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THE DEVELOPMENT OF SEX DIFFERENCES IN THE DEMETHYLATION OF ETHYLMORPHINE AND IN ITS INTERACTION WITH COMPONENTS OF THE HEPATIC MICROSOMAL CYTOCHROME P450 SYSTEM IN MICE*

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Abstract—The development of sex differences in ethylmorphine *N*-demethylation and several components of the reaction chain were studied in hepatic microsomes from mice of the CPB-SE strain between 3–11 weeks of age. Sex-specific changes were observed in demethylation rate, type 1 spectral interaction, cytochrome P450 content, and ethylmorphine-induced stimulation of NADPH-cytochrome P450 reductase activity. These changes occurred mainly between weeks 3–7 and were confined to females. It is concluded that the development of the cytochrome P450 system is repressed by androgen during sexual maturation. The kinetic constants of demethylation developed differently from ethylmorphine binding constants. Changes in demethylase were mainly restricted to K_m, whereas the changes in type 1 binding only involved the maximum spectral change. In combination with differences observed between the developmental patterns of demethylation rate and cytochrome P450 reductase activities, this demonstrated that the reduction of cytochrome P450-substrate complex is not rate-limiting in ethylmorphine demethylation. The type 1 spectral change was correlated with the amount of cytochrome P450 only when a large portion of the cytochrome was considered inactive in ethylmorphine binding. It is suggested that immature animals possess a low basal level of ethylmorphine binding type 1 sites, which is elevated selectively in females during sexual maturation.

The development of hepatic microsomal drug metabolizing enzyme activity (cytochrome P450 system) varies with species and substrate. Nevertheless, monoxygenase activity is usually hardly detectable during foetal life (with the exception of primates) and increases postnatally to reach a plateau at adulthood. Results of investigations in this area have recently been reviewed by several authors [1–3].

Some substrates (type 1 substrates, defined by their spectral interaction with cytochrome P450 [4]) show sex-dependent metabolism. These sex differences appear to occur primarily in rats (see [5] for review). In this species, males exhibit higher hydroxylating activity than females. On the basis of the results of castration and testosterone treatment, this was attributed to an androgenic action [5–7]. The development of the sex difference in demethylation was found to run parallel with the onset of sexual maturity, and was mainly the result of an increase in enzyme activity in the male between approximately 3–7 weeks of age. This was observed for aminopyrine [8, 9] as well as ethylmorphine [10, 11]. Studies which included only male rats also revealed sudden increases in ethylmorphine demethylation [12, 13].

Some strains of mice also exhibit a sex difference in the metabolism of type 1 substrates [14], which has been the subject of a number of studies in our laboratory [15–19]. Interestingly, in the CPB-SE strain, metabolism is faster in females than in males. Although this difference is opposite to that found in rats, it appeared to be abolished by treatment of females with testosterone and castration of males, thus suggesting that this sex dependency is also due to androgenic action. Pomp *et al.* [20] demonstrated that mice were not able to *O*- or *N*-demethylate *p*-nitroanisol or monomethyl-*p*-nitraniline, respectively, at birth. The development of these activities was followed for a period of 4 weeks thereafter and found to be completed at approximately 3 weeks of age. No specification was given as to the sex of the animals. It was therefore of interest to study the demethylation of ethylmorphine in the CPB-SE strain during sexual development.

In a previous investigation [19] the roles of type 1 binding and NADPH-cytochrome P450 reductase in the demethylation of ethylmorphine were examined in two strains of mice. It had been proposed by others [21–23] that type 1 binding might reflect the formation of enzyme-substrate complex, whereas the substrate-induced stimulation of NADPH-cytochrome

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P450 reductase activity would represent the reduction of this complex by the reductase, which probably constitutes the rate-limiting step. Both mouse strains exhibited considerable sex differences in type 1 binding and reductase stimulation, these parameters being highly correlated. As demethylation was not sex-dependent in the CPB-V strain, the reduction of cytochrome P450-substrate complex can not be rate-limiting, but this could not be excluded in the CPB-SE strain. In order to further elucidate the nature of this sex difference the development of NADPH-cytochrome P450 reductase activities and cytochrome P450 were studied as well as the kinetic constants of ethylmorphine demethylation and type 1 binding.

