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PERINATAL DEVELOPMENT OF HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE ACTIVITY IN SWINE*

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Abstract—Hepatic microsomal cytochrome P-450, cytochrome b_5, NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase levels were measured in fetal (107-days gestation), newborn and 1-, 2-, 3-, 4- and 6-week-old swine. Cytochrome P-450 levels and NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase activities increased in near parallel with ethylmorphine demethylase (V_{max}) activity between the first and the sixth postnatal week. The activities or levels of all parameters measured appeared to plateau between the fourth and sixth week postpartum. The only qualitative change observed after 1 week of age was a slight increase in the K_m for ethylmorphine demethylation. NADPH-cytochrome *c* reductase activity of fetal liver was relatively high, being approximately 40 per cent of the values attained at 6 weeks of age. This was in contrast to very low levels of NADPH-cytochrome P-450 reductase activity and cytochrome P-450 content of fetal liver. Clearly the activity of the flavoprotein NADPH-cytochrome *c* reductase does not limit the rate of reduction of cytochrome P-450 in the microsomal fraction of fetal liver. The possibility that cytochrome P-450 exists in a different form, or ratio of forms, in fetal liver could not be ascertained from carbon monoxide (CO) or ethylisocyanide (EtCN) difference spectra of fetal microsomal preparations. However, the dithionite difference CO spectra of cytochrome P-450 did not change with age.

THE GENERAL deficiency of hepatic microsomal mixed function oxidase (MFO) activity in the near-term fetal and newborn animal is well established. Enzyme activity in the rat, mouse, guinea pig and rabbit has been found to increase gradually after birth to a maximum at 3–6 weeks of age.[1–6] It has generally been assumed that the attainment of maximal activity is coincident with the onset of puberty,[7] except in the male rat in which activity continues to increase past that point. That this correlation is not universal, however, has been demonstrated in swine, a specie in which the level of MFO activity begins to plateau at about 4-weeks postpartum and in which puberty is achieved at 6–8 months of age.[8,9]

Steroid hormones are metabolized by the MFO system and influence its activity. Changes in the activity of this system which occur during the first 3–6 weeks after birth may be influenced to a considerable degree by alterations in androgen or estrogen levels in species in which birth and puberty are separated by no more than

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4- or 6-weeks' time. The swine was employed as a model in the present study primarily to determine the rate of development of components of the MFO system in a specie in which such development was apart from puberty-linked changes in sex steroid levels.

The several components of the MFO system include NADPH as a source of reducing equivalents, the flavoprotein NADPH-cytochrome *c* reductase, which is involved in the transfer of reducing equivalents, and the terminal oxidase cytochrome P-450. NADPH-cytochrome P-450 reductase is considered to be the flavoprotein, NADPH-cytochrome *c* reductase.[10,11] Measurement of the rate of reduction of cytochrome P-450 (NADPH-cytochrome P-450 reductase) generally provides a better correlation with the rate of substrate oxidation than does measurement of the rate of cytochrome *c* reduction.[12–14] A non-heme iron flavoprotein (microsomal Fe_x)[15] has been proposed as an electron carrier interposed between NADPH-cytochrome *c* reductase and cytochrome P-450, though its existence or function in the hepatic microsomal MFO system is uncertain. A possible additional component depicted in schema of electron flow is cytochrome b_5, a major hemoprotein of the microsomal fraction of the hepatocyte.[16] This cytochrome may be involved in the transfer of a second reducing equivalent to the cytochrome P-450 substrate complex.

The present paper compares the rates of postnatal increase in the activities or levels of NADPH-cytochrome *c* reductase, cytochrome P-450 reductase, cytochromes P-450 and b_5, and ethylmorphine demethylase. Changes in the carbon monoxide (CO) and ethylisocyanide (EtCN) spectra of microsomal preparations during the early perinatal period are described.

