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SEX-DEPENDENT DIFFERENCES IN DRUG METABOLISM IN THE RAT

III. Temporal Changes in Type I Binding and NADPH-Cytochrome, P-450 Reductase during Sexual Maturation

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ABSTRACT

Microsomal ethylmorphine N-demethylase activity increased with age in male rats, but decreased with age in female rats. Relative to the cytochrome P-450 content of microsomes, there was no temporal change or sex difference in the magnitudes of the ethylmorphine binding spectrum (type I) and NADPH-cytochrome P-450 reductase activities. Thus, neither of these parameters correlated with observed temporal changes and sex differences in ethylmorphine N-demethylase activity. On the other hand, ethylmorphine-stimulated NADPH-cytochrome P-450 reductase activity was markedly greater in microsomes from males than from females, and the difference widened as the rats grew older. This was due entirely to an increase in activity in the microsomes from male rats. Differences in rates of ethylmorphine N-demethylation correlated well with differences in Δ reductase activity (reductase activity in the presence of ethylmorphine minus reductase activity in the absence of ethylmorphine). A 1:1 relationship was observed between Δ reductase activity and the rate of ethylmorphine N-demethylation. NADH synergism of NADPH-dependent ethylmorphine N-demethylation changed little with age but was greater in microsomes from male than from female rats.

Sex differences in the abilities of rats to metabolize certain drugs and other foreign compounds have been attributed to sex hormones (1, 2), to substrate binding to cytochrome P-450 (3–5), to basal and substrate-stimulated rates of NADPH-cytochrome P-450 reductase activities (6–8), and to qualitative differences in cytochrome P-450 (9, 10). In the present study, an evaluation was made of the roles that substrate binding to cytochrome P-450 and substrate stimulation of NADPH-cytochrome P-450 reductase activity might play in mediating the sex difference in drug metabolism. Ethylmorphine was selected for this study because its rate of metabolism is sex-dependent (4, 5, 9, 10), it is a type I compound (6, 11), and type I compounds are known to stimulate NADPH-cytochrome P-450 reductase activity (6, 7, 12); aniline was selected because its rate of metabolism is not sex-dependent (8, 9, 13), it is a type II compound (14), and it does not stimulate NADPH-cytochrome P-450 reductase activity (6, 7, 12). These studies employed male and female rats between the ages of 21 and 56 days.

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Materials and Methods

Male and female Holtzman rats were used as described in the preceding communication (9). The microsomes were prepared as described previously (15) except that the 9,000*g* supernatant fraction was centrifuged in a Beckman L-2B ultracentrifuge at 105,000-g_{av} (rotor No. 60 Ti) for 60 min and the microsomal pellet was suspended in 0.05 M PO_4 (pH 7.4) and 1.15% KCl solution. The N-demethylation of ethylmorphine (2 mM) was measured as described by Anders (15), except that nicotinamide was omitted from the incubation medium. NADH (0.4 mM) was added in some experiments.

The difference spectra of drugs binding to oxidized microsomes (1–1.5 mg of protein per ml) were deter-

mined as described by Remmer *et al.* (14); ethylmorphine (2 mM) was the type I compound and aniline (9.9 mM) was the type II compound. NADPH-cytochrome P-450 reductase activity was determined at 37°C in the presence of carbon monoxide by recording the increase in optical density between 450 and 490 nm as described by Gigon *et al.* (7). The reaction was initiated by the rapid addition of 10 μl of 0.25 M NADPH in a plunger to 3 ml of the microsomal suspension (1 mg of protein per ml) contained in an Aminco anaerobic cuvette (A1-65 085) in the presence or absence of either ethylmorphine (2 mM) or aniline HCl (0.2 mM). The cytochrome P-450 content of the suspension was measured at the end of the reaction by removing the plunger, adding a few milligrams of sodium dithionite, and recording the $\Delta A_{450-490}$. The initial rates of reduction and the amounts of cytochrome P-450 were calculated using an extinction coefficient of 91 mM^{-1} cm^{-1} (16). The NADPH-cytochrome P-450 reductase activity was measured in a split-beam, dual-wavelength recording spectrophotometer (Aminco DW-2) in the dual-wavelength mode. The binding of drugs to oxidized microsomes was measured in either a Shimadzu MPS 50L or an Aminco DW-2 spectrophotometer with the split-beam mode.