MATERIALS AND METHODS

Animals. Immature male and female mice of the CPB-SE strain (2 weeks) were obtained from the Central Animal Breeding Station TNO, Zeist, The Netherlands. The mice of each sex were randomly divided into five groups, which were sacrificed when the animals reached the age of 3, 5, 7, 9, and 11 weeks respectively. All animals were kept under similar conditions in Makrolon cages, with pinewood shavings, and received standard food pellets (Hope Farms) and water *ad lib.*

Chemicals. Ethylmorphine was obtained from Brocacef; $NADP^+$ (grade 1), NADPH (grade 1), glucose-6-phosphate (disodium salt), and glucose-6-phosphate dehydrogenase (grade 1) were obtained from Boehringer, and bovine serum albumin from Poviet. All other chemicals used were at least reagent grade.

Preparation of microsomes. After starving overnight, the animals were killed by a blow on the head. Livers were homogenized in 3 volumes of 0.1 M phosphate buffer (pH 7.4), using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged for 20 min at 9000 *g* and the microsomal fraction was sedimented from the resulting supernatant be centrifuging at 75,000 *g* for 90 min. The pellet was suspended in 0.1 M phosphate buffer (pH 7.4). For each determination the livers of several animals were pooled.

Enzyme assays. Ethylmorphine *N*-demethylation was assayed by measuring the formation of formaldehyde. Microsomes were incubated with ethylmorphine (0.4, 0.5, 0.67, 1.0, and 2.0 mM) for 10 min at 37°, with shaking, air being freely admitted. The incubation mixture further contained $NADP^+$ (0.75 μmole), glucose-6-phosphate (12.5 μmoles), glucose-6-phosphate dehydrogenase (1 i.u.), semicarbazide (12.5 μmoles), nicotinamide (5 μmoles), $MgCl_2$ (12.5 μmoles), and microsomes, in a total volume of 3 ml. The reaction was initiated by the addition of 0.5 ml of microsomal suspension (10 mg protein/ml) and stopped by consecutive additions of 0.5 ml 40% $ZnSO_4$ and 1 ml saturated $Ba(OH)_2$. After centrifugation, 2 ml of the supernatant were mixed with 1 ml double strength Nash reagent [24], and incubated at 60° for 30 min. The amount of HCHO formed was estimated by determining the absorbance at 415 nm relative to that at 500 nm, and subtracting the amount of Nash-positive material formed in the appropriate blank. The apparent Michaelis–Menten constant (K_m) and maximum velocity of demethylation (V_{max}) were calculated according to the method of Wilkinson [25].

Difference spectra were recorded on an Aminco-Chance dual wavelength spectrophotometer in the split beam mode. Cuvettes contained 3 ml of microsomal suspension (3 mg/protein/ml). After establishing a baseline of equal absorbance, ethylmorphine (0.33, 0.4, 0.5, 0.67, 1.0, and 2.0 mM) was added to the sample cuvette and the difference spectrum was recorded. The peak-to-trough difference (385–420 nm) was taken as the magnitude of spectral interaction, and expressed in absorbance units. The spectral dissociation constant (K_s) and maximum spectral change (ΔA_{max}) were calculated according to the method of Wilkinson [25].

NADPH-cytochrome P450 reductase was assayed under anaerobic conditions in a carbon monoxide atmosphere at 30° essentially as described by Gigon *et al.* [13]. The formation of the reduced cytochrome P450-CO complex was recorded as the absorbance change at 450 nm relative to 490 nm, using an Aminco DW-2 UV-VIS spectrophotometer in the dual wavelength mode. The reaction was initiated by the addition of 50 μl of NADPH-solution (20 mg/ml) to 2.5 ml of a microsomal suspension containing about 1 mg protein/ml. The initial rate of reduction was expressed as nmoles cytochrome P450 reduced/min/mg protein using 91 $cm^{-1}mM^{-1}$ [26] as the value of the absorption coefficient. Reductase stimulation (Δ_{red}) represents the difference between the rates of reduction in the presence and in the absence of ethylmorphine (endogenous or basal reduction rate), which were determined in duplicate and triplicate, respectively. Total cytochrome P450 content was determined by completely reducing the cuvette contents with sodium dithionite.