MATERIALS AND METHODS

Animals. Twelve Duroc sows were bred over a period of approximately 3 months. Individual offspring were sacrificed on the day of birth, and at 1, 2, 3, 4 and 6 weeks of age. One or two animals were sacrificed from one litter at each age so that mean values at each age were obtained on pigs from at least three litters. The pigs were weaned gradually over the course of the experiment. They began to eat the sows' diet (Purina complete sow chow) at about 1 week of age and were completely weaned by the sixth to seventh week. Caesoratomies were performed on two sows on day 107 of gestation (7 days before term). Tissues from all of the fetuses delivered by Caesarean section were assayed at the same time. Pigs of both sexes were used as no sex differences in oxidative metabolism were found in neonatal pigs in a previous study.[8]

Tissue preparation. The pigs were sacrificed by stunning and jugular venasection. The livers were immediately excised, and after removal of the gall bladder, were immersed in 1·15% KCl–0·05 M Tris–HCl, pH 7·4. All subsequent tissue manipulations were conducted at 2–4°. Tissue samples were weighed and homogenized with 3 vol. of 1·15% KCl–0·05 M Tris–HCl, pH 7·4. The microsomal fraction was prepared by differential centrifugation as described previously.[8]

Enzyme assays. Ethylmorphine demethylase activity was measured by incubation of the substrate with an aliquot of the microsomal fraction and an NADPH generating system. Each reaction vessel contained 2 mg/ml of microsomal protein, 120 mM Tris–HCl buffer, pH 7·4, an NADPH generating system (0·37 mM NADPH, 2 mM MgCl, 10 mM isocitric acid and 1·8 units of isocitric dehydrogenase), substrate and 1·15% KCl in a final volume of 5 ml. Ethylmorphine was incubated at eight different

concentrations, ranging from 0·64 to 8 mM. The formaldehyde formed via ethylmorphine demethylation was measured by the procedure of Nash.[17]

NADPH-cytochrome P-450 reductase and NADPH-cytochrome *c* reductase were measured as described by Gigon *et al.*[18] using an Aminco anaerobic spectrophotometric cell. Only the rapid initial reduction (5 sec) of cytochrome P-450 was employed in calculating NADPH-cytochrome P-450 reductase activity. The rate of cytochrome *c* reduction was linear for at least 30 sec. The microsomal suspension in the above assays contained 3 mg of protein/ml and 0·5 mg of protein/ml, respectively. Cytochrome P-450 was measured by reduction with NADPH, while cytochrome b_5 was measured using NADH as the reducing agent. The microsomal suspension contained 2 mg of protein/ml. All of the above assays were performed on a Shimadzu MPS-50L recording spectrophotometer using 1-cm^2 cuvettes. NADPH-cytochrome P-450 reductase and NADPH-cytochrome *c* reductase were assayed at 37°. The carbon monoxide (CO) difference spectrum of cytochrome P-450 and the spectrum of cytochrome b_5 were assayed at 25°. Extinction coefficients of 18,500, 91,000 and 185,000 cm^{-1} were used to estimate reduced cytochrome *c*, and cytochromes P-450 and b_5 respectively.

Ethylisocyanide spectra. The ethylisocyanide (EtCN) spectra of cytochrome P-450 was measured at several pH values ranging from 6·8 to 8·5 in a microsomal suspension containing 2 mg of protein/ml at 25° as described by Imai and Sato.[19] Data were presented as the ratio ΔA 430–490/ΔA 455–490 nm as a function of pH. EtCN was synthesized according to the method of Jackson and McKusick.[20] The identity and purity of the redistilled product were confirmed by infra-red and NMR spectroscopy.

Protein was measured by the method of Lowry *et al.*[21] using crystalline bovine albumin as a standard. The difference between two means was analyzed for significance ($P < 0·05$) by the Student's *t*-test.[22] Enzyme kinetic constants were obtained by least squares regression analysis of the data points. Linear correlation was calculated by standard methods.