Microsomal protein was estimated by the method of Lowry *et al.* (17).

Data were analyzed by analysis of variance and each treatment mean was compared with every other treatment mean by Duncan's new multiple range test (18). Paired and unpaired Student *t* tests were also used when appropriate.

Results

Ethylmorphine N-Demethylation. The sex difference in microsomal ethylmorphine N-demethylation activity previously observed in young adult or mature rats by several investigators (4, 5, 11) is shown in fig. 1 to result from both an increased enzyme activity in male rats during the developmental period between 21 and 56 days and a decreased enzyme activity in female rats during the same period. These results obtained with microsomes are similar to those obtained with the 9000*g* supernatant fraction in the preceding communication (9). At 21 and 28 days of age no sex difference was observed in rates of ethylmorphine N-demethylation, but thereafter, microsomes from males exhibited a significantly higher activity than those from females. Similar results were obtained whether enzyme activities were expressed as nanomoles of HCHO formed per min per mg of protein or as nanomoles of HCHO formed per min per nmol of cytochrome P-450.

NADH supports microsomal N-demethylation of ethylmorphine only to about 10% of the rate achievable when NADPH is the source of electrons; when the nucleotides are used together, rates of ethylmorphine N-demethylation are about 135% of that obtained with NADPH alone (19, 20). Temporal and sexual aspects of this synergistic effect of NADH were studied (fig. 2). The mean

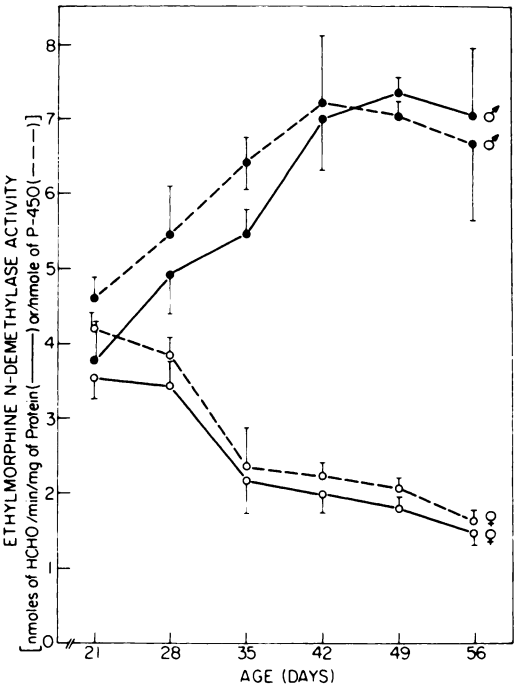

FIG. 1. *Effect of age on ethylmorphine N-demethylase activity in hepatic microsomes from male and female rats.*

Duncan's new 5% multiple-range test (18) was used for the following statistical analysis (based on data relative to cytochrome P-450 concentration):

Age and sex

56♀ 49♀ 42♀ 35♀ 28♀ 21♀ 21♂ 28♂ 35♂ 56♂ 49♂ 42♂

Animal groupings not underscored by the same line are significantly different whereas those underscored by the same line are not significantly different. For example: a) the 42♂ is significantly greater than the 28♂, 21♂, 21♀, 28♀, 35♀, 42♀, 49♀, and 56♀, but it is not significantly different from the 49♂, 56♂, and 35♂; b) the 28♂ is (1) significantly greater than the 35♀, 42♀, 49♀, and 56♀; (2) significantly less than the 42♂ and 49♂, and (3) not significantly different from the 35♂ and 56♂. The animal groupings are arranged in ascending order of their mean values from *left* to *right*. Similar results were obtained when enzyme activity was expressed relative to protein content. *Vertical bars* represent the mean ± SE of 4 experiments.

NADH synergism, expressed as percentage of control value, was significantly greater for all males used in the study than that for all females ($F_{1,36} = 49.97$; $F_{1,36,\alpha=0.95} = 4.12$). With microsomes from females, the NADH effect was temporally biphasic; values declined between 21 and 42 days and increased thereafter, whereas values with microsomes from males remained essentially constant throughout the experimental period.

