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SEX-DEPENDENT DIFFERENCES IN DRUG METABOLISM IN THE RAT

I. Temporal Changes in the Microsomal Drug-metabolizing System of the Liver during Sexual Maturation

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ABSTRACT

The temporal aspect of the well known sex difference in drug metabolism in rats was studied by evaluating the drug-metabolizing system in liver preparations from animals that ranged in age from 21 to 56 days. The following observations were made. a) As male rats matured, N-demethylating activity generally increased in 9000*g* fractions from male rats, whereas decreases were seen in fractions from female rats. b) Rates of aniline hydroxylation declined with age and no sex difference was observed. A temporal increase in the degree of acetone enhancement of aniline hydroxylation was observed in males, but not in females. c) Changes in NADPH-cytochrome *c* and -neotetrazolium reductase, NADPH oxidase, and lipid peroxidase activities did not relate to changes in drug metabolism. d) Cytochrome P-450 levels of microsomes increased in males and remained constant in females, but the increase did not account for all of the increase in N-demethylating activity. e) Specific activities of cytochrome P-450, as related to N-demethylating activity, generally increased with age in males and decreased in females. f) The magnitude of the 430-nm maximum of the ethyl isocyanide spectrum increased with age with microsomes from males, but not from females. The pH at which the heights of the 430- and 455-nm peaks of the ethyl isocyanide difference spectrum were equal increased with age in males, but not in females. g) The apparent Michaelis constant for the N-demethylation of ethylmorphine decreased in males between the ages of 21 and 56 days, but remained unchanged in females. It is concluded a) that sex differences in drug metabolism observed in mature rats result not only from temporal increases in drug metabolism in males, but also from temporal decreases in females, and b) that these changes are due to qualitative changes in cytochrome P-450 in both males and females.

As early as 1927, Winton (1) reported a greater response to red squill in female than in male rats. Since then, numerous examples have been given of a greater duration of drug action in female rats (2–4). In 1958, Quinn and associates (5) provided an explanation for at least some of these sex differences when they showed that the biological half-life of hexobarbital was significantly longer in female than in male rats, and that the activities of the hexobarbital-, aminopyrine-, and anti-pyrine-metabolizing enzymes of the liver were lower in female than in male rats. Since then a variety of other drugs have been shown to be metabolized in rats at different rates by the two sexes (2–4). Among laboratory animals, the phenomenon seems to be peculiar to rats (6), although there is evidence of a limited degree of sex difference in drug metabolism in mice (2).

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Hepatic microsomes of newborn animals have little or no ability to metabolize drugs (7, 8). During the first 4 weeks after birth the increase in the drug-metabolizing activities of the microsomes from male and female rats is about the same (2); after 4 weeks sex differences begin to appear, becoming maximal at about 50 days (5, 9, 10). Sex-related events can be expected to occur during this time course because rats begin assuming sexual maturity at about 4 weeks of age. The first two of this series of four communications is concerned with changes in the microsomal drug-metabolizing system which occur throughout the period in the life-span of male and female rats beginning at 21 days (weaning) and ending at 56 days (early adulthood). Several recent publications have dealt with temporal and sex-related differences in the hepatic drug-metabolizing system, but none has considered both during the period between 21 and 56 days of age. Thus, while Basu *et al.* (11) studied biphenyl hydroxylation, nitroreductase and glucuronylsynthetase activities, and cytochrome P-450 content of microsomes from rats which ranged in age from 6 to 100 days, only male rats were studied after 21 days of age. Similarly, Gram *et al.* (12) studied ethylmorphine N-demethylase and aniline hydroxylase activities and cytochrome P-450 content of microsomes from animals 1–84 days of age, but again, only males were employed. Henderson (13) studied temporal changes in aminopyrine N-demethylase activity in microsomes from male and female rats from birth to 120 days of age, but did not study the components of the enzyme system involved. MacLeod *et al.* (14) studied aminopyrine N-demethylase, aniline hydroxylase, NADPH-cytochrome *c* reductase and NADPH cytochrome P-450 reductase activities, and the cytochrome P-450 contents of microsomes from developing male and female rats, but the study did not include animals older than 49 days of age. In this communication, liver fractions from rats that ranged in age from 21 to 56 days were evaluated at weekly intervals for drug-metabolizing activities with the use of aminopyrine, aniline, codeine, ethylmorphine, N-methylaniline, and 3-methyl-4-methylaminoazobenzene as substrates for NADPH-cytochrome *c* and -neotetrazolium reductase activities, for NADPH oxidase and lipid peroxidase activities, for cytochrome P-450 content, and for microsomal binding to ethyl isocyanide. The apparent Michaelis constant for ethylmorphine N-demethylation was determined using microsomes from 21- and 56-day-old male and female rats.

Materials and Methods

Chemicals and Enzymes. Ethylmorphine HCl was obtained from Mallinckrodt Chemical Works (New York, N. Y.); codeine phosphate from Merck and Co. (Rahway, N. J.); N-methylaniline HCl, 4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (aminopyrine), and neotetrazolium chloride from K & K Laboratories (Plainview, N. Y.); $NADP^+$, glucose 6-phosphate (G-6-P), cytochrome *c*, ADP, and bovine serum albumin from Sigma Chemical Co. (St. Louis, Mo.); glucose 6-phosphate dehydrogenase (G-6-PDH) from Sigma or from Boehringer-Mannheim Corp. (New York, N. Y.); and 2-thiobarbituric acid and aniline HCl from Eastman Organic Chemicals Co. (Rochester, N. Y.). Ethyl isocyanide and 3-methyl-4-methylaminoazobenzene (3-MMAB) were prepared by Dr. Don Shoeman. All chemicals were used as obtained by the supplier with the exception of 2-thiobarbituric acid, which was purified with charcoal.

Animals. Holtzman strain rats of specified ages were obtained from the supplier and kept at least 1 week before killing. In the case of weanling rats (21 days old), mothers were obtained from the supplier along with their 14-day-old offspring and the young were killed 7 days later. During this time they were fed water and a standard diet *ad libitum*. Animals were killed at 21, 28, 35, 42, 49, and 56 days of age.