RESULTS

Hepatic microsomal cytochrome content. The specific content (nanomoles per milligram of microsomal protein) of cytochrome P-450 increased almost 10-fold in a nearly linear manner during the first 4 postnatal weeks and then appeared to plateau between the fourth and sixth weeks (Fig. 1). Only about 0·075 nmole of cytochrome P-450/mg of protein was found in the microsomal fraction of the near-term fetal pig (−7 days). Cytochrome b_5 levels were approximately one-half of the cytochrome P-450 levels from the first through the sixth postnatal week. The ratio of cytochrome P-450 to cytochrome b_5 was reversed, however, in the fetal microsomal fraction, and there were approximately equal amounts of the two hemoproteins at birth. Thus the specific content (nanomoles per milligram of protein) of cytochrome P-450 increased markedly after birth, while the specific content of cytochrome b_5 increased only about 200 per cent between birth and 4 weeks of age.

NADPH-cytochrome reductases. The patterns of development of NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reduction were similar after birth (Fig. 2). However, there was relatively more NADPH-cytochrome *c* reductase than NADPH-cytochrome P-450 reductase activity in fetal liver. Thus, a very low rate of NADPH reduction of cytochrome P-450 coincided with a comparatively high

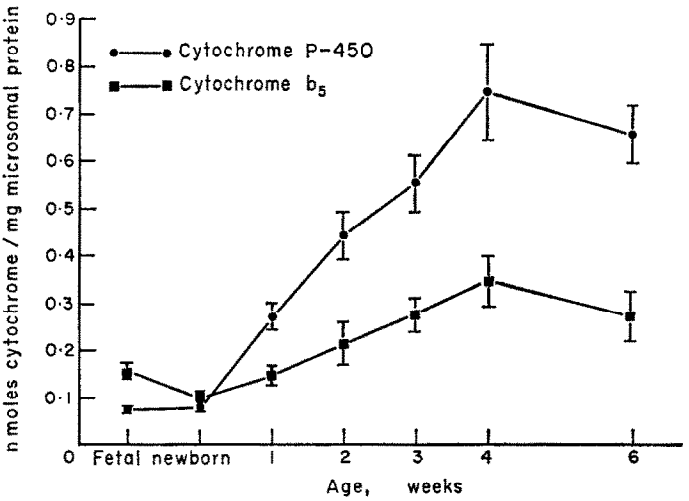

FIG. 1. Hepatic microsomal cytochrome P-450 and cytochrome b_5 levels as a function of perinatal age. Each value represents the mean ± S. E. M. for determinations on five or six fetal (107-days gestation) or neonatal pigs.

NADPH-cytochrome *c* reductase activity (about 40 nmoles of cytochrome *c* reduced/mg/min) in fetal liver, indicating an age-related change in the reducibility of cytochrome P-450 by NADPH-cytochrome *c* reductase.

The age-related change in the ratio of reductase activity as measured by cytochrome *c* reduction and the rate of cytochrome P-450 reduction became more apparent when the rate of reduction of the two hemoproteins was presented as a function of cytochrome P-450 content. Figure 3 depicts the rates of cytochrome *c* and P-450 reduction in terms of nanomoles of cytochrome reduced per nanomole of cytochrome P-450 per min. A marked increase in the reducibility of cytochrome P-450 with NADPH

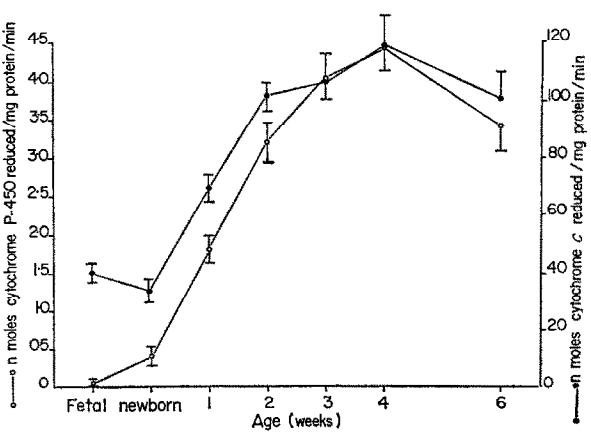

FIG. 2. Hepatic microsomal NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase activities as a function of perinatal age. Each value represents the mean ± S. E. M. for determinations on five or six fetal (107-days gestation) or neonatal pigs.

