Changes in Certain Kinetic Properties of Hepatic Microsomal Aniline Hydroxylase and Ethylmorphine Demethylase Associated with Postnatal Development and Maturation in Male Rats

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(Received 3 March 1969)

1. Changes in certain kinetic properties ($V_{\max}$ and apparent K_m) of hepatic microsomal mixed-function oxidases have been studied as a function of postnatal development and maturation in male rats. 2. Microsomal cytochrome *P*-450 content changed only slightly between 1 and 12 weeks of age. 3. Aniline hydroxylase activity ($V_{\max}$) increased abruptly between 1 and 2 weeks of age to greater than adult activities and then returned to a plateau value between 4½ and 12 weeks of age. Ethylmorphine demethylase activity remained low and relatively constant between 1 and 3 weeks of age and then increased markedly (~100%) between 3 and 4½ weeks. 4. The apparent Michaelis constant (K_m) for aniline hydroxylation increased almost linearly with time between 1 and 6 weeks of age and tended to reach a plateau value thereafter. The apparent K_m for ethylmorphine demethylation increased between 1 and 3 weeks of age and then decreased abruptly to a constant value between 6 and 12 weeks. 5. The data indicate that developmental changes in the activity of these microsomal oxidases do not correlate temporally with each other or with changes in microsomal cytochrome *P*-450 content. 6. The most dramatic changes in enzyme activity were associated with early development (1–3 weeks) and weaning (3–4 weeks). 7. Changes in weight of seminal vesicle, a criterion of sexual maturation in male rats, were most prominent between 6 and 8 weeks of age and thus appeared to be separated in time from the prominent changes in enzyme activity.

It is generally believed that the mammalian hepatic microsomal mixed-function oxidases are present at low or undetectable activities in foetal and newborn animals and gradually increase thereafter, reaching adult activities at some time near puberty (Jondorf, Maickel & Brodie, 1958; Fouts & Adamson, 1959; Brodie & Maickel, 1962; Bresnick & Stevenson, 1968). Fouts & Adamson (1959) reported that newborn rabbits were markedly deficient in their ability to metabolize several drugs, and Jondorf *et al.* (1958) described a similar inability in newborn guinea pigs and mice. Both groups reported that, after birth, enzyme activity appeared gradually, reaching adult activities at some time between 3 and 8 weeks of age. Similarly, Kato, Vassanelli, Frontino & Chiesara (1964) found that the microsomal drug-metabolizing activity was very low in 1-day-old rats, that it increased to a maximum at about 30 days of age and tended to decline thereafter.

The present paper shows that although the hepatic microsomal ethylmorphine *N*-demethylase

activity in 2-week-old rats is below that found in adults, the microsomal aniline hydroxylase activity in these animals is significantly higher than in adult animals. Moreover, the apparent Michaelis constant (K_m) for aniline hydroxylation is lower in microsomes from young animals than in microsomes from adults.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats from the N.I.H. colony were used in the experiments. The 1- and 2-week age groups were unselected as to sex. Males were employed exclusively at all other times. In preliminary experiments we found no sex differences in microsomal cytochrome *P*-450 content or in $V_{\max}$ or K_m for either ethylmorphine or aniline in 2-week-old rats.

To provide normal laboratory conditions, newborn animals were caged with their mothers until the time of natural weaning (3–4 weeks of age in our colony), at which time they were allowed access to Purina Laboratory Chow. Tap water was available at all times.

Preparation of microsomes. Animals were killed by decapitation and their livers were immediately excised and

chilled in crushed ice. All subsequent tissue manipulations were conducted at 0–4°. In experiments involving very young animals it was necessary to pool livers from many animals to obtain sufficient tissue for a single kinetic analysis. For example, at 1 week of age each group consisted of livers pooled from about 40 animals, and five such groups were examined. At other ages, six groups were used. Livers were homogenized in 2 vol. of 1.15% KCl soln. containing 0.02M-tris-HCl (pH 7.4) and microsomes were prepared by differential centrifugation as described by Gram, Guarino, Greene, Gigon & Gillette (1968).