Cytochrome P-450 Levels. Because in the present study it was considered desirable to express enzyme activities in terms of the cytochrome P-450 content as well as the protein content of microsomes, it was necessary to repeat that part of the preceding study (9) which dealt with the cytochrome P-450 content of microsomes. Results summarized in fig. 3 are in reasonable agreement with those published previously (8, 9). Cytochrome P-450 levels increased slightly in microsomes from males, but not in those from females, and levels

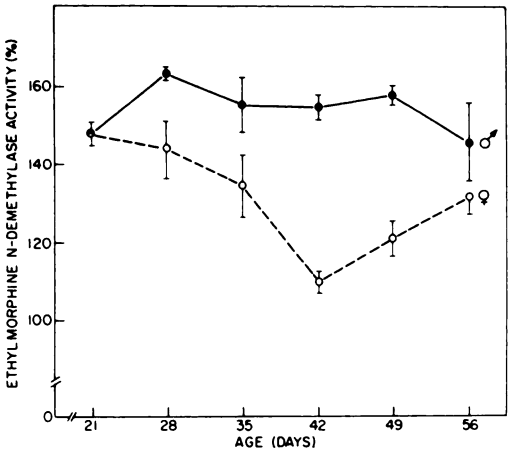

FIG. 2. *Effect of age on NADH synergism of NADPH-dependent ethylmorphine N-demethylation in microsomes from male and female rats.*

Results are expressed as percentages of the activities observed in the presence of NADPH alone, which are given in fig. 1. Duncan's new 5% multiple range test (18) was used for the following statistical analysis (based on data relative to protein concentration):

Age and sex

42♀ 49♀ 56♀ 35♀ 28♀ 56♂ 21♀ 21♂ 35♂ 42♂ 49♂ 28♂

For explanation of notation see fig. 1. *Vertical bars* represent the mean ± SE of 4 experiments. Animals were those used to obtain data for fig. 1.

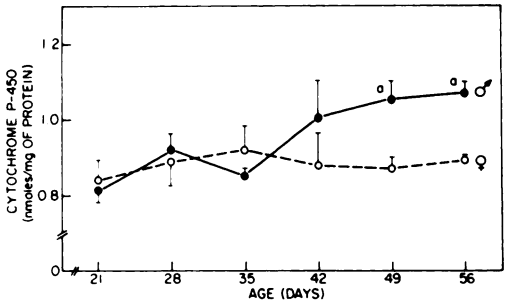

FIG. 3. *Effect of age on cytochrome P-450 levels in microsomes from male and female rats.*

Vertical bars represent the mean ± SE of 4 experiments. Animals were those used to obtain data for fig. 1. *a*, significantly different from the corresponding female with an unpaired *t* test ($p < 0.05$).

were significantly higher in males than females at 49 and 56 days. In agreement with our earlier observation (9) and those of others (6, 7, 11), levels of cytochrome P-450 did not correlate with respective increases or decreases in rates of ethylmorphine N-demethylation (compare figs. 1 and 3), again illustrating that the sex difference in metabolism resides in some feature of the mixed-function oxidase system other than the amount of cytochrome P-450.

Drug Binding. Schenkman *et al.* (3) showed that liver microsomes from adult male rats elicited binding spectra with type I drugs such as hexobarbital and aminopyrine with magnitudes more than twice those observed with microsomes from adult female rats. It was therefore of interest to study the temporal aspects of this sex difference in drug binding. When results were expressed relative to protein concentration, there was an increase in ethylmorphine binding (type I) in microsomes from male rats with age, but this increase was not apparent when the results were expressed relative to cytochrome P-450 concentration (fig. 4). In agreement with the observation of Davies *et al.* (11) there is no correlation between the magnitude of the ethylmorphine-binding spectrum and the ethylmorphine N-demethylating activity of microsomes from male rats (compare figs. 1 and 4). Microsomes from female rats showed no increase in the magnitude of the ethylmorphine binding spectrum with age regardless of how the results were expressed. Thus the temporal decrease in ethylmorphine N-demethylation by microsomes from female rats did not correlate with the lack of change in the binding spectrum (compare figs. 1 and 4). However, in agreement with other workers (11), the magnitude of the ethylmorphine binding

