7].

In the experiments reported here, the above question was approached by measuring monooxygenase levels and activities in foetal rat livers during artificially prolonged gestation periods; prolongation was achieved by progesterone. The original prolongation method as used by Rosso [11] was substantially modified according to the kind advice of Dr. I. Chahoud of the Dept. of Embryopharmacology, Free University of Berlin.

Induction was attempted by the conventional inducer phenobarbital and also by alpha-hexachlorocyclohexane [8–10] which precipitates a strong early-postnatal increase in inducibility and because of its lipophilicity exerts a long-standing effect after a single prenatal dose.

The presented results suggest that postnatal inducibility seems to be triggered by the end of the normal gestation period rather than by the parturition itself.

MATERIALS AND METHODS

Animals. Female albino Wistar rats, 250–300 g, were purchased from the Zentralinstitut für Versuchstiere, Hannover, West Germany. After a two week acclimatisation period animals were mated overnight (four females with one male rat). The next day was considered day 1 of pregnancy. Pregnancy was ascertained by comparing the weights two weeks after breeding. If the increase in weight was at least 20 g, pregnancy was assumed. The method of vaginal inspection for spermatozoa was considered to be too

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† Abbreviations: 3-MC, 3-methylcholanthrene; PB, phenobarbital; α-HCH, alpha-hexachlorocyclohexane; *p*-NA, *p*-nitroanisole.

uncertain, because shortly after copulation sperm always will be found in the vagina.

As soon as pregnancy was ascertained, rats were housed separately. The constant environmental conditions were 23° and a 12 hr light/dark cycle. The pregnant rats had free access to tap water and lab chow ("Ssniff", Versuchstierdiäten GmbH, Soest/Westfalen, West Germany).

Animal experiments. The pregnant rats were divided into groups that were treated in the following way. One group stayed untreated as control after it was shown that application of solvents does not affect the parameters measured. Another group received sodium phenobarbital 40 mg/kg, dissolved in 0.9% saline which was injected intraperitoneally once a day during the last 3 days preceding sacrifice; to a further group alpha-hexachlorocyclohexane, 200 mg/kg, dissolved in olive oil, was administered via stomach tube in one dose at the fourth day before death.

All foetuses were obtained by Caesarean section. Foetal livers were collected on days 20, 21, 22, 23, 24 and 25 post conception. As the 22nd day of pregnancy normally is the day of parturition, gestation was prolonged to the days 23–25. To extend pregnancy we injected progesterone, 25 mg/kg, intramuscularly once a day, beginning with day 21 until one day before sacrifice [11]. The progesterone-treated animals were again divided into different groups that were treated with inducers in the same way as described above. One additional group consisted of neonates two days after spontaneous delivery.

Materials. Sodium phenobarbital was purchased from Merck, Darmstadt, West Germany. α-Hexachlorocyclohexane was a gift from Dr. Schulte-Hermann of the Institute of Toxicology and Pharmacology in Marburg; benzphetamine HCl was obtained from Temmler-Werke, Marburg, *p*-nitroanisole and *p*-nitrophenol were purchased from Merck, Darmstadt; NADP, NADPH, glucose-6-phosphate and glucose-6-phosphate dehydroxygenase were supplied by Boehringer, Mannheim; progesterone = Proluton® ampoules 10 mg/1 ml was obtained from Schering AG, Berlin.

Liver preparation. Pregnant animals were anesthetized with ether, decapitated and exsanguinated. Foetuses were removed *in utero* and decapitated; livers were excised and immediately pooled by litter and weighed. They were washed twice in ice cold 1.15% KCl and then homogenised in five volumes of a buffer of 20 mM Tris in 1.15% KCl. The homogenate was centrifuged for 20 min at 10,000 *g*. The supernatant was again centrifuged for 60 min at 105,000 *g*. Each microsomal pellet was completed to the original liver-weight with a buffer of 0.25 M sucrose and 20 mM Tris, pH 7.4, then resuspended. Livers from 2-day-old neonates (after spontaneous delivery) were processed in the same way. Protein content was determined according to Lowry *et al.* [12] using Folin's reagent [13].

Determination of cytochrome P-450. Assays of cytochrome P-450 were performed according to the procedure of Omura and Sato [14]. The microsomal suspension, diluted with Tris buffer 0.66 M, pH 7.4 (1:8) was distributed into two cuvettes. After bubbling the sample cuvette with CO, the reducing agent sodium dithionite was added to both cuvettes. The characteristic difference spectrum of cytochrome P-450 was recorded in the wavelength area from 400–500 nm. This experiment was carried out using an Aminco DW2 spectrophotometer.