during the last week of fetal life and the first-week postpartum was evident. This is in contrast to a significant decrease in NADPH-cytochrome *c* reductase activity relative to cytochrome P-450 content.

Ethylmorphine metabolism. Ethylmorphine demethylase activity per unit of cytochrome P-450 did not change from the first through the sixth postpartum week (Fig. 3). Thus the rate of hydroxylation of this spectral type I substrate could be a function of total cytochrome P-450 content during this period. The sensitivity of the analytical procedures was not sufficient to determine kinetic parameters of metabolism of this compound in fetal or newborn microsomal preparations. Table 1 illustrates that the V_{max} for ethylmorphine demethylation increased in a fashion similar to that of the other parameters; in addition, there was a slight increase in K_m for ethylmorphine metabolism between 1 and 6 weeks of age.

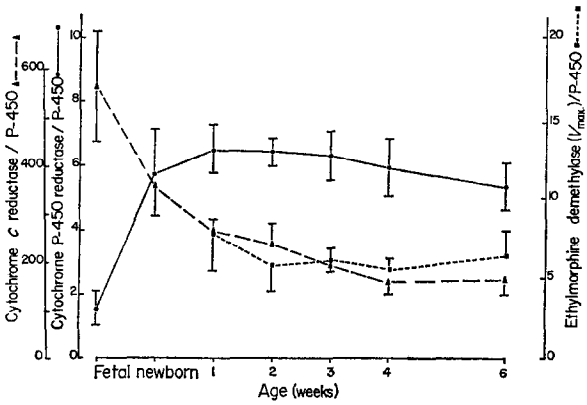

FIG. 3. Age-related change in hepatic microsomal NADPH-cytochrome *c* reductase, NADPH-cytochrome P-450 reductase and ethylmorphine demethylase (V_{max}) activities as a function of cytochrome P-450 levels. Reductase activities are presented as nanomoles of cytochrome reduced per nanomole of cytochrome P-450 per min. Ethylmorphine demethylase activity is presented as nanomoles of formaldehyde formed per nanomole of cytochrome P-450 per min. Each value represents the mean ± S. E. M. for determinations on five or six fetal (107-days gestation) or neonatal pigs.

The comparative rates of development of the several parameters studied are presented in Fig. 4. Substrate oxidation, cytochrome P-450 content and cytochrome reductase activities are presented as the per cent activity or level attained at 6 weeks of age. It was apparent that no single component involved in electron flow could be singled out as rate limiting to substrate oxidation between 1 and 6 weeks of age. High correlation coefficients were obtained between the V_{max} for ethylmorphine demethylation and cytochrome P-450 content ($r = +0{\cdot}97$), NADPH-cytochrome *c* reductase activity ($r = +0{\cdot}88$) and NADPH-cytochrome P-450 reductase activity ($r = +0{\cdot}90$) between 1 and 6 weeks of age, and all activities appeared to attain maximal levels at approximately the same age.

Ethylisocyanide spectrum of microsomal preparation. The pH-dependent EtCN spectrum of cytochrome P-450 has been employed as evidence for the formation of a new form of the hemoprotein (P_1-450, P-448, P-466) after induction with polycyclic hydrocarbons, particularly 3-methylcholanthrene (3-MC).[19,23] The pH intercept of the ΔA 430–490/ΔA 455–490 nm ratio varies with the specie from approximately 7·4

TABLE 1. KINETIC CONSTANTS FOR HEPATIC MICROSOMAL ETHYLMORPHINE DEMETHYLASE AS A FUNCTION OF AGE*

Postnatal age (weeks)	V_{max}†	K_m (mM)
1	0·82 ± 0·17	0·40 ± 0·10
2	2·28 ± 0·24	0·54 ± 0·16
3	3·56 ± 0·27	0·55 ± 0·09
4	3·72 ± 0·63	0·54 ± 0·16
6	4·17 ± 0·81	0·71 ± 0·15‡

* Each value represents the mean ± S. E. M. for determinations on five or six animals.