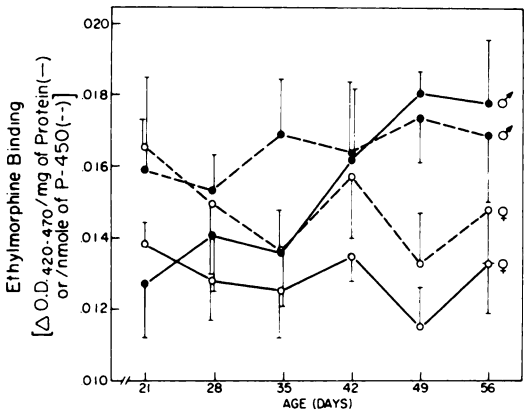

FIG. 4. *Effect of age on ethylmorphine binding to hepatic microsomes of male and female rats.*

Duncan's new 5% multiple range test (18) was used for the following statistical analysis (based on data relative to protein concentration):

Age and sex

49♀ 35♀ 21♂ 28♀ 56♀ 42♀ 35♂ 21♀ 28♂ 42♂ 56♂ 49♂

When the results were expressed relative to cytochrome P-450 concentration, differences were not significant. For explanation of notation see fig. 1. *Vertical bars* represent the mean ± SE of four experiments. Animals were those used to obtain data for fig. 1.

spectrum was significantly greater with microsomes from male rats than with those from female rats when results were expressed relative to protein concentration ($F_{1,36} = 10.72$; $F_{1,36,\alpha=0.95} = 4.12$), but not when expressed on the basis of the content of cytochrome P-450 ($F_{1,36} = 2.93$).

No consistent sex difference was observed in the magnitude of the aniline binding spectrum (type II) whether results were expressed relative to protein or to cytochrome P-450 content of microsomes (fig. 5). The magnitudes of the aniline binding spectra with microsomes from both sexes, increased with age but the increases were not significant when pooled results obtained at all ages were expressed relative to cytochrome P-450 concentration ($F_{5,36} = 2.47$; $F_{5,35,\alpha=0.95} = 2.48$) or on the basis of protein content ($F_{5,36} = 2.32$).

NADPH-Cytochrome P-450 Reductase Activity. When expressed relative to protein concentration, the basal NADPH-cytochrome P-450 reductase activity of microsomes from male rats increased slightly with age, but no increase was apparent when activity was expressed relative to cytochrome P-450 content (figs. 6 and 7). No change with age was observed with microsomes from females when results were expressed either way. There was also no significant sex difference in basal reductase activity. On the other hand, ethylmorphine-stimulated NADPH-cytochrome P-450 reductase activity was markedly greater in microsomes from male than in those from female rats. This agrees with Gigon *et al.* (6, 7), who found similar sex differences in substrate-stimulated NADPH-cytochrome P-450 reductase activity of microsomes from 180–200-g male and 160–170-g female Sprague-Dawley rats. The sex difference, which became significantly different at 35 days, widened as the rats grew older. The increase in sex difference with age was due entirely to increases in activity in microsomes from male rats; activity remained essentially unchanged in microsomes from female rats throughout the entire experimental period, or perhaps even decreased slightly.

Similar patterns of ethylmorphine-stimulated NADPH cytochrome P-450 reductase and ethylmorphine N-demethylase activities were observed regardless of whether results were expressed relative to protein (compare figs. 1 and 6) or cytochrome P-450 concentration (compare figs. 1 and 7). The rates of both basal and ethylmorphine-stimulated NADPH reductase activities were

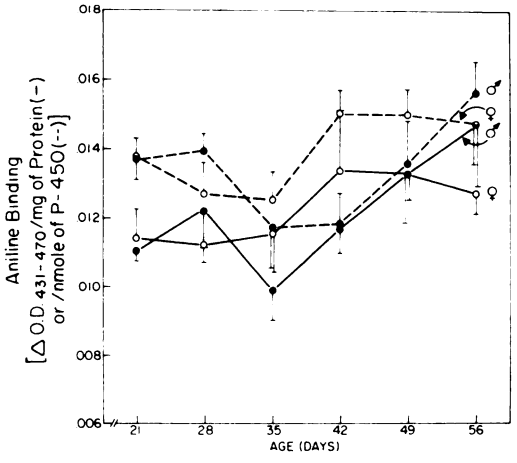

FIG. 5. *Effect of age on aniline binding to hepatic microsomes of male and female rats.*

Duncan's new 5% multiple range test (18) was used for statistical analysis. When expressed relative to cytochrome P-450 content, values for 56-day-old males and 42- and 49-day-old females were significantly greater than the values for 35- and 42-day-old males. No other significant differences were seen even when the aniline binding was expressed relative to protein concentration. *Vertical bars* represent the mean ± SE of 4 experiments. Animals were those used to obtain data for fig. 1.