Microsomal protein was assayed according to the method of Lowry *et al.* [27], using crystalline bovine serum albumin as a standard.

Statistics. Two-way analysis of variance was employed to analyze all data for effects of age, sex, and interaction (age–sex), the latter representing the degree of dissimilarity between the developmental patterns. In the case of significant effects of sex, the sex differences at all ages were tested separately against the residual variance. K_m values could not be analyzed this way, because of a significant difference between the male and female population variances. Sex differences were, therefore, tested by Student's *t*-test or Wilcoxon test where appropriate. $P < 0.05$ was taken as the lowest level of significance. Additionally, the significance of differences at the $P < 0.01$ level has been indicated. The number of experiments was four, except in the case of 3 week old females, where this number was three.

RESULTS

Kinetics of ethylmorphine demethylation. The maximum rate of demethylation (V_{max}) decreased between 3–5 weeks in both sexes, and then remained constant (Fig. 1). The developmental patterns were not significantly different. However, taking all ages together, males had a significantly lower (about 20 per cent) V_{max} than females ($P < 0.01$).

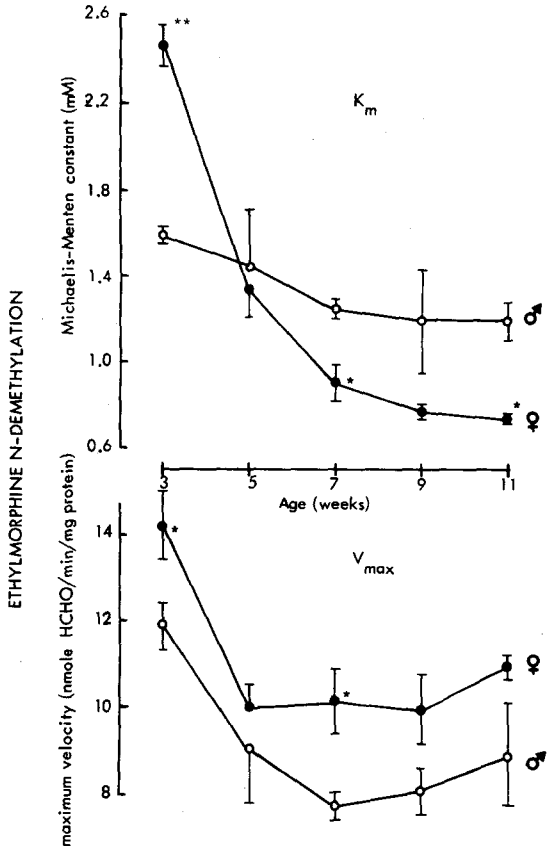

Fig. 1. Effects of age on the kinetics of ethylmorphine demethylation in liver microsomes from male and female mice. * Indicates a significant sex difference, $P < 0.05$; ** id. $P < 0.01$. Values represent means ± S.E.

The apparent K_m decreased by a factor of 3, in females, whereas no statistically significant change was observed in males. Age-dependent changes in K_m have also been observed in rats [7, 12, 28]. The sex dependency of K_m was rather complicated. At 3 weeks, females had a higher K_m whereas the opposite was observed at 11 weeks, when normal, adult values were reached [18, 29]. These developmental patterns of V_{max} and K_m imply that an increasing sex difference is apparent when measured at low substrate concentrations, which might be of significance in the *in vivo* situation.

Type I binding of ethylmorphine. The development of the maximum spectral change (ΔA_{max}) was highly sex-dependent (Fig. 2). In females, a dramatic increase was observed, whereas ΔA_{max} remained at a constant level in males. At 3 weeks no sex difference existed, but at 11 weeks females exhibited a 3-fold higher ΔA_{max} than males. ΔA_{max} does not correlate with the demethylation rate (Fig. 1), which appears to disagree with the concept that the spectral interaction reflects the formation of enzyme-substrate complex [21].