Preparation of Microsomes. The rats were stunned by a blow on the head, decapitated, and exsanguinated, and their livers quickly perfused *in situ* with ice-cold isotonic KCl solution (1.15%). The livers were squeezed and blotted to remove excess perfusion fluid, excised, and weighed. Thereafter all procedures were carried out in the cold (0–4°C). The livers were homogenized in 2 volumes of isotonic KCl solution using a Dounce homogenizer fitted with a loose plunger (16 strokes). The homogenates were diluted with isotonic KCl solution so that each milliliter contained the equivalent of 250 mg of liver, wet weight, and then centrifuged for 20 min in a refrigerated Lourdes centrifuge, model LRS (rotor No. 9RA). The 9,000*g* supernatant fraction was used for all determinations of drug metabolism, but all other measurements were made with the 105,000*g* microsomal fraction, which was prepared by centrifugation of the 9,000*g* supernatant fraction in a Spinco model L ultracentrifuge at $105{,}000g_{av}$ (rotor No. 50) for 60 min. The microsomal pellet was suspended in isotonic KCl solution and diluted so that each milliliter of suspension contained the equivalent of 250 mg of liver. Further dilutions were sometimes made to accommodate assay procedures. All microsomal fractions were used the day they were prepared except when lipid peroxidation and protein assays were performed, in which cases they were stored at −25°C for 1–4 days. No change in lipid peroxidation occurred during storage.

Measurement of Enzyme Activities. The reaction mixture used to determine the activities of hepatic drug-metabolizing enzymes contained the following constituents in 5 ml of 0.15 M KCl-0.2 mM phosphate buffer, pH 7.4, (μmol): $NADP^+$, 2; G-6-P[3], 20; nicotinamide, 20; magnesium chloride, 10; semicarbazide, 37.5; drug substrate, and 9,000*g* supernatant fraction equivalent to 250 mg of fresh liver. When microsomal preparations (105,000*g* pellet equivalent to 250 mg of fresh liver) were used, 2 units of G-6-PDH were included in the mixture. Nicotinamide was omitted from the medium when aniline hydroxylation was measured. NADPH-generating enzymes were determined to be present in adequate amounts in the 9,000*g* supernatant fractions from animals of both sexes of all ages except those that were 3 weeks old. In some cases, the liver preparations from 21-day-old animals were supplemented with 1 unit of G-6-PDH. Substrates were employed in saturating, or near saturating, concentrations, namely (mM), aminopyrine, 5; aniline, 0.2; codeine, 3; ethylmorphine, 10; N-methylaniline, 10; and 3-MMAB, 0.2. Formaldehyde produced by the N-demethylation of aminopyrine, ethylmorphine, and N-methylaniline and the N- and O-demethylations of codeine were determined by the method of Nash (15) as reported previously (16). The formaldehyde derived from 3-MMAB was measured by the chromotropic acid method as described by MacFayden (17). Aniline *p*-hydroxylation was determined by the method of Kato and Gillette (18). All reaction mixtures were incubated (37°C) for periods which had been previously shown to ensure linear reaction rates, namely 10 min for ethylmorphine and 3-MMAB, 15 min for aniline, codeine, and N-methylaniline, and 20 min for aminopyrine.

NADPH-cytochrome *c* reductase and NADPH-neotetrazolium reductase were determined at 28° and 37°C, respectively, by the methods of Dallner (19) and Lester and Smith (20, 21), respectively. NADPH oxidase was determined as described by Gillette and co-workers (22) except that nicotinamide was omitted from the incubation mixture and the temperature was kept at 28°C. Lipid peroxidase activity was measured by the method of Hochstein and Ernster (23) at 37°C. A Beckman model B spectrophotometer was used for the NADPH-neotetrazolium reductase assay and a Beckman model DB spectrophotometer was used for the NADPH oxidase and NADPH-cytochrome *c* reductase assays.

Spectral Measurements. All spectral measurements were conducted with suspensions of microsomes (105,000*g* pellet).

Cytochrome P-450 was determined by the method of Omura and Sato (24). Ethyl isocyanide difference spectra were obtained as described previously (25) with buffered microsomal preparations which ranged between pH 6.5 and 8.5. The pH at which the 430-nm and 455-nm peaks were of equal height is referred to as the "pH intercept." A Beckman model DB spectrophotometer was employed for the determination of cytochrome P-450 and the recording of the ethyl isocyanide spectra.

Determination of Protein. The protein contents of the liver preparations were determined by the method of Lowry *et al.* (26).

Data Processing and Statistics. The Student's *t* distribution was used as a test of the null hypothesis (27). Data used in the determination of Michaelis constants were analyzed by the method of Wilkinson (28) with use of a Fortran program written by Cleland (29).

[3] The abbreviations used are: G-6-P, glucose 6-phosphate; G-6-PDH, glucose 6-phosphate dehydrogenase; 3-MMAB, 3-methyl-4-methylaminoazobenzene.

Results

Body Weight, Liver Weight, and Protein Contents of Liver Fractions. Table 1 summarizes data obtained from the 21- to 56-day-old male and female rats regarding body weight, weight of liver relative to body weight, and protein contents of the 9,000*g* supernatant and 105,000*g* microsomal liver fractions. In rats of both sexes the ratio of liver weight to body weight increased between 21 and 28 days of age, and then gradually decreased until the end of the experiment. Kato *et al.* (30) observed similar changes. Female rats showed significantly higher liver weight to body weight ratios than male rats at 21, 42, and 56 days of age. A gradual increase in protein content of the 9,000*g* fraction was seen throughout the experimental period, but no sex difference was observed. However, a significant increase in the protein content of the 105,000*g* microsomal fraction from male rats over that from female rats was observed at 49 and 56 days of age. This prompted us to express results not only relative to liver weight, but also to protein concentration as well.

Demethylase Activity. No sex-dependent differences were seen in any of the N-demethylase activities studied in liver preparations from 21-day-old rats except for the small, but statistically significant difference observed when codeine was the substrate (figs. 1-5). It is to be noted, however, that 10-20% increases in N-demethylase activities were obtained when the 9000*g* supernatant fractions from livers of rats of both sexes were supplemented with 1 unit of G-6-PDH. No such evidence of a limitation of NADPH-generating enzymes was seen in the liver fractions from animals 28 days old or older. This observation was made after much of the metabolism work had been completed; therefore, the 9000*g* fractions from 21-day-old animals were not supplemented with G-6-PDH when the metabolism of aminopyrine, ethylmorphine, and N-methylaniline was studied.