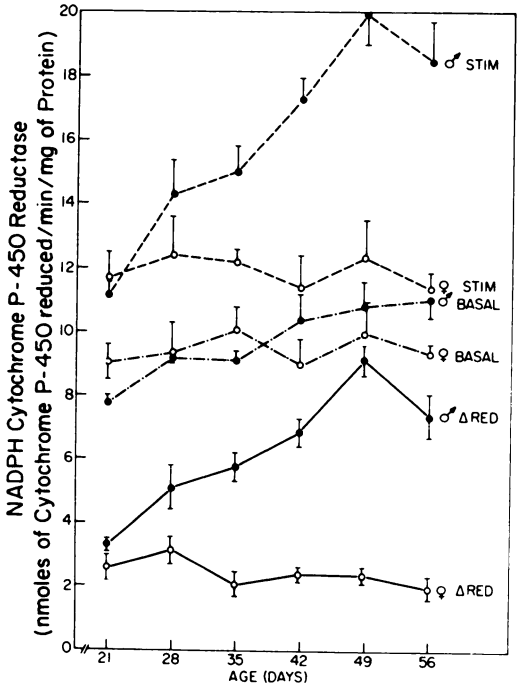

FIG. 6. *Effect of age on NADPH cytochrome P-450 reductase activities relative to the protein content of hepatic microsomes from male and female rats.*

Duncan's new 5% multiple range test (18) was used for the statistical analysis of temporal and sexual differences in basal (*BASAL*) and ethylmorphine (2 mM)-stimulated (*STIM*) NADPH-cytochrome P-450 reductase activity. The only significant difference in *BASAL* values was that between 21- and 56-day-old males. The following differences in *STIM* activities were noted:

Age and sex

21♂ 56♀ 42♀ 21♀ 35♀ 49♀ 28♀ 28♂ 35♂ 42♂ 56♂ 49♂

For an explanation of the notation see fig. 1. *Vertical bars* represent the mean ± SE of 4 experiments. Animals were those used to obtain data for fig. 1. Δ *RED* = *STIM* value minus *BASAL* value.

much higher than respective rates of HCHO production. However, as has been observed by others (6, 7, 21–23), Δ reductase activity (activity in the presence of ethylmorphine minus activity in the absence of ethylmorphine) and the rate of HCHO formation showed excellent correlation (r = 0.92) with a slope of 1.04 (fig. 8).

In contrast to type I compounds, which cause a stimulation of NADPH-cytochrome P-450 reductase activity, type II compounds have been reported to cause a decrease in this activity (6, 7, 12). The same concentration of aniline (0.2 mM), used in previous metabolism studies (9), was used to study the effect of a type II compound on the reductase. This concentration of aniline produced a significant decrease in NADPH-cytochrome P-450 reductase activity only in microsomes from 21-day-old females and 42-day-old males and females ($p < 0.05$ using a paired t test) (table 1). There was no apparent correlation between aniline p-hydroxylation (9), aniline binding (fig. 5), and effect of aniline on NADPH-cytochrome P-450 reductase activity (table 1).

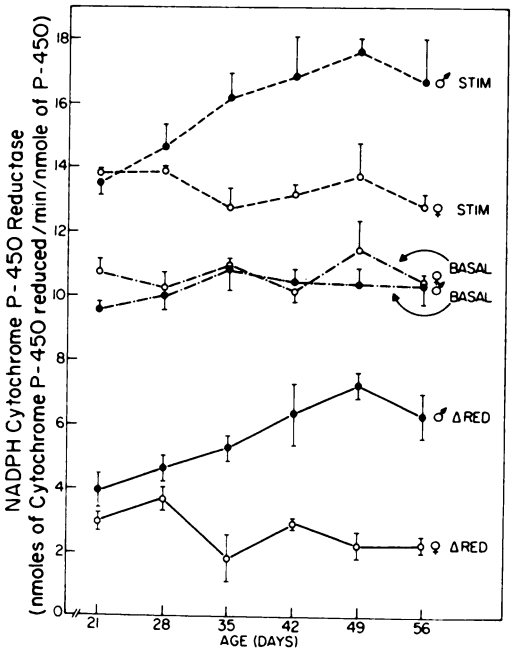

FIG. 7. *Effect of age on NADPH cytochrome P-450 reductase activities relative to cytochrome P-450 content of hepatic microsomes from male and female rats.*