Enzyme assays. Incubations were carried out aerobically with shaking at 37° in a Dubnoff apparatus. Reaction mixtures consisted of the following components: microsomal protein (2mg./ml.), substrate in varying amounts, tris-HCl buffer, pH 7.4 (120 mM), an NADPH-generating system consisting of NADP (0.4 mM), glucose 6-phosphate (10 mM), MgCl₂ (5 mM), glucose 6-phosphate dehydrogenase (yeast) (2 units) and sufficient 1.15% KCl soln. to make a final volume of 5 ml. Mixtures were incubated for 10 min.; the reactions were linear with time and with protein concentration under these conditions. The substrates, aniline and ethylmorphine, were present in concentrations ranging from 0.01 to 2.0 mM for the former and 0.2 to 8.0 mM for the latter. Seven different substrate concentrations were used.

Analytical methods. Microsomal aniline hydroxylase activity was assayed by the method of Kato & Gillette (1965) as described in detail by Guarino, Gram, Gigon, Greene & Gillette (1969). Ethylmorphine demethylase activity was assayed by the method of Nash (1953) as described by Holtzman, Gram, Gigon & Gillette (1968). Standards (*p*-aminophenol and formaldehyde respectively) were carried through the procedures with each assay and

were employed to obtain quantitative results from enzymic activity. Cytochrome *P*-450 was determined by the method of Omura & Sato (1964) as described by Holtzman *et al.* (1968). Microsomal protein was assayed as described by Lowry, Rosebrough, Farr & Randall (1951). Enzyme kinetic constants were obtained by a computerized least-squares method as reported previously (Davies, Gigon & Gillette, 1968; Inturrisi & Titus, 1968); all experimentally obtained values were retained regardless of the standard error.

RESULTS

Changes in rat liver microsomal enzyme activities and in cytochrome P-450 content as a function of age. The data presented in Fig. 1 indicate that ethylmorphine *N*-demethylase and aniline hydroxylase activities (V_{max}) undergo marked changes during the first 4½ weeks of life. During this period these changes are not synchronously related either to each other or to concentrations of cytochrome *P*-450. Indeed, the changes in enzyme activity were in opposite directions and occurred at different times. Aniline hydroxylase activity nearly doubled between 1 and 2 weeks of age (Fig. 1); from this peak, activity tended to decline slightly to a constant value between 3 and 8 weeks and then to a slightly lower value at 12 weeks. By contrast, ethylmorphine *N*-demethylase activity remained relatively low and constant between 1 and 3 weeks of age but between 3 and 4½ weeks of age activity increased by about

Fig. 1. Developmental changes in microsomal mixed-function oxidase activity (V_{max}) towards aniline (●) and ethylmorphine (■) (left ordinate) and in cytochrome *P*-450 content (▲, right ordinate) in rat liver. Conditions of incubation and assay are described in the Materials and Methods section. Enzyme activities are expressed as nmoles of formaldehyde formed/mg. of microsomal protein/10 min. (ethylmorphine) or as nmoles of *p*-aminophenol formed/mg. of microsomal protein/10 min. (aniline). Cytochrome *P*-450 content is expressed as $\Delta E_{450} - \Delta E_{490}$ /mg. of microsomal protein. Values are means from five or six groups of animals; vertical bars represent one S.D.

Fig. 2. Developmental changes in apparent Michaelis constant (K_m) of microsomal enzymes of rat liver toward aniline (●) and ethylmorphine (■). Conditions of incubation and assay are described in the Materials and Methods section. Values are means from five or six groups of animals; vertical bars represent one S.D.

100%. The tendency toward a decrease in activity observed after 4½ weeks was not statistically significant. Concomitant with these dramatic changes in microsomal enzyme activities, concentrations of cytochrome *P*-450, although tending to rise, changed only slightly.

Changes in the apparent substrate affinity of rat liver microsomal enzymes as a function of age. During the first 3 weeks of life the apparent K_m for both aniline hydroxylation and ethylmorphine demethylation increased significantly (Fig. 2). Thereafter, the former continued to increase and reached a plateau value approximately three- to four-fold that obtained in 1-week-old animals and the latter diminished to a value about 30% below that of the 1-week-old animals (Fig. 2). The apparent K_m for ethylmorphine demethylation increased by about 60% between 1 and 3 weeks of age and then abruptly declined.

Time of sexual maturation in male rats. It is known that newborn animals are deficient in microsomal mixed-function oxidase activity (Fig. 1; see also Fouts & Adamson, 1959; Jondorf *et al.* 1958) and it has been generally assumed that the increase in activity to adult values occurs at puberty. To test this assumption, we examined changes in weights of seminal vesicle (Eisenberg & Gordan, 1950) and testicle as indices of sexual maturation. The data (Table 1) indicate that the most significant changes in seminal vesicular weight (expressed as mg. per unit of body weight) occurred between 6 and 8 weeks of age, whereas the testicular

Table 1. Age-related changes in weights of seminal vesicle, testes and liver in rats

Results are given as means $\pm$ S.D. for five animals in each group.