TABLE 1

Temporal changes in body and liver weights of male and female rats and in the protein contents of liver fractions from the same animals

Age (days) and Sex	Body Weight (N = 8–28)	Liver Weight / Body Weight × 100 (N = 8–28)	Protein Content	
			9,000g fraction (N = 8–19)	105,000g fraction (N = 8–21)
	g		*mg/g liver*	
21				
M	46 ± 1	4.14 ± 0.05[b]	75.3 ± 2.1	17.0 ± 0.9
F	43 ± 2	4.45 ± 0.11	75.8 ± 2.2	17.7 ± 0.9
28				
M	89 ± 2[a]	4.95 ± 0.07	82.4 ± 2.2	19.1 ± 0.8
F	80 ± 1	5.04 ± 0.08	80.3 ± 2.0	18.4 ± 0.7
35				
M	130 ± 3[a]	4.74 ± 0.07	82.0 ± 2.0	19.2 ± 0.6
F	111 ± 2	4.86 ± 0.08	81.6 ± 1.0	18.7 ± 0.6
42				
M	168 ± 1[a]	4.32 ± 0.07[a]	88.9 ± 2.6	20.9 ± 0.8
F	144 ± 3	4.59 ± 0.08	90.7 ± 3.5	20.8 ± 1.0
49				
M	224 ± 3[a]	4.49 ± 0.06[b]	87.1 ± 2.2	23.2 ± 1.2[b]
F	161 ± 2	4.67 ± 0.07	79.4 ± 0.8	20.2 ± 1.3
56				
M	265 ± 5[a]	4.22 ± 0.07	92.5 ± 2.9	24.1 ± 0.9[a]
F	181 ± 1	4.32 ± 0.06	93.1 ± 2.7	19.6 ± 0.6

[a] M *vs.* F ($p < 0.01$).
[b] M *vs.* F ($p < 0.05$).

At 28 days, male rats had a greater capacity than female rate to demethylate aminopyrine and ethylmorphine (figs. 1 and 2). At 35 days old and older, male rats had a consistently higher ability than female rats to demethylate all five of the substrates studied (figs. 1–5). This was generally the case regardless of whether the results were expressed relative to liver weight or to protein concentration. In male rats, maximum N-demethylase activities were reached by day 49 and significant increases in all demethylase activities were observed between 21 and 49 days. In female rats, maximum N-demethylase activities were seen at 28 days, and activities decreased thereafter.

The pattern of demethylating activity as a function of age and sex varied with the substrate. For example, at 49 days, the livers from male rats exhibited six times more ethylmorphine-metabolizing activity than those from female rats, but only three times more aminopyrine-metabolizing activity.

Aniline Hydroxylase Activity. In contrast to the demethylation reactions, no sex difference was observed in aniline hydroxylation throughout the experimental period (fig. 6). This is in agreement with the observation of Kato and Gillette (18), who employed rats that are estimated from their weights to be about 60 days old and MacLeod *et al.* (14), who observed no sex difference in aniline

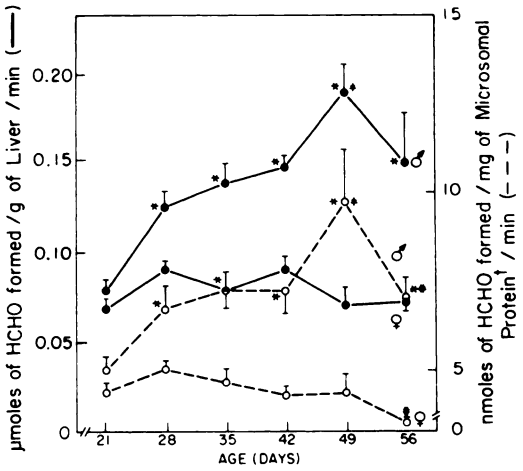

FIG. 1. *Aminopyrine N-demethylase activity of 9000g supernatant fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 7 to 8 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, calculations are based on the determined protein content of microsomes contained in the 9000g supernatant fraction; ‡, significantly different from value at 21 days ($p < 0.05$); #, significantly different from value at 28 days ($p < 0.05$).

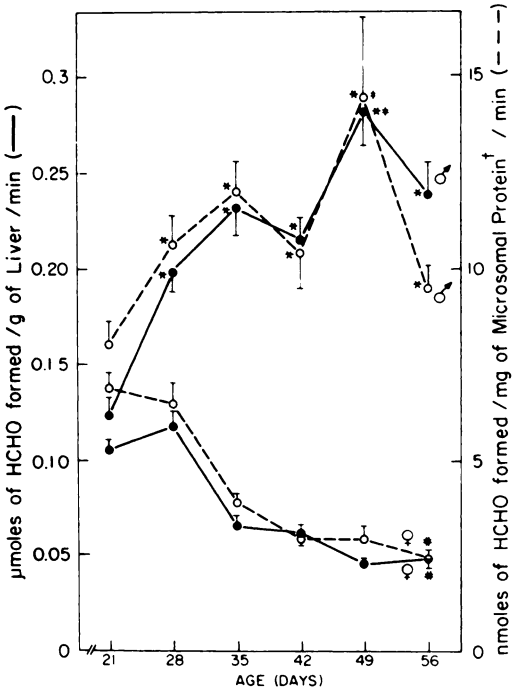

FIG. 2. *Ethylmorphine N-demethylase activity in 9000g supernatant fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 7 to 8 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, calculations are based on the determined protein content of microsomes contained in the 9000*g* supernatant fraction; ‡, significantly different from value at 21 days ($p < 0.05$); #, significantly different from value at 28 days ($p < 0.05$).

hydroxylation in 35-day-old and mature (10 weeks or older) rats. In contrast to the demethylation reactions, where rates of metabolism generally increased in males and decreased in females with age, rates of aniline hydroxylation decreased in rats of both sexes with age. This is in agreement with Gram *et al.* (12), who employed male rats only, but in disagreement with MacLeod, who observed increases in aniline hydroxylation in both male and female rats as they aged.

Anders (31) observed that acetone enhances rates of aniline hydroxylation by rat microsomes. Similar acetone enhancement was observed using 9000*g* supernatant fractions (fig. 7). The percentage of enhancement in both sexes increased with age regardless of sex and no overall sex difference was observed. However, when aniline hydroxylation in the presence of acetone was expressed as the amount of product formed rather than as percent enhancement, a temporal sex difference was observed; the degree of acetone enhancement of aniline hydroxylation increasd with age in males, but not in females.