There was no significant effect of age on K_s, neither in females nor in males (Fig. 2), nor were the developmental patterns significantly different. A significant sex difference was found at 9 weeks only. In other experiments we have sometimes noticed that adult animals do show a small sex difference in K_s, females having lower values [18]. In this study, also, a significant sex difference was found when K_s was tested at all ages simultaneously ($P < 0.05$). Nevertheless, a large discrepancy between the developmental patterns of K_m and K_s is apparent.

Cytochrome P450 content. The sex difference in cytochrome P450 content in adult animals appeared to be mainly the result of developmental changes occurring in females (Fig. 3). Between 3–11 weeks an increase of 41 per cent was observed. A significant sex difference in favour of females did not arise until the age of 7 weeks. At 3 weeks, even an opposite sex difference was noticed. The cytochrome P450 content does not seem to be related to the rate of demethylation, which is in agreement with our previous findings, as well as with those obtained in rats by many other authors [9–12, 22, 30, 31].

Relationship between ΔA_{max} and cytochrome P450. The development of sex differences in ΔA_{max} and cytochrome P450 content is mainly the result of age-dependent increases occurring in females (Figs 2 and 3). The maximum spectral change is an expression of the total amount of binding sites involved in ethylmorphine binding. If all cytochrome P450 is involved in this binding, then ΔA_{max} and cytochrome P450 content should be related. The data presented, however, show that the relative changes in cytochrome P450 content are much smaller than those in ΔA_{max}. A better proportionality can be obtained when the increments in female values are calculated at all time intervals, with the situation at 3 weeks as a reference level. Furthermore, when the female values are corrected for fluctuations occurring in the male, a linear proportionality between ΔA_{max} and ΔP450 is observed (Fig. 4). This suggests that, until the age

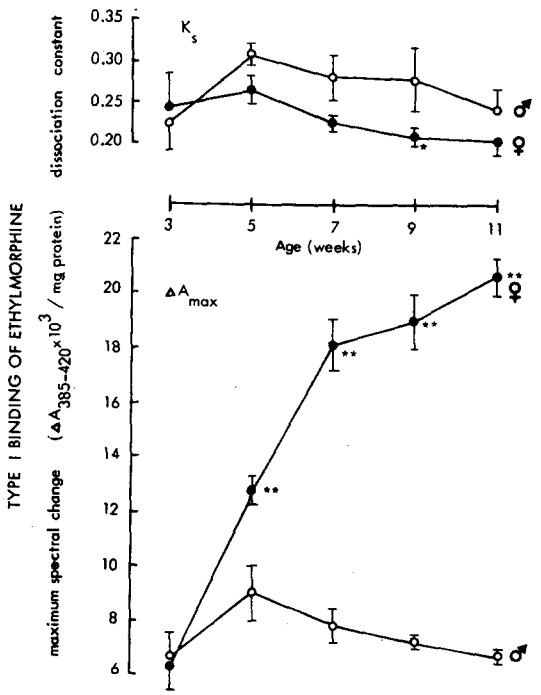

Fig. 2. Effects of age on the kinetics of type 1 binding of ethylmorphine in liver microsomes from male and female mice. * Indicates a significant sex difference, $P < 0.05$; ** id. $P < 0.01$. Values represent means ± S.E.