Age (weeks)	Wt. of seminal vesicle (g./100g. body wt.)	Wt. of testes (g./100g. body wt.)	Wt. of liver (g./100g. body wt.)
3	0.024 $\pm$ 0.005	0.44 $\pm$ 0.02	4.9 $\pm$ 0.4
4	0.054 $\pm$ 0.010	0.84 $\pm$ 0.09	4.8 $\pm$ 0.2
6	0.086 $\pm$ 0.025	1.24 $\pm$ 0.18	4.5 $\pm$ 0.5
8	0.221 $\pm$ 0.038	1.27 $\pm$ 0.15	4.6 $\pm$ 0.4
9	0.263 $\pm$ 0.047	1.26 $\pm$ 0.04	4.4 $\pm$ 0.3

weight changes were most pronounced between 3 and 6 weeks of age.

DISCUSSION

Our results indicate that, in male rats during the first 3 weeks of life, kinetic constants (V_{max} and K_m) for hepatic microsomal aniline hydroxylase and ethylmorphine demethylase undergo marked changes. These changes occur independently of each other, both temporally and directionally. Moreover, they did not correlate with changes in concentration of microsomal cytochrome *P*-450. Aniline hydroxylation (V_{max}) reaches its highest values in 2-week-old animals and gradually declines thereafter, whereas ethylmorphine demethylation remains low for 3 weeks and then increases abruptly.

to a peak at about 4½ weeks of age and declines only slightly thereafter. Microsomal cytochrome *P*-450 tends to increase slightly during the first 12 weeks of life. Apparent Michaelis constants (K_m) for these two substrates increased markedly during the first 3 weeks of life; thereafter the K_m for aniline tended to reach a plateau whereas that for ethylmorphine decreased sharply.

The finding of differential rates of post-natal enzyme development towards various substrates, although perhaps novel for the hepatic microsomal 'drug-metabolizing' enzymes, only confirms numerous similar observations made with other systems. Microsomal UDP-glucuronyltransferase activity is known to display different developmental patterns towards several substrates (Dutton, 1966a). Flint, Lathe, Ricketts & Silman (1964) observed that in rabbit liver the glucuronyltransferase activity towards bilirubin reached adult activities by 5–7 days of age, whereas about 30 days were required for activity towards *o*-aminophenol to reach equivalent values. Dutton, Langelaan & Ross (1964) and Dutton (1966b) reported that whereas glucuronyltransferase activity towards phenolphthalein was low in newborn rats and reached adult activities in about 5 days, the activity towards *p*-nitrophenol in newborn rats was twice that in adults and decreased to adult values thereafter.

The developmental pattern of microsomal aniline hydroxylase differs markedly from that of ethylmorphine demethylase. Contrasts between these substrates have been noted previously: (1) they give different types of difference spectra when added to microsomal suspensions in the absence of NADPH (Schenkman, Remmer & Estabrook, 1967; Gigon, Gram & Gillette, 1968); (2) they produce opposite effects on microsomal NADPH-cytochrome *P*-450 reductase activity (Gigon *et al.* 1968); (3) the apparent K_m for aniline hydroxylation changes significantly upon pretreatment with phenobarbital (Guarino *et al.* 1969), whereas this pretreatment has no effect on K_m for ethylmorphine, hexobarbital, aminopyrine or certain other compounds (Rubin, Tephly & Mannering, 1964); (4) in rats, there is relatively little sex difference in the microsomal metabolism of aniline, but for ethylmorphine, aminopyrine and hexobarbital males possess two- to four-fold the activity shown by females (Kato & Gillette, 1965).

Ethylmorphine demethylase activity was found to increase abruptly between 3 and 4 weeks of age, concomitant with weaning (Fig. 1). These data should not, of course, be taken to indicate that weaning itself stimulates enzyme activity. Whether the increased enzyme activity is caused by weaning, the presence of enzyme inducers in the commercial laboratory food or other unknown factors is not clear. If these differences are due to inducers in the

diet, however, these inducers would be unusual in that they selectively increase ethylmorphine *N*-demethylase and do not increase the cytochrome *P*-450 content.