Microsomal NADPH Oxidase, NADPH-

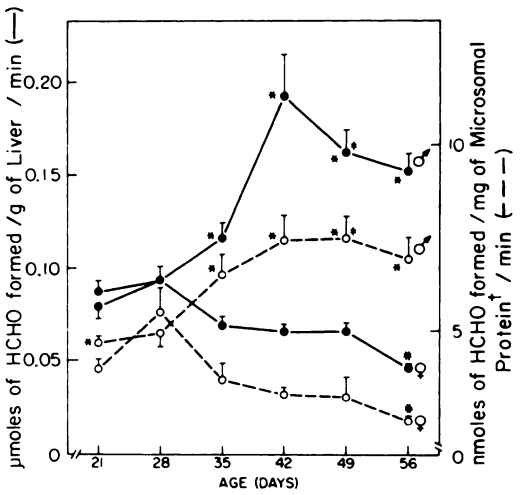

FIG. 3. *Codeine demethylase activity in 9000g supernatant fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 4 to 9 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, calculations are based on the determined protein content of microsomes contained in the 9000*g* supernatant fraction; ‡, significantly different from value at 21 days ($p < 0.05$); #, significantly different from value at 28 days ($p < 0.05$).

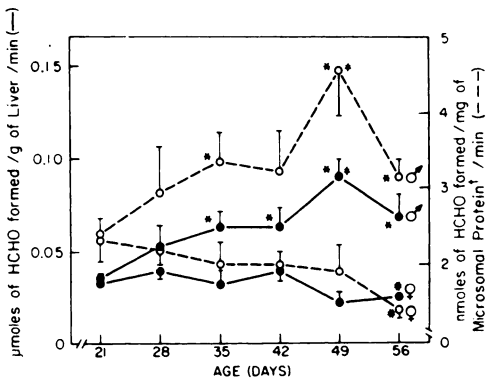

FIG. 4. *N-Methylaniline demethylase activity in 9000g supernatant fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 7 to 8 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, calculations are based on the determined protein content of microsomes contained in the 9000*g* supernatant fraction; ‡, significantly different from value at 21 days ($p < 0.05$); #, significantly different from value at 28 days ($p < 0.05$).

Neotetrazolium Reductase, and NADPH-Cytochrome c Reductase Activities. When evaluated relative to liver weight, no sex differences were observed in the NADPH oxidase, NADPH-neotetrazolium reductase, and NADPH-cytochrome *c* reductase activities of liver fractions until the 49th and 56th days (figs. 8–10). When data were evaluated relative to protein concentration, no sex differences were seen throughout the experimental period. No consistent temporal differences were

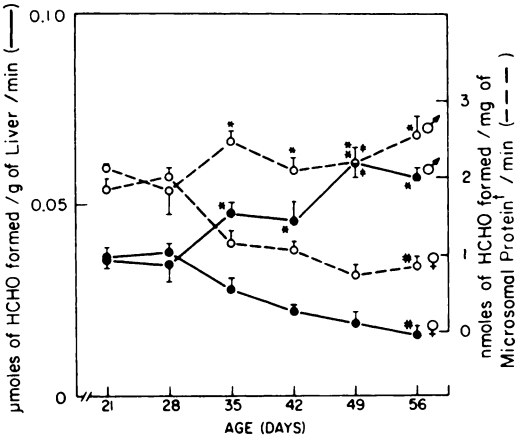

FIG. 5. *3-MMAB N-demethylase activity in 9000g supernatant fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 4 to 8 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, calculations are based on the determined protein content of microsomes contained in the 9000*g* supernatant fraction; ‡, significantly different from value at 21 days ($p < 0.05$); #, significantly different from value at 28 days ($p < 0.05$).

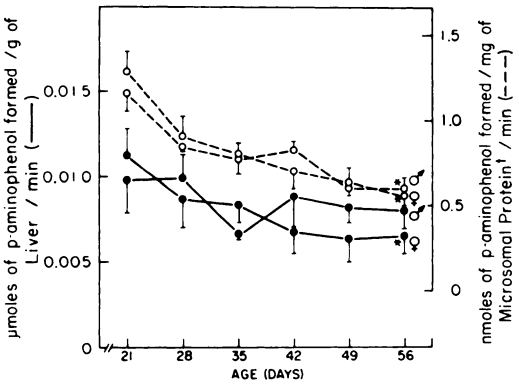

FIG. 6. *Aniline p-hydroxylation in 9000g supernatant fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 4 experiments. *, significantly different from value at 21 days; †, calculations are based on the determined protein content of microsomes contained in the 9000*g* supernatant fraction.

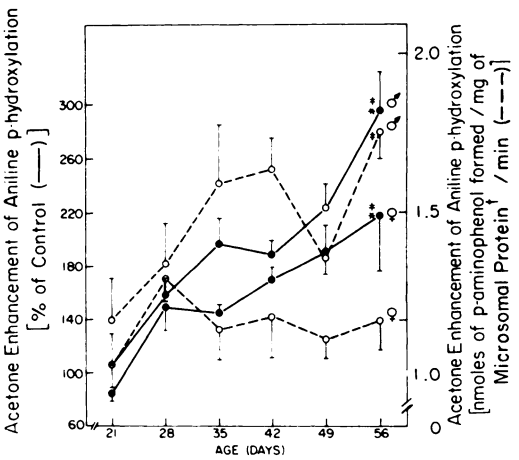

FIG. 7. *Enhancement of aniline hydroxylation by acetone (0.69 M) in 9000g supernatant fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 4 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, calculations are based on the determined protein content of microsomes contained in the 9000*g* supernatant fraction; ‡, significantly different from value at 21 days ($p < 0.05$); #, significantly different from value at 28 days ($p < 0.05$).

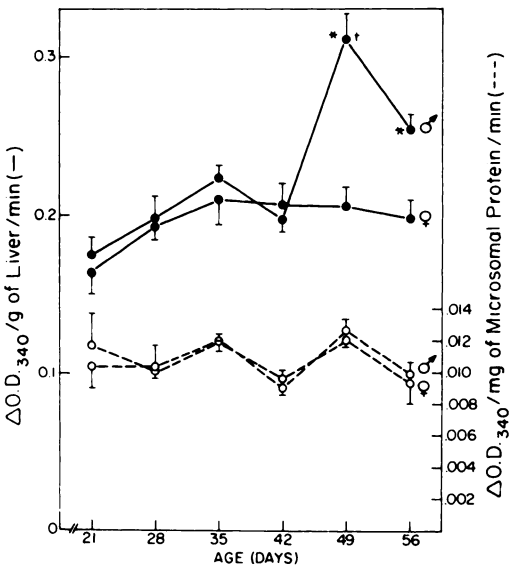

FIG. 8. *NADPH oxidase activity in microsomal fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 5 to 7 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, significantly different from value at 21 days ($p < 0.05$).

seen in the activities of these enzymes.