Enzyme assays. NADPH cytochrome *c*-reductase: the rate of cytochrome *c*-reduction has been followed by observing the increase in optical density at 550 nm with time; calculations were carried out with the extinction-coefficient of $1.91 \times 10^4\ mM^{-1}cm^{-1}$ [15, 16]. *p*-Nitroanisole-*O*-demethylation assay was performed according to the method of Netter (1960) [17]. Benzphetamine-*N*-demethylase activity was measured by formaldehyde production [16]; formaldehyde was determined according to Nash [18].

Statistics. For the determination of the significance of differences between means, Student's *t*-test was employed.

Influence of progesterone on formation of cytochrome P-450 and its activities. Several authors pointed out that progesterone possesses an inhibitory effect on drug metabolism [5, 19–22]. In order to assess this possible influence under our conditions of dosage and sex, respective experiments were carried out with liver microsomes from young virgin and older rats which previously had delivered at least one litter. Their design corresponded to those in foetal livers as described above.

When the activities of both above mentioned demethylation reactions were related to the content of cytochrome P-450 (in nmoles) the molecular activities obtained after pretreatment with progesterone were not significantly changed. This was also found after previous induction by PB or α-HCH.

RESULTS

Perinatal development and inducibility of cytochrome P-450

By administration of 25 mg/kg progesterone, starting on day 21 of pregnancy, the gestation was prolonged to maximally 25 days. During this time-period the cytochrome P-450 content per mg microsomal protein rose continously from barely measurable concentrations on day 21 to about 0.1 nmoles per mg protein.

The inducers phenobarbital (PB) and alpha-hexachlorocyclohexane (α-HCH) in a dosage of three times 40 mg/kg i.p. and once 200 mg/kg orally, respectively, lead to a marked increase in cytochrome P-450 concentration that becomes apparent on day 24 and 25 for PB (Fig. 1). α-HCH affects an earlier increase (at day 23) which, however, subsides on day 25. The inducer effects for PB are significant on day 24 ($P < 0.002$) and 25 ($P < 0.01$) and those for α-HCH on day 23 ($P < 0.02$) and 24 ($P < 0.01$) but not on day 25. All induced values have been compared with those of the controls of the day referred to.

Monooxygenase activities in fetal rat livers

(a) *NADPH-cytochrome c-reductase.* When expecting an increase in metabolic activities that is comparable to the increase in cytochrome P-450, the question arose whether the rate of cytochrome P-

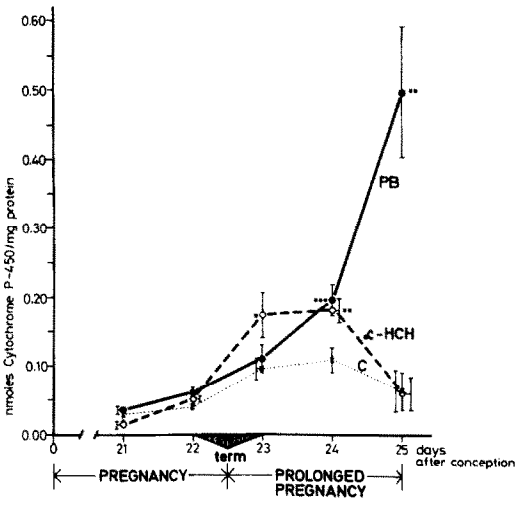

Fig. 1. Perinatal development of the cytochrome P-450 concentration and inducibility by phenobarbital (PB) and alpha-hexachlorocyclohexane (α-HCH). In order to prolong the gestation period pregnant rats were injected with 25 mg/kg progesterone in oil. Animals were killed at the times indicated, livers of all foetuses from one litter were pooled and processed as in Materials and Methods. Term was assumed to be between day 22 and 23; therefore, animals were sacrificed always at midday. C = controls, untreated; PB = phenobarbital (40 mg/kg, in saline, i.p., daily for three days before killing); α-HCH (200 mg/kg in oil by gavage, 4 days before sacrifice). * = $P < 0.02$, ** = $P < 0.01$, *** = $P < 0.002$. The number of experiments (litters) is given in the following list for the respective durations of gestation.

Days of pregnancy	Controls	Phenobarbital treated	α-HCH treated
21	4	6	4
22	13	11	10
23	6	3	4
24	12	13	8
25	3	3	3

450 reduction would also be elevated. Respective measurements of NADPH-cytochrome *c*-reductase showed a very marked increase in activity per mg protein. Activities on day 22 were compared with those on day 24 (Fig. 2). As can be seen there is a doubling of the activity in control animals. Upon induction, however, the activity is three or four times as high as in the respective conditions on day 22. This finding attests to perinatal inducibility of cytochrome P-450 reduction by NADPH.