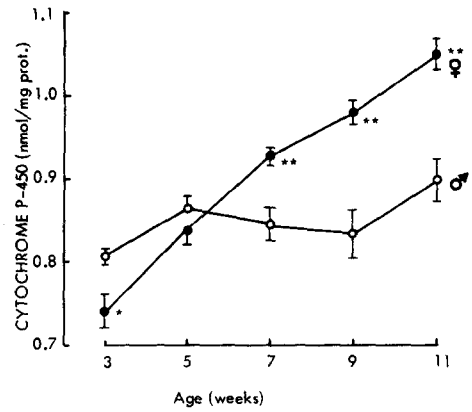

Fig. 3. Effects of age on the cytochrome P450 content of liver microsomes from male and female mice. * Indicates a significant sex difference, $P < 0.05$; ** id. $P < 0.01$. Values represent means ± S.E.

of 3 weeks, a low basal level of type 1 binding activity has developed in both sexes, which does not involve the total amount of cytochrome P450. Thereafter, an additional ethylmorphine binding capacity develops selectively in females. This might be due to synthesis of a cytochrome P450 fraction with high specific binding activity, which can be calculated (using Fig. 4) to be about seven times higher than that of the cytochrome already present.

NADPH-cytochrome P450 reductase activity. High basal activities of NADPH-cytochrome P450 reductase were found at 3 weeks, which subsequently decreased to the same degree in males and females, 37 and 36 per cent respectively (Fig. 5). A sex difference

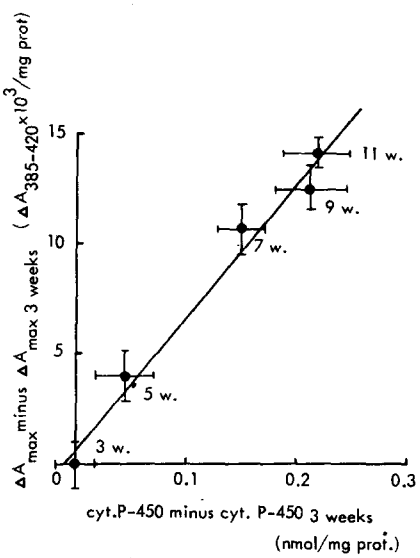

Fig. 4. Relationship between the sex-specific increases in maximum spectral change (ΔA_{max}) and cytochrome P450 content in liver microsomes from female mice between 3–11 weeks of age. Values were calculated from the data presented in Figs 2 and 3, by taking the differences between the male and female values at all ages and defining the value at 3 weeks as zero level. Standard errors were obtained using the weighted mean variances of the male and female series of experiments. The line shown was obtained by linear regression analysis.

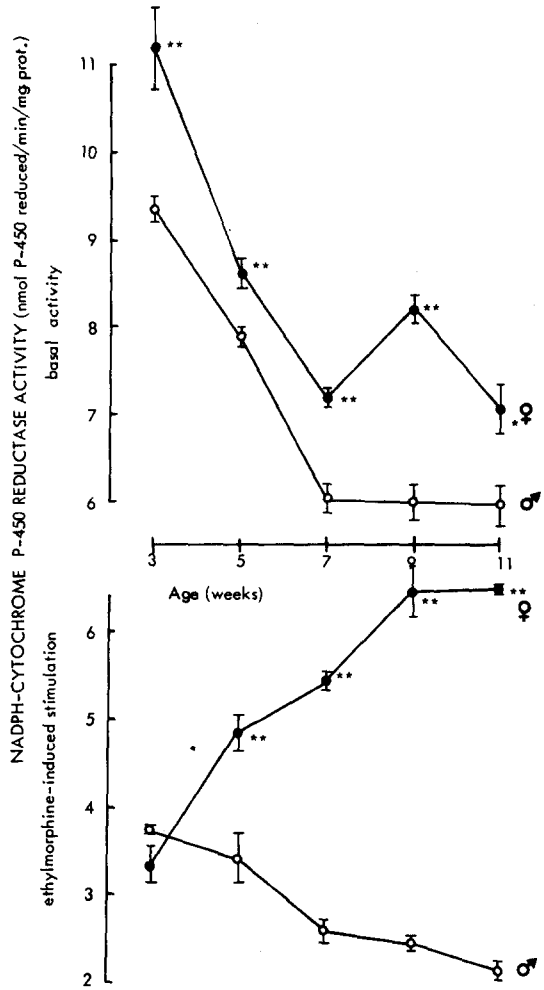

Fig. 5. Effects of age on basal NADPH-cytochrome P450 reductase activity (upper part) and on ethylmorphine (1 mM)-induced stimulation of NADPH-cytochrome P450 reductase activity (lower part) in male and female mice. * Indicates a significant sex difference, $P < 0.05$; ** id. $P < 0.01$. Values represent means ± S.E.

was already apparent at 3 weeks and remained constant up to 11 weeks (male values approximately 85 per cent of those in females). The development of basal reductase activity resembled, to a certain extent, that of V_{max} of ethylmorphine demethylation. The decrease which appeared between 5–7 weeks, however, was not observed with V_{max}.