Microsomal Lipid Peroxidase. Peroxidase activity varied greatly from week to week and showed no temporal trend; thus, activity at 21 days was not significantly different from that at 56 days (fig. 11). Higher activities were observed in liver preparations from male rats than in those from female rats at 49 and 56 days.

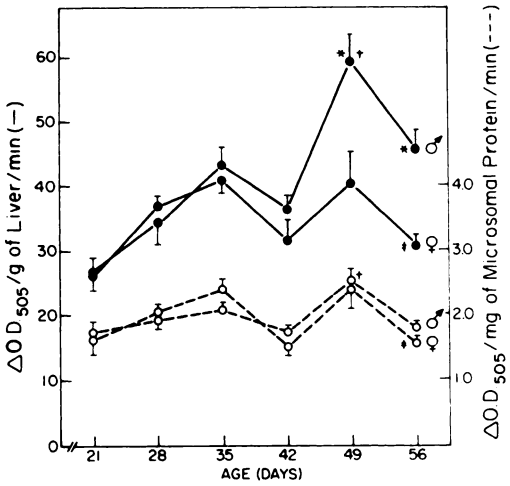

FIG. 9. *NADPH-neotetrazolium reductase activity in microsomal fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 5 to 7 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, significantly different from value at 21 days ($p < 0.05$); ‡, significantly different from value at 28 days ($p < 0.05$).

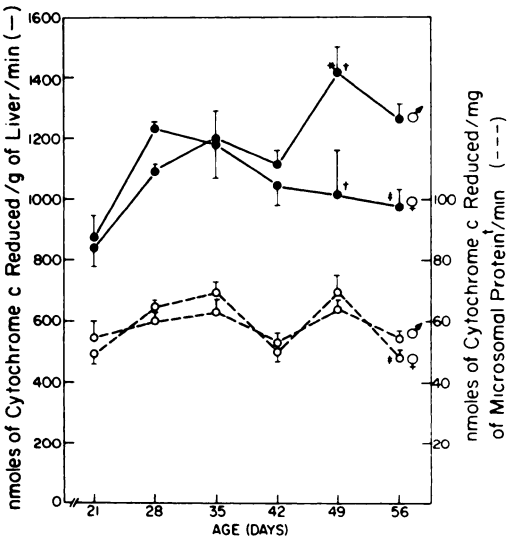

FIG. 10. *NADPH-cytochrome c reductase activity in microsomal fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 4 to 8 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, significantly different from value at 21 days ($p < 0.05$); ‡, significantly different from value at 28 days ($p < 0.05$).

Cytochrome P-450 Content of Liver Microsomes. The cytochrome P-450 content of microsomes from livers of female rats remained constant throughout the experimental period, but that of microsomes from male rats increased (fig. 12). Values for males were significantly greater than those for females at 42, 49, and 56 days whether calculated relative to liver weight (21, 38, and 42%, respectively) or to protein concentration (20, 21, and 37%, respectively).

Specific Activity of Cytochrome P-450. When figs. 1–5 are compared with fig. 12, it is apparent that the increases in demethylating capacity of liver preparations of male rats with age are greater than can be accounted for by increases in microsomal cytochrome P-450, thus demonstrating a qualitative as well as a quantitative sex difference with the onset of puberty. In figs. 13 and 14 the specific activities of cytochrome P-450 (nanomoles of drug metabolized per min per nmol of cytochrome P-450) are given for liver preparations from animals of both sexes in each age group with data from figs. 1–6 and fig. 12. At 21 days there was no sex difference in the specific activities of cytochrome P-450, irrespective of the substrate used. At 28 days there was a sharp increase in the

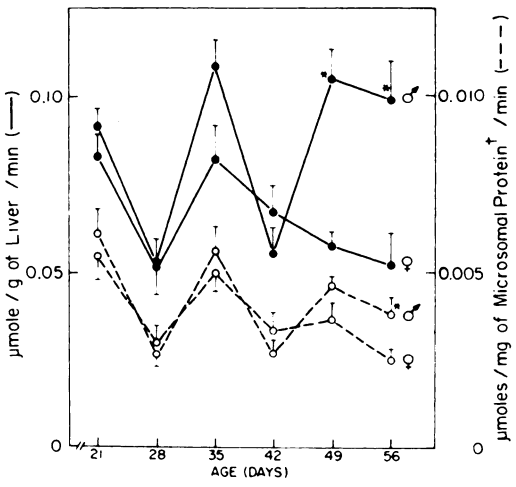

FIG. 11. *Lipid peroxidation activity in microsomal fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 4 to 8 experiments. *, significantly different from the corresponding female ($p < 0.05$).

specific activity of cytochrome P-450 from male rats when ethylmorphine, aminopyrine, and N-methylaniline were substrates, no appreciable increase with codeine and 3-MMAB, and a decrease with aniline. In females, there was a general, but irregular and variable, decline in the specific activities of cytochrome P-450 with all substrates except when aminopyrine was employed, in which case the specific activity remained essentially constant throughout the experimental period. Although the specific activity of cytochrome P-450 from males remained higher than that from females throughout the remaining experimental period, it varied greatly with time and with the substrate employed. Thus values declined after 28 days when ethylmorphine, codeine, and 3-MMAB were the substrates, but the value remained essentially constant when aminopyrine was the substrate. The specific activity of cytochrome P-450 from male rats rose markedly on day 42 when codeine was the substrate, but values on other days varied little from that observed on day 21.

These results suggest that qualitative changes in cytochrome P-450 occur in both sexes during puberty.

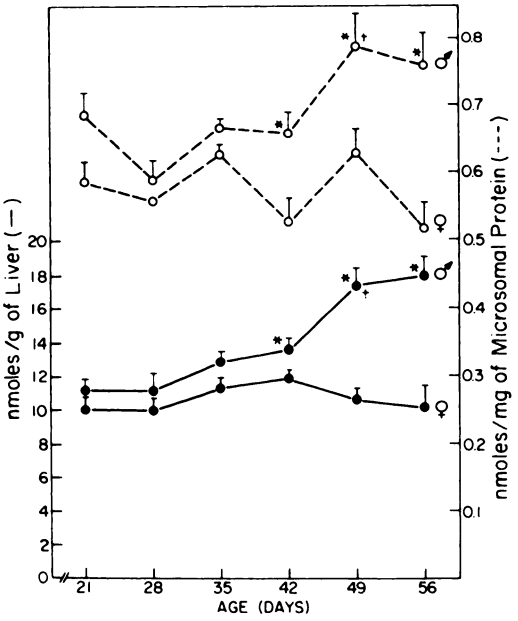

FIG. 12. *Levels of cytochrome P-450 in microsomal fractions from livers of male and female rats.*

Vertical bars represent the mean ± SE of 8 to 21 experiments. *, significantly different from the corresponding female ($p < 0.05$); †, significantly different from value at 21 days ($p < 0.05$).