(b) *p-Nitroanisole-demethylase.* As an example for the xenobiotic metabolic activity *O*-demethylation of *p*-nitroanisole was measured and related to the various stages of perinatal development. It was subsumed that this represents the oxidative metabolism of a substrate that binds to cytochrome P-450 according to type I. Based on microsomal protein content the activity increases in a manner similar to that described previously for the cytochrome P-450 content. Controls and induced animals show the same pattern as in Fig. 1.

However, when values are based on cytochrome P-450 itself (molecular activity), a different picture evolves (Fig. 3). One day prior to term there is no detectable activity at all; subsequently in controls it rises again continously until day 24. When the inducers are employed, the situation changes drastically in that PB on day 24 and 25 causes a decrease in molecular activity that is statistically significant.

In contrast, α-HCH pretreatment seems to generally lower the molecular activity and to depress it below control level. Thus, the inducers exert basically different effects on the monooxygenase.

(c) *Benzphetamine-N-demethylase.* An altogether different situation exists for the *N*-demethylation of the model compound benzphetamine. Firstly, the molecular activity tends to be about one tenth only of that for *p*-nitroanisole, qualitatively an even more drastic alteration has been observed in the fact that on day 21 (prenatally) there is considerable molecular activity in all three groups. This activity decreases as term appproaches and remains low in controls and α-HCH treated animals. The PB group (four experiments), however, shows a greatly increased molecular activity on day 24 and thus exhibits a clear biphasic alteration of molecular activity (Fig. 4). This behaviour suggests similar alterations affecting molecular activity as seen with *p*-nitroanisole, whereby the respective activities undergo changes in opposite directions. The difference between the two inducers, therefore, is reflected also with benzphetamine as a substrate.

When, however, activity is related to microsomal protein instead of to cytochrome P-450, α-HCH clearly increases the benzphetamine demethylase activity: on day 24 controls show 0.14 nmole formaldehyde per mg protein per min, while α-HCH animals produce 0.25 nmole formaldehyde per mg per min. Phenobarbital raises this value about tenfold to 1.5 nmole/mg/min.

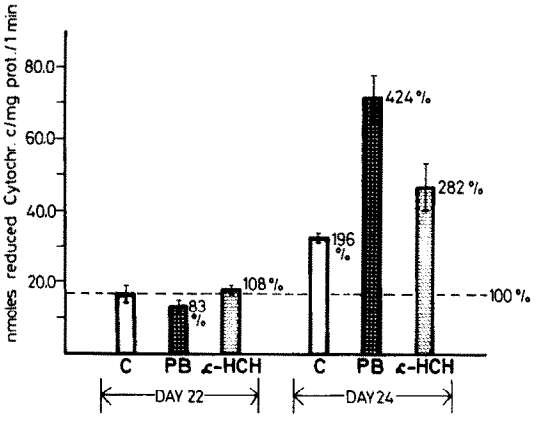

Fig. 2. Activity of the NADPH-cytochrome *c*-reductase in foetal rat livers on days 22 and 24 of pregnancy with or without induction; pregnant rats were treated with PB (40 mg/kg daily for three days) or with α-HCH (200 mg/kg once four days before sacrifice; progesterone treatment was as in Fig. 1. $N = 3$–5.

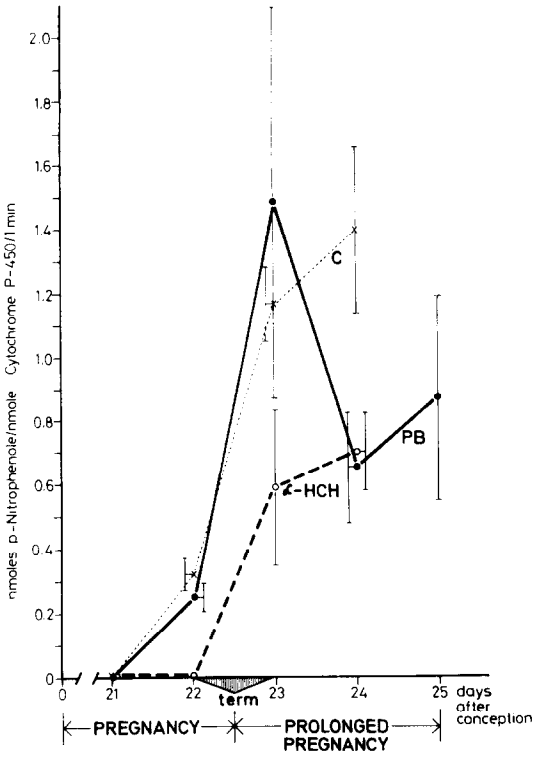

Fig. 3. Molecular activity of microsomal *p*-nitroanisole-*O*-demethylase at various stages of foetal development. Treatment and symbols are the same as in Fig. 1; number of experiments between 3 and 12. In PB-animals the decrease from day 23 to 24 is significant ($P < 0.05$).