The sex difference in ethylmorphine-induced stimulation of NADPH-cytochrome P450 reductase activity, observed previously [19], seems to arise from developmental changes in both sexes. Reductase stimulation increased by 95 per cent in females, whereas a gradual 43 per cent decrease was noticed in males. There appears to be no correlation with V_{max} values. The concentration of ethylmorphine used (1 mM), however, did not produce maximal stimulation. The use of still higher concentrations is hampered by the fact that the initial rate of reduction becomes too fast to be measured accurately. When the demethylation rate is examined at 1 mM ethylmorphine a developmental increase of approximately

50 per cent is observed in females (due to the decreasing K_m), whereas hardly any change is noticed in males. Thus reductase stimulation does not correlate with the demethylation rate, in contrast to the results obtained with rats [11, 22, 23, 32].

Relationship between reductase stimulation and type 1 binding. The magnitude of spectral interaction in males did not change between 3–11 weeks, whereas a substantial decrease in reductase stimulation was noticed (Figs 2 and 5). Furthermore, the increases in these parameters in females were parallel. Interestingly, however, the sex differences developed simultaneously and to the same extent (Table 1). This suggests that Δ_{red} and ΔA are intrinsically related, which would confirm our previous observations [19], but it indicates that this relationship is masked by a factor which affects one of these parameters in both sexes to the same degree. This points to the decline in basal NADPH-cytochrome P450 reductase activity, which amounted to 36 per cent in males and 37 per cent in females (Fig. 5). This decline is proportional to the decline in Δ_{red} values in males. The assumption that Δ_{red} was similarly affected in females satisfactorily explains the discrepancy between the increases in Δ_{red} and ΔA in this sex, and at the same time the highly-correlated development of sex differences in these parameters.

DISCUSSION

The present study demonstrates a sex-dependency in the development of ethylmorphine *N*-demethylation, ethylmorphine binding to cytochrome P450, cytochrome P450 content and ethylmorphine-induced stimulation of NADPH-cytochrome P450 reductase activity in mice. Adult values are reached at about 11 weeks of age, thus coinciding with sexual maturation. The time course of this development is similar to that found in rats, but the sex differences tend towards the opposite direction. We have demonstrated that, in mice, castration of males and testosterone-treatment of females abolish the sex differences [17, 19]. These findings indicate an inhibitory effect of androgen, whereas, in rats, the androgen effect is presumed to be stimulatory [5]. Androgen apparently prevents the stimulation of enzyme activity seen in female mice, which is probably the basic developmental pattern in this species. The responses to androgen might be enzyme-specific [33–35] as well as species-specific [36], as observed with some enzymes involved in steroid metabolism.

The different developmental patterns of the kinetic constants of the demethylation reaction and type 1 binding present a problem concerning the mechanism of the reaction. The concept that type 1 binding represents the formation of enzyme–substrate complex, which is subsequently reduced by NADPH-cytochrome P450 reductase in a rate-limiting reaction [21, 37], is apparently not applicable. In a previous study we observed that, in mice, the stimulation of cytochrome P450 reductase activity by type 1 substrates was correlated with the magnitude of spectral interaction, thus confirming results obtained with rats [22, 23], although certain discrepancies have been reported [11]. The results presented in Figs 1 and 5 apparently contradict the earlier findings. The data given in Table 1 and Fig. 5, however, indicate that this paradox may be explained on the basis of developmental changes in basal reductase activities. These changes might reflect changes in the (pseudo first order) rate constant of cytochrome P450 reduction, which alter the absolute value of Δ_{red} independently from ΔA. The correlation between reductase stimulation and type 1 binding suggests that type 1 binding represents the formation of a functional cytochrome P450-substrate complex.