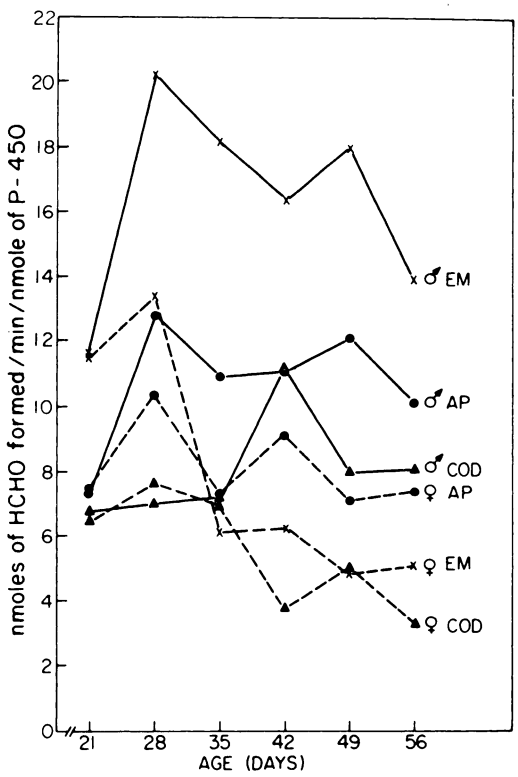

FIG. 13. *Specific activities of cytochrome P-450 with respect to aminopyrine (AP), ethylmorphine (EM), and codeine (COD) metabolism in developing male and female rats.*

Values were calculated from data given in figs. 1, 2, 3, and 12.

Ethyl Isocyanide Difference Spectrum of Reduced Liver Microsomes. Consideration of the relative magnitudes of the 430- and 455-nm maxima of the difference spectrum produced by the combination of ethyl isocyanide with reduced microsomes is of particular interest because changes in the ratios of the two peaks are thought to reflect qualitative differences in P-450 hemoproteins (25). Fig. 15 shows the variation with age of the cytochrome P-450 content of microsomes and of the magnitudes of the 430-nm and 455-nm peaks of the ethyl isocyanide difference spectra of microsomes at pH 7.4. In microsomes from male rats, the magnitude of the 430-nm peak increased temporally in parallel with the increase in cytochrome P-450, with only a small increase in the height of the 455-nm peak. Little or no change with age was seen in the heights of either of the peaks in microsomes from female rats. The ratios of the 455:430 peak heights are not sex-dependent at 21 and 28 days; as the age of the males

increases, ratios decrease, whereas no appreciable change is seen in females throughout the experimental period. This disparity in the changes in the heights of the 430-nm and 455-nm peaks was further manifested when peak heights were measured over a pH range of 7.0 8.0 (fig. 16). It is seen that the pH intercept (the pH at which the 430-nm and 455-nm peaks are of equal height) observed with microsomes from female rats remained at about 7.5 at 21, 42, and 56 days, but that obtained with microsomes from male rats increased from 7.6 at 21 days to 8.1 at 56 days. These results add to the preceding evidence that a qualitative change in cytochrome P-450 occurs in male rats during puberty, but do not reflect the qualitative change in cytochrome P-450 in females suggested by the preceding specific activity studies.

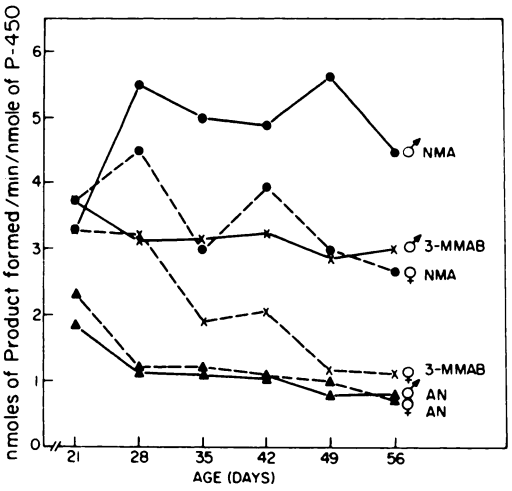

FIG. 14. *Specific activities of cytochrome P-450 with respect to aniline (An), N-methylaniline (NMA), and 3-MMAB metabolism in developing male and female rats.*

Values were calculated from data given in figs. 4, 5, 6, and 12.

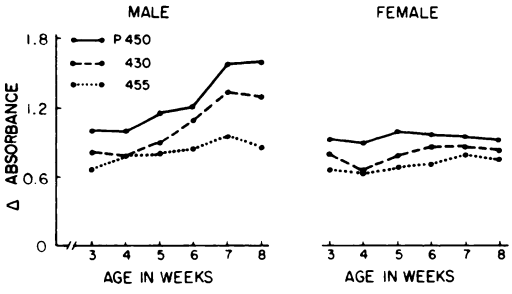

FIG. 15. *Temporal changes in the magnitudes of the 430- and 455-nm peaks of the ethyl isocyanide difference spectra and of the 450-nm peak of the CO difference spectra of microsomes from male and female rats.*

All spectra were recorded with microsomes suspended in a medium buffered at pH 7.4.

Michaelis Constant for Ethylmorphine N-Demethylase. In accordance with the generalization that qualitative changes in enzyme systems are frequently accompanied by changes in the kinetics of the reactions with which the systems are involved, the apparent Michaelis constant of the N-demethylation of ethylmorphine by microsomes (105,000*g* pellet) from 21- and 56-day-old male and female rats was determined (table 2). The K_M obtained with microsomes from 56-day-old male rats is seen to be about one-half those obtained with microsomes from female rats of both ages and from 21-day-old males.