Duncan's new 5% multiple range test (18) was used for the statistical analysis of temporal and sexual differences in basal (*BASAL*) and ethylmorphine (2 mM)-stimulated (*STIM*) NADPH-cytochrome P-450 reductase activity. The only significant difference in *BASAL* values was that between 21-day-old males and 49-day-old females. The following differences in *STIM* activities were noted:

Age and sex

35♀ 56♀ 42♀ 21♂ 49♀ 21♀ 28♀ 28♂ 35♂ 56♂ 42♂ 49♂

For an explanation of the notation see fig. 1. *Vertical bars* represent the mean ± SE of 4 experiments. Animals were those used to obtain data for fig. 1. Δ *RED* = *STIM* value minus *BASAL* value.

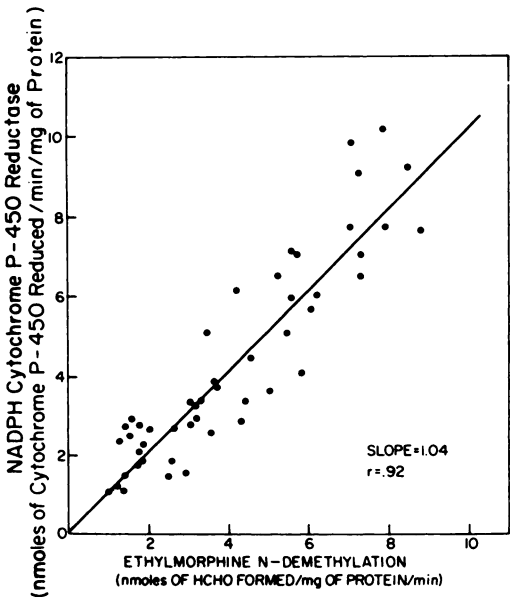

FIG. 8. *Relationship of ethylmorphine N-demethylation to ethylmorphine-stimulated cytochrome P-450 reductase activity of hepatic microsomes from male and female rats.*

Values were obtained from data presented in figs. 1, 6, and 7. Δ NADPH cytochrome P-450 reductase = activity in presence of ethylmorphine minus activity in the absence of ethylmorphine.

TABLE 1

Effect of aniline (0.2 mM) on NADPH-cytochrome P-450 reductase activity

Values are means of four experiments ± SE. Animals were those used to obtain data for fig. 1.

Age	Male		Female	
	NADPH-cytochrome P-450 reductase	+ Aniline	NADPH-cytochrome P-450 reductase	+ Aniline
days				
21	7.79 ± 0.44	7.47 ± 0.64	9.05 ± 0.56	7.99[a] ± 0.28
28	9.19 ± 0.35	9.01 ± 0.70	9.26 ± 1.07	8.43 ± 0.51
35	9.19 ± 0.58	8.87 ± 0.57	10.11 ± 0.68	9.15 ± 0.33
42	10.39 ± 0.81	9.89[a] ± 0.80	8.97 ± 0.83	8.15[a] ± 0.61
49	10.83 ± 0.77	10.08 ± 0.60	9.97 ± 1.06	9.06 ± 0.68
56	11.05 ± 0.56	10.67 ± 0.61	9.36 ± 0.26	8.65 ± 0.33

[a] Different from corresponding control ($p < 0.05$ using a paired t test)

Discussion

The sex difference in ethylmorphine N-demethylase activity of microsomal preparations from mature rats, reported by several investigators (4, 5, 10), was shown in the preceding communication (9) to develop during the period between weaning and young adulthood not only as a result of increased demethylating activity in microsomes from males, but also because of decreased activity in microsomes from females. Evidence was presented which suggested that the sex difference might be explained by developmental qualitative changes in cytochrome P-450. Sex differences in drug metabolism have been attributed to differences in drug binding to cytochrome P-450 (3-5) and to differences in drug-stimulated rates of NADPH cytochrome P-450 reductase activity (6, 7), both of which might reflect qualitative differences in cytochrome P-450. The major objective of the current study was to evaluate the roles of binding and NADPH-cytochrome P-450 reductase in the development of the sex difference in drug metabolism in rats which occurs between the ages of 21 and 56 days.