Several authors have observed significant correlations between Δ_{red} and substrate oxidation in rats [11, 22, 23, 32], but other reports have indicated that the reductase does not control the rate of substrate oxidation [38–40]. Previously we could not exclude this possibility, as any one of the accessible and routinely measured parameters (endogenous or basal reduction rate, substrate-induced or total reduction rate, or reductase stimulation) might not be totally representative of the rate of reduction of the cytochrome P450-substrate complex, formed by type 1 binding [19]. From the present results it is evident that cytochrome P450 reduction is not correlated with ethylmorphine demethylation, whichever parameter of reductase activity is used.

If the reductase step is not rate-limiting, changes in K_m do not necessarily reflect changes in K_s. In the case of a reaction mechanism which is not completely ordered (as discussed by Gillette *et al.* [41]) K_m may approximate the dissociation constant of the oxygenated or the active oxygen cytochrome P450-substrate complex rather than K_s. A changing K_m may be indicative of conformational changes or of a shift in the rate-limiting step. According to the most plausible models, however, V_{max} should be related to ΔA_{max}. If the intermediate forms of cytochrome P450 reversibly interact with the substrate and if the unbound forms of the cytochrome are reduced and oxygenated at rates which differ considerably from those of the bound intermediates, plots of 1/v vs 1/S will be curves. This curvature may not be detected and may lead to V_{max} and K_m values which are dependent on the substrate range chosen. Although in that case, these parameters would be ill-defined, it is unlikely that this explains the highly diverging ΔA_{max} and V_{max} observed during development. Hayes *et al.* [42] have suggested the existence of two demethylases with different kinetic constants (in rats). This might introduce a discrepancy between ΔA_{max} and V_{max}. Our results may indicate the presence of two demethylases in female mice, one of which operates at a negligible rate, although they are characterized by cytochromes P450 which have a similar ability to form type 1 complexes and to activate the reductase. This is suggestive of ethylmorphine functioning as a (partial) uncoupler of monoxygenase activity [43–45]. Further work is needed to resolve these questions.

An increasing body of evidence suggests the existence of multiple forms of cytochrome P450, which have distinct (but sometimes overlapping) specificities with regard to substrate or ligand interaction, and substrate hydroxylation [46–53]. The demonstration of different classes of type 1 binding sites is of particular interest [48]. Our study indicated that type 1 binding of ethylmorphine is restricted to a limited

Table 1. The development of sex differences in ethylmorphine-induced stimulation of NADPH-cytochrome P450 reductase activity and magnitude of type 1 spectral interaction

Age (weeks)	Reductase stimulation* (males/females × 100%)	Type 1 spectrum* (males/females × 100%)
3	112	106
5	71	68
7	48	40
9	38	35
11	33	31

* Values were calculated from the data shown in Figs 2 and 5.

amount of cytochrome P450. This was deduced from the data presented in Figs 2–4, which demonstrate a correlation between the sex-specific increases in the extent of spectral interaction (ΔA_{max}) and cytochrome P450, and not between the absolute values of these parameters. This correlation might indicate either specific synthesis of the ethylmorphine-binding cytochrome P450 already present in small amounts in immature animals, or generalized synthesis of cytochrome P450 together with a process of differentiation. The latter possibility is supported by the work of Levin *et al.* [54], who observed a sex-dependent differentiation of rat liver cytochrome P450 into two fractions with different rates of turnover. It might be speculated that, in female mice, a type of ethylmorphine-binding cytochrome P450 develops, which is different with respect to functions other than type 1 binding (e.g. at the stage of the oxygenated complex), this accounting for the kinetic anomalies discussed above.

Different levels of tightly-binding endogenous substrates may explain differences in type 1 binding [23, 55]. We have obtained evidence that this is not the case with the observed sex-difference (Van den Berg *et al.*, unpublished observations).

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