Comparison of drug oxidative parameters in postnatal intra- and extrauterine life

In order to delineate possible factors responsible for enzyme induction immediately after term, a comparison of cytochrome P-450 content and enzyme activity was made between rat embryos whose intrauterine period was artificially prolonged and embryos born at term, suckling for two days. In each case drugs were administered to the mother only, and comparisons were made between day 24 of sustained pregnancy and the second day of extrauterine life.

Determination of cytochrome P-450 shows the results given in Fig. 5. In intrauterine animals on day 24 cytochrome P-450 is roughly doubled by PB as well as α-HCH-pretreatment; these values are identical to those in Fig. 1. When, however, one compares them to the same figures obtained from two day old rats, which had been born normally, it appears that enzyme induction is markedly more efficient in extrauterine conditions. In these newborn animals inducibility is about twice as high as in their intrauterine companions.

Enzyme activity based on microsomal protein yields a very similar picture. However, the molecular activities for *p*-nitroanisole and benzphetamine metabolism show a much more varied picture: molecular *O*-demethylase activity decreases to about half upon intrauterine induction by PB or α-HCH, which is in contrast to the findings reported above for cytochrome P-450. During extrauterine induction by α-HCH and in controls there is a decrease in molecular activity which again is in the order of about 50%. An exception is the induction by PB, which increases molecular activity over that of the intrauterine partners. This situation seems to be substrate specific because molecular activities of benzphetamine-*N*-demethylation follow a different pattern. In agreement with Fig. 4, intrauterine molecular activity is greatly enhanced in PB pretreated foetuses, while α-HCH leads on the contrary to a decrease. Furthermore, in distinct contrast to *O*-demethylation of *p*-NA, extrauterine controls show a very much lower molecular activity, again pointing to a distinct influence of the particular substrate. This also applies to the conditions after PB induction, where intrauterine molecular activity is about six times higher than extrauterine. The exception is again α-HCH, which in this case causes higher activities in extrauterine newborns (176%).

DISCUSSION

In the past many data on perinatal activities of xenobiotic metabolising cytochrome P-450 dependent monooxygenases have been published [1–6, 23–28]. They unanimously stress the fact that prenatally there is very little activity, at least in rodents, while after birth the rate of metabolism rises steeply. Most activity comparisons are based on microsomal protein or on liver weight [2]. At the same time most authors agree that prenatal enzyme induction by phenobarbital and other inducers is not achievable, even after high doses of inducers [2, 4]. After parturition, however, inducibility appears very soon and often quite dramatically [1, 2, 4–7].

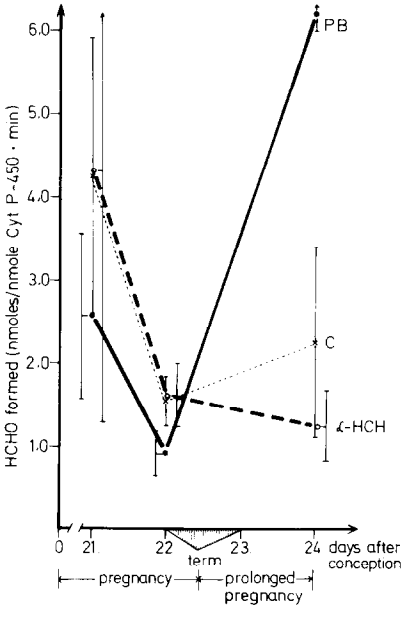

Fig. 4. Benzphetamine-*N*-demethylase in foetal livers at various perinatal stages. Results are expressed as molecular activity and related to perinatal age. Induction and symbols are as in Fig. 1. $N = 3$–8.