The magnitude of the ethylmorphine binding spectrum (type I) relative to the cytochrome P-450 content of microsomes changed little with age in rats of either sex and therefore did not correlate with the observed marked increase in males and decrease in females of ethylmorphine N-demethylase activity. For example, a sex difference in ethylmorphine metabolism was seen at 56 days, but not at 21 days (fig. 1), yet no sex difference in binding was observed at either age (fig. 4).

Basal NADPH-cytochrome P-450 reductase activity (fig. 7) did not vary with age and did not correlate with the sex difference in ethylmorphine metabolism. With a different substrate (aminopyrine) and different strain of rats (Long-Evans), MacLeod *et al.* (8) showed a parallelism between the maturational increases in drug metabolism and the mean specific activity of NADPH-cytochrome P-450 reductase. In our experiments, the age-dependent changes in ethylmorphine stimulation of the rate of NADPH-cytochrome P-450 reductase (figs. 6 and 7) paralleled the changes in ethylmorphine metabolism in both sexes (fig. 1). Similarly, the sex-dependent differences in the Δ reductase activity (the difference in the activity in the presence and absence of ethylmorphine) paralleled the sex differences in the N-demethylation of ethylmorphine (fig. 1), which agrees with the observations of Gigon *et al.* (6). The slope of the plot of the velocity of N-demethylation of ethylmorphine (nanomoles of HCHO per mg of protein per min) against Δ reductase activity (nanomoles of P-450 reduced per mg of protein per min) was 1.04 (fig. 8); this suggests a stoichiometric relationship between the two parameters,

thus confirming previous observations by Holtzman and associates (21-23). Although Gigon *et al.* (6, 7) observed a parallelism between the sex differences in ethylmorphine metabolism and NADPH-cytochrome P-450 reductase activity, a stoichiometric relationship was not seen, possibly because the concentration of ethylmorphine (0.4 mM) used in their studies was not saturating.

Several investigators (6, 7, 12) observed that type I compounds such as ethylmorphine enhance the rate of cytochrome P-450 reduction by combining with cytochrome P-450 to produce a complex that is more readily reduced by NADPH-cytochrome P-450 reductase than cytochrome P-450 itself (6, 12). Our observation that stimulation of NADPH-cytochrome P-450 reductase is age-dependent, but that type I binding is not, suggests that factors other than type I spectral binding are responsible for the stimulation of the reductase. This suggestion is compatible with the conclusion of Holtzman and Rumack (23) that spectral binding and reductase activity involve different binding sites.

Aniline, a type II compound, has been reported to lower NADPH-cytochrome P-450 reductase activity (6, 12). However, at the 0.2 mM concentration used in our studies, a statistically significant lowering of reductase activity occurred only in 21-day-old females and 42-day-old males and females. The suggestion has been made that aniline retards the rate of reduction by binding to the heme of cytochrome P-450 (12). In the present study, no relationship was observed between aniline spectral binding, aniline hydroxylation, or inhibition of reductase activity by aniline. Similarly, other workers (23) have reported a lack of agreement between K_s and K_M values for aniline.

The observation that NADH synergizes NADPH-dependent microsomal metabolism of certain drugs has suggested a role for cytochrome b_5 in these reactions (19, 25-27). In male rats the degree of NADH synergism of ethylmorphine N-demethylation did not vary with age, whereas in female rats it decreased from 128% at 21 days to a minimum of 110% at 42 days, and then increased slightly at 56 days (fig. 2). A comparison of the temporal relationships of ethylmorphine-stimulated reductase and NADH synergism, particularly in males, suggests that different factors are responsible for each phenomenon. Correia and Mannering (20) showed that the degree of NADH synergism of ethylmorphine metabolism correlated with the availability of type I binding sites. The finding that ethylmorphine binding and NADH synergism were significantly greater in males than females supports their observations. However, because type I binding and NADH synergism did not vary with the ages of the animals, no positive conclusions can be drawn about their relationship, if any exists.

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