† Enzyme activity is expressed as nanomoles of formaldehyde formed per milligram of protein per min.

‡ Significantly ($P < 0·05$) higher than value for 1 week of age.

in untreated rats[24] to about 7·9 in human liver microsomes.[25] After 3-MC induction in rats, the pH intercept has been found to shift to approximately 6·9. The significance of this shift is not clear, but it has been equated with an increase in the amount of high-spin iron hemoprotein.[26]

Microsomal EtCN spectra were measured in the present study to determine whether or not any age-related change in the form of cytochrome P-450 could be detected. It was found that the pH intercept did not vary significantly between 2 and 6 weeks of age (Fig. 5). An average pH intercept of 7·94 was observed during this period. This value compared very well with a pH intercept of 7·9 obtained on adult (8-month-old)

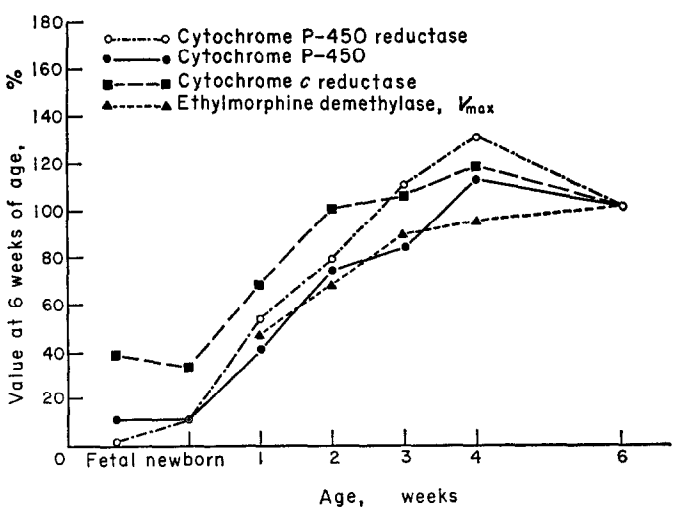

FIG. 4. Hepatic microsomal NADPH-cytochrome *c* reductase, NADPH-cytochrome P-450 reductase, and ethylmorphine demethylase (V_{max}) activities and cytochrome P-450 content relative to the value obtained at 6 weeks of age. Each point represents the mean value per unit of microsomal protein (see text) for determinations on five or six fetal (107-days gestation) or neonatal pigs.

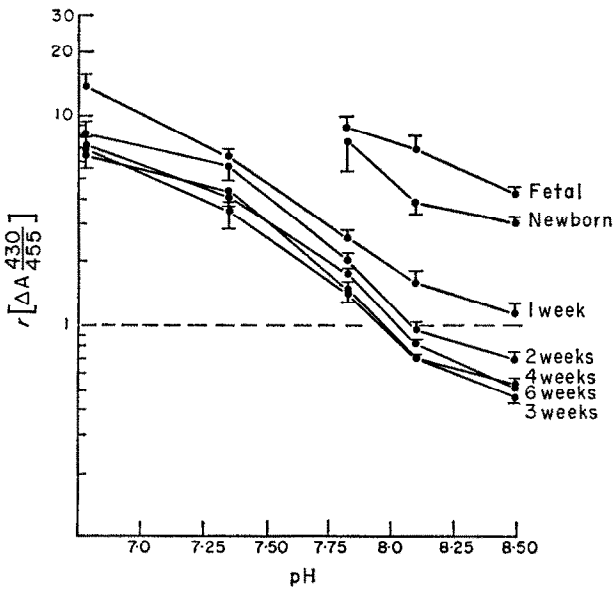

FIG. 5. Ratio of 430 nm to 455 nm peak heights for ethylisocyanide spectra of the microsomal fraction of pig liver as a function of pH and perinatal age. Each value represents the mean ratio ΔA 430–490/ΔA 455–490 nm ± S. E. M. for determinations on five or six fetal (107-days gestation) or neonatal pigs.