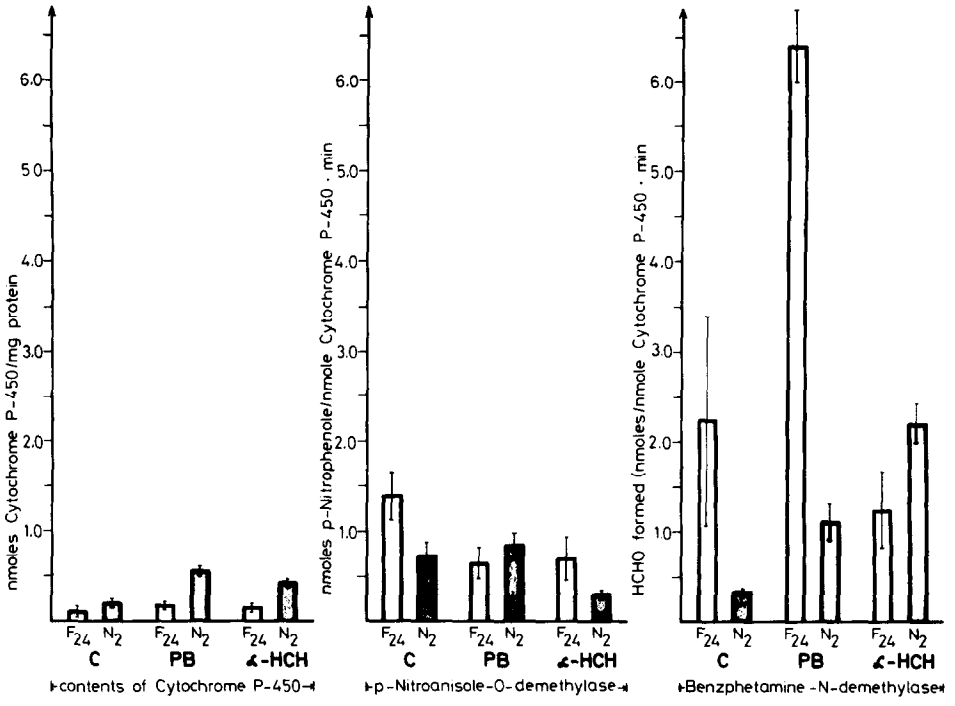

Fig. 5. Comparison of drug oxidative parameters in foetal livers and neonatal livers. Enzymatic parameters were compared for foetal livers from animals at day 24 of gestation = F_{24} (2 days overdue) and for livers of 2 day old newborns = N_2 (24 days after conception). Transmission of the inducers to the newborns was by maternal milk. N = 5–13. Open bars represent intrauterine activities and hatched bars represent newborns.

Thus, comparison of cytochrome P-450 contents and respective activities between foetuses of day 21–22 and newborn rats of 1–2 days of age shows a continuous increase in cytochrome P-450 per g liver. Benzo(a)pyrene oxidation as well as aminopyrine and ethylmorphine demethylation show absolutely no prenatal but steeply rising postnatal activity [2]. Phenobarbital generally leads to a measurable increase over control activities only after at least the first day of extrauterine life. The same is true for aminopyrine-demethylation after induction by α-HCH [6].

It could be concluded, that prenatally an endogenous factor suppresses cytochrome P-450 formation and its inducibility and that this factor disappears after birth [2]. In order to test this hypothesis, the gestation period was prolonged by about 2–3 days through the application of progesterone to pregnant rats. The results presented here show that the above hypothesis is not quite correct. Spontaneous formation of cytochrome P-450 as well as inducibility by PB and α-HCH occur *in utero* under these conditions. From this observation it is concluded that birth *per se* does not trigger these two events but that they rather depend on an inborn time signal which is given automatically and at which the inhibitory 'endogenous factor' is removed. Phenobarbital induction seems to be suppressed until term, although PB given to the mother reaches the foetus in appreciable concentrations transplacentally long before this date [29]. These authors found that PB accumulates in foetal rat livers to almost the same extent as in the maternal liver. The nature of the apparent suppressive factor remains open to speculation.

Determination of the molecular activity based on cytochrome P-450 content uncovers another interesting periodicity in perinatal events. *p*-Nitroanisole-*O*-demethylase activity rises sharply from intrauterine day 22–24 in controls as well as in α-HCH treated animals. In PB-treated rats after a steep increase from day 22 to 23 there is an equally steep decrease in molecular activity on day 24. This must be interpreted as indicative of drastic changes in the cytochrome, either by appearance or disappearance of new 'isoenzymes' or by structural changes in the apoprotein during this critical life period.

When benzphetamine is the substrate, molecular activity changes in almost the exactly opposite way. Here the initial activity in all three conditions (control, PB, α-HCH) is high on day 21 of gestation and falls at about term to about a fifth of its starting value. On the intrauterine day 22 activity in both controls and α-HCH treated animals remains low. But after PB-treatment a very markedly higher molecular activity is formed. Thus, phenobarbital-induced perinatal cytochrome P-450 seems to interact with both substrates in a manner that is basically different from that in controls and α-HCH-treated animals.

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