Discussion

Most studies of the sex difference in the abilities of rats to metabolize certain drugs have been made with animals of a selected age, usually young adult

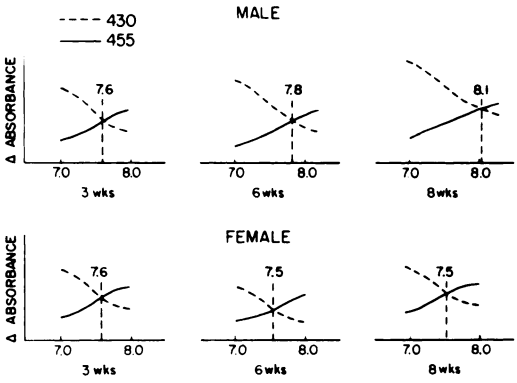

FIG. 16. *Temporal changes in the effect of pH on the magnitudes of the 430- and 455-nm peaks of the isocyanide difference spectra of microsomes from male and female rats.*

TABLE 2

Apparent Michaelis constants for the N-demethylation of ethylmorphine by microsomes from 21- and 51-day-old male and female rats

Rats are those from which other data were derived for table 1. Results are expressed as the mean ± SE; N = 4–9.

Sex	Age	K_M
	days	*mM*
Male	21	0.41 ± 0.07[a]
Female	21	0.47 ± 0.05[b]
Male	56	0.26 ± 0.03[a, b, c]
Female	56	0.49 ± 0.06[c]

[a, b, or c] Significantly different from each other, $p < 0.05$.

or mature animals. The use of animals of different ages over a given period of development provides the opportunity to observe changes as well as differences. In accordance with the principle that parallel sex and temporal changes in features of the drug-metabolizing system with changes in drug-metabolizing activities of liver preparations might reflect causal relationships, a number of the biochemical and spectral properties of liver preparations were studied.

The current study of male and female rats during the period between weaning and young adulthood revealed that sex differences in the ability of microsomal preparations from sexually mature rats to metabolize drugs results from changes in the female as well as in the male during puberty and that the direction of the changes depend upon the substrate. Thus, rates of N-demethylation of aminopyrine, codeine, N-methylaniline, and 3-MMAB increased in males and decreased in females with age and the metabolism of aniline declined in both sexes.

No consistent sex difference in NADPH oxidase activity was observed when specific activities were related to protein concentration, but when related to liver weight, activities were slightly higher in males at 49 and 56 days. This agrees with the observation of Kato and Gillette (18) that NADPH oxidase activity based on liver weight was about 38% higher in males than in females whose ages are estimated to have been 42–49 days. The observed absence of a temporal change in NADPH oxidase activity is consistent with the observation of Dallner and co-workers (32) that adult levels of the enzymes are reached when the animals are 7 days old.

Slightly higher activities of NADPH-cytochrome *c* and NADPH-neotetrazolium reductases were seen in males than in females, but only when values were based on liver weight. Kato and Gillette (18) and Davies *et al.* (33) reported similar results in studies with adolescent rats. Activities of the two reductases varied considerably, but in parallel, throughout the experimental period without remarkable changes in either sex except in 49- and 56-day-old males when increased activity was observed. Dallner and associates (32) reported that by the 1st week of age. NADPH-neotetrazolium reductase had reached only 40% of the adult level. MacLeod and co-workers (14) observed no sex difference in NADPH-cytochrome *c* reductase activity, but in their studies, activity increased until the 5th week of age.

Because the metabolism of aminopyrine, codeine, ethylmorphine, N-methylaniline, and 3-MMAB increased with age in males and decreased in females, and aniline metabolism decreased in both sexes, and because these changes did not parallel changes in activities of NADPH oxidase, NADPH-cytochrome *c* reductase, or NADPH-neotetrazolium reductase, it is concluded that temporal and sex-dependent changes in these latter enzyme activities do not account for the observed changes in rates of drug metabolism observed in developing male and female rats.

Lipid peroxidase activity of liver preparations was studied because of the effect it might have in lowering rates of drug metabolism by channeling electron flow or by altering enzyme activities, in which case higher peroxidase activity in female liver preparations as compared to that in preparations from male rats might explain the sex difference in drug metabolism. Cytochromes are among the most potent catalysts of lipid peroxidation; they catalyze the homolytic scission of peroxides into free radicals which initiate further reaction chains and thereby damage enzymes (32, 34). Orrenius *et al.* (35) and Gram and Fouts (36) found that the initiation of lipid peroxidation in liver microsomes interfered with drug-metabolizing activity. That lipid peroxidation cannot account for the sex difference in drug metabolism was demonstrated in the current study by the observation that lipid peroxidase activity in liver preparations from female rats is actually lower than that in preparations from males. In passing, it should be mentioned that conditions employed for the measurement of lipid peroxidase activity are different enough from those employed for the measurement of drug-metabolizing activity that studies which attempt to relate the two activities may not be valid. When peroxidase activity is measured, the microsomes are supplied with ADP, ferrous iron, and high oxygen tension; none of these is supplied when rates of drug metabolism are being measured.

Cytochrome P-450 content of microsomes from male rats increased slightly with age, particularly near the end of the experimental period, but no important temporal change was seen in female rats. Gigon *et al.* (37) and Davies *et al.* (33), with the use of rats that weighed about the same as our 42- and 49-day-old rats, observed about the same sex difference in cytochrome P-450 content of microsomes (about 20%, based on nanomoles of dithionite-reducible cytochrome P-450 per mg of protein). Gram *et al.* (12) observed only a slight increase in microsomal cytochrome P-450 in male

rats as they aged from 7 to 84 days, and Basu *et al.* (11), who also employed male rats, observed little or no change in the cytochrome P-450 content of microsomes from animals that ranged in age from 20 to 100 days. MacLeod *et al.* (14) observed no temporal change in microsomal cytochrome P-450 in male and female rats between the ages of 21 and 70 days, but microsomes from female rats contained only about 60% of the cytochrome P-450 found in microsomes from male rats.

Several kinds of evidence contribute to the conclusion that a temporal qualitative change in cytochrome P-450 occurs in the male rat. a) The increase in cytochrome P-450 in males did not match the much greater increase in drug metabolism seen with certain substrates, particularly ethylmorphine, and the decline of the rate of metabolism of certain substrates such as ethylmorphine with age was not consistent with the unchanging level of cytochrome P-450 seen in females. These differences in specific activity of cytochrome P-450 (figs. 13 and 14) varied in magnitude and with age, but in general, there was an increase in the specific activities in males with aging and a decrease in females, thus suggesting the occurrence of qualitative changes in cytochrome P-450 in both sexes during puberty. b) The height of the 430-nm peak of the ethyl isocyanide difference spectra relative to cytochrome P-450 was seen to increase with age in males, but remained essentially unchanged in females; little change in the 455-nm peak with age was seen with either sex. It is of some interest that when administered to rats, 3-methylcholanthrene causes increases in the 455 nm:430 nm ratio largely by increasing the height of the 455-nm peak (25). c) The Michaelis constant for the N-demethylation of ethylmorphine decreased between the ages of 21 and 56 days when microsomes from male rats were used, but remained unchanged during this time period when microsomes from female rats were employed (table 2).