Ordinarily the marked increase in the rate of ethylmorphine demethylation and the decrease in the apparent K_m value that occurred between 3 and 4½ weeks would be related to development of the sex difference in the metabolism of this substrate (Davies *et al.* 1968; Gigon *et al.* 1968). Indeed these results are in accord with the finding of Quinn, Axelrod & Brodie (1958) that the sex difference in hexobarbital sleeping times is manifested between the fourth and fifth weeks and the finding of Kato, Chiesara & Vassanelli (1962) that appearance of the sex difference in strychnine metabolism by rat liver microsomes occurs between 4 and 8 weeks of age. However, Resko, Feder & Goy (1968) have examined post-natal changes in concentrations of plasma testosterone in male rats and found that although measurable increases occurred between 4 and 6 weeks of age, peak values were not reached until 8–16 weeks of age. These data correlate reasonably well with our finding that maximal changes in seminal vesicle weight occurred between 6 and 8 weeks of age (Table 1).

Thus the increase in microsomal ethylmorphine demethylase activity (Fig. 1) appears to precede sexual maturity as measured by marked increases in seminal vesicle weight (Table 1).

The authors gratefully acknowledge the skilful technical assistance of Miss Jacqueline B. Call of the National Cancer Institute, National Institutes of Health. T.E.G., A.M.G. and D.H.S. are Research Associates in the Pharmacology-Toxicology Program, National Institute of General Medical Sciences, National Institutes of Health.

REFERENCES

- Bresnick, E. & Stevenson, J. G. (1968). *Biochem. Pharmacol.* **17**, 1815.
- Brodie, B. B. & Maickel, R. P. (1962). *Proc. 1st int. Pharmacol. Meet.*, p. 299. Ed. by Brodie, B. B. & Erdos, E. G. New York: Pergamon Press Inc.
- Davies, D. S., Gigon, P. L. & Gillette, J. R. (1968). *Biochem. Pharmacol.* **17**, 1865.
- Dutton, G. J. (1966a). In *Glucuronic Acid, Free and Combined*, p. 186. Ed. by Dutton, G. J. New York: Academic Press Inc.
- Dutton, G. J. (1966b). *Biochem. Pharmacol.* **15**, 947.
- Dutton, G. J., Langelaan, D. E. & Ross, P. E. (1964). *Biochem. J.* **93**, 4r.
- Eisenberg, E. & Gordan, G. S. (1950). *J. Pharmacol.* **99**, 38.
- Flint, M., Lathe, G. H., Ricketts, T. R. & Silman, G. (1964). *Quart. J. exp. Physiol.* **49**, 66.
- Fouts, J. R. & Adamson, R. H. (1959). *Science*, **129**, 897.
- Gigon, P. L., Gram, T. E. & Gillette, J. R. (1968). *Biochem. biophys. Res. Commun.* **31**, 558.
- Gram, T. E., Guarino, A. M., Greene, F. E., Gigon, P. L. & Gillette, J. R. (1968). *Biochem. Pharmacol.* **17**, 1769.

- Guarino, A. M., Gram, T. E., Gigon, P. L., Greene, F. E. & Gillette, J. R. (1969). *Molec. Pharmacol.* **5**, 131.
- Holtzman, J. L., Gram, T. E., Gigon, P. L. & Gillette, J. R. (1968). *Biochem. J.* **110**, 407.
- Inturrisi, C. E. & Titus, E. O. (1968). *Molec. Pharmacol.* **4**, 591.
- Jondorf, W. R., Maickel, R. P. & Brodie, B. B. (1958). *Biochem. Pharmacol.* **1**, 352.
- Kato, R., Chiesara, E. & Vassanelli, P. (1962). *Jap. J. Pharmacol.* **12**, 26.
- Kato, R. & Gillette, J. R. (1965). *J. Pharmacol.* **150**, 279.
- Kato, R., Vassanelli, P., Frontino, G. & Chiesara, E. (1964). *Biochem. Pharmacol.* **13**, 1037.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Nash, T. (1953). *Biochem. J.* **55**, 416.
- Omura, T. & Sato, R. (1964). *J. biol. Chem.* **239**, 2379.
- Quinn, G. P., Axelrod, J. & Brodie, B. B. (1958). *Biochem. Pharmacol.* **1**, 152.
- Resko, J. A., Feder, H. H. & Goy, R. W. (1968). *J. Endocrin.* **40**, 485.
- Rubin, A., Tephly, T. R. & Mannering, G. J. (1964). *Biochem. Pharmacol.* **13**, 1007.
- Schenkman, J. B., Remmer, H. & Estabrook, R. W. (1967). *Molec. Pharmacol.* **3**, 113.