Additional evidence for a sex-dependent, temporal qualitative change in cytochrome P-450 has been presented by Stripp *et al.* (38), who reported a lower apparent extinction coefficient of microsomal cytochrome P-450 from 6-week-old male rats than that of cytochrome P-450 from female rats of the same age. Schenkman *et al.* (39) reported sex-dependent differences in microsomal drug metabolism as being due to differences in the magnitudes of the type I spectral shift produced by binding of substrates to cytochrome P-450. Gigon *et al.* (37) related sex-dependent differences in drug metabolism to the greater ability of type I substrates to stimulate NADPH-cytochrome P-450 reductase activity in male rats. Differences in type I binding and the stimulation of NADPH-cytochrome P-450 reductase activity by type I substrates may reflect qualitative differences in cytochrome P-450.

Developmental changes in cytochrome P-450 might occur in any or all of several ways: a) an "immature" P-450 hemoprotein might develop into a "mature P-450" in the male, but not in the female; b) two or more P-450 hemoproteins may exist in immature rats and the proportions of each might change in the two sexes during development; this concept has been suggested by Stripp *et al.* (38); c) the environment of cytochrome P-450 in the microsomal membrane might change with sexual development in a manner that would affect the specific activity of cytochrome P-450 differently in the two sexes; d) different activators or inhibitors of the system may be produced by male and female rats.

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References

1. F. B. Winton, *J. Pharmacol. Exp. Ther.* **31,** 123 (1927).
2. A. H. Conney, *Pharmacol. Rev.* **19,** 317 (1967).
3. J. R. Gillette, *Progr. Drug Res.* **6,** 11 (1963).
4. J. R. Gillette, D. C. Davis, and H. A. Sasame, *Annu. Rev. Pharmacol.* **12,** 57 (1972).
5. G. P. Quinn, J. Axelrod, and B. B. Brodie, *Biochem. Pharmacol.* **1,** 152 (1958).
6. R. Kato and K. Onoda, *Jap. J. Pharmacol.* **16,** 217 (1966).
7. J. R. Fouts and R. H. Adamson, *Science* **129,** 897 (1959).
8. W. R. Jondorf, R. P. Maickel, and B. B. Brodie, *Biochem. Pharmacol.* **1,** 352 (1959).
9. R. Kato, E. Chiesara, and G. Frontino, *Biochem. Pharmacol.* **11,** 221 (1962).
10. S. D. Murphy and K. P. Dubois, *J. Pharmacol. Exp. Ther.* **124,** 194 (1958).
11. T. K. Basu, J. W. T. Dickerson, and D. V. M. Parke, *Biochem. J.* **124,** 19 (1971).
12. T. E. Gram, A. M. Guarino, D. H. Schroeder, and J. R. Gillette, *Biochem. J.* **113,** 681 (1969).
13. P. Th. Henderson, *Biochem. Pharmacol.* **20,** 1225 (1971).
14. S. M. MacLeod, K. W. Renton, and N. R. Eade, *J. Pharmacol. Exp. Ther.* **183,** 489 (1972).
15. T. Nash, *Biochem. J.* **55,** 416 (1953).
16. M. W. Anders and G. J. Mannering, *Mol. Pharmacol.* **2,** 319 (1966).

17. D. A. MacFayden, *J. Biol. Chem.* **158,** 107 (1945).
18. R. Kato and J. R. Gillette, *J. Pharmacol. Exp. Ther.* **150,** 279 (1965).
19. G. Dallner, *Acta Pathol. Microbiol. Scand.* (suppl. 166), 1–24 (1963).
20. R. L. Lester and A. L. Smith, *Fed. Proc.* **19,** 34 (1960).
21. R. L. Lester and A. L. Smith, *Biochim. Biophys. Acta* **47,** 475 (1961).
22. J. R. Gillette, B. B. Brodie, and B. N. La Du, *J. Pharmacol. Exp. Ther.* **119,** 532 (1957).
23. P. Hochstein and L. Ernster, *Biochem. Biophys. Res. Commun.* **12,** 388 (1963).
24. T. Omura and R. Sato, *J. Biol. Chem.* **239,** 2370 (1964).
25. N. E. Sladek and G. J. Mannering, *Mol. Pharmacol.* **5,** 186 (1969).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and J. L. Randall, *J. Biol. Chem.* **193,** 265 (1951).
27. W. J. Dixon and F. G. Massey, "Introduction to Statistical Analysis," McGraw-Hill, New York, 1957.
28. G. N. Wilkinson, *Biochem. J.* **80,** 324 (1961).
29. W. W. Cleland, *Nature* **198,** 463 (1963).
30. R. Kato, P. Vassanelli, G. Frontino, and E. Chiesara, *Biochem. Pharmacol.* **13,** 1037 (1964).
31. M. W. Anders, *Arch. Biochem. Biophys.* **126,** 269 (1968).
32. G. Dallner, P. Siekevitz, and G. E. Palade, *Biochem. Biophys. Res. Commun.* **20,** 135 (1965).
33. D. S. Davies, P. L. Gigon, and J. R. Gillette, *Life Sci.* **8,** 85 (1969).
34. F. Bernheim, K. M. Wilbur, and C. B. Kenaston, *Arch. Biochem. Biophys.* **38,** 177 (1952).
35. S. Orrenius, G. Dallner, and L. L. Ernster, *Biochem. Biophys. Res. Commun.* **14,** 329 (1964).
36. T. E. Gram and J. R. Fouts, *Arch. Biochem. Biophys.* **114,** 331 (1966).
37. P. L. Gigon, T. E. Gram, and J. R. Gillette, *Mol. Pharmacol.* **5,** 109 (1969).
38. B. Stripp, F. E. Greene, and J. R. Gillette, *Pharmacology* **6,** 56 (1971).
39. J. B. Schenkman, H. Remmer, and R. W. Estabrook, *Mol. Pharmacol.* **3,** 113 (1967).