swine. The intercept at 1 week of age would have averaged slightly higher than pH 8·5, and no intercept could be obtained from microsomes of fetal or newborn pigs. It is probable that the EtCN spectra obtained from the microsomes of fetal and newborn animals were not the spectra of cytochrome P-450 alone, but were a composite of spectra of this cytochrome and another hemoprotein. It was found that the decrease in the 430 nm peak was not proportional to the increase in 455 nm peak at these ages. The changes in peak heights should have been proportional if only cytochrome P-450 were being measured.[19] Specifically, the 430 nm peak decreased very little with increasing pH. Two other ferrohemoproteins which may be present in microsomal preparations, cytochrome P-420 and hemoglobin, yield EtCN difference spectra characterized by a 430–433 nm peak. The CO difference spectra of fetal and neonatal preparations were observed to have an appreciable 420 nm peak, which indicated that either or both of these hemoproteins were present (Fig. 6). The occurrence of the EtCN peak at 429–430 nm would tend to implicate hemoglobin, as the maxima for cytochrome P-420 is closer to 433 nm.[19]

In order to rule out the effect of hemoglobin, EtCN spectra were remeasured on microsomal fractions which were prepared in a manner that would normally ensure that hemoglobin contamination was minimal. The livers of two fetal and two newborn pigs were perfused *in situ* with 1·15% KCl, removed, diced, weighed and a microsomal fraction was prepared. The microsomal fraction was carefully suspended and recentrifuged at 105,000 *g*. It was then "washed" a second time, and an EtCN spectrum was obtained at several pH values. A CO difference spectrum was obtained after each

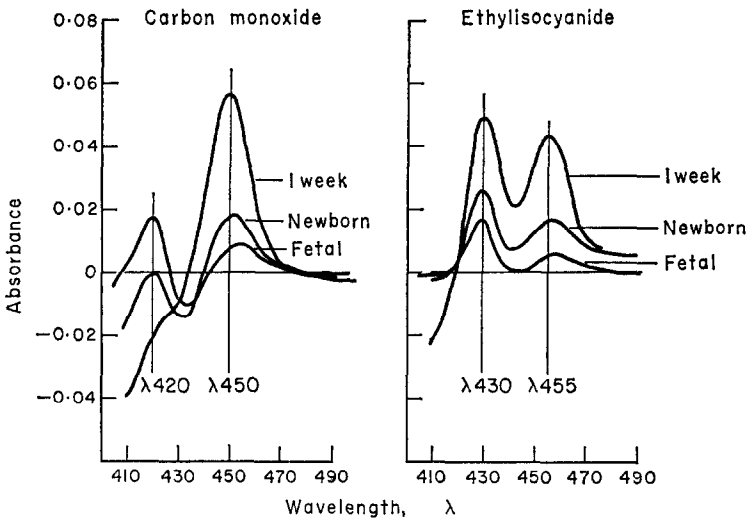

FIG. 6. Carbon monoxide (CO) and ethylisocyanide (EtCN) difference spectra of hepatic microsomal preparations of one representative fetal (107-days gestation), newborn and 1-week-old pig. The microsomal fraction was reduced with sodium dithionite and CO or EtCN was added to the sample cuvette as described in Methods.

hourly centrifugation. It was found that "washing" the microsomal preparation produced very little decrease in the magnitude of the 420 nm peak of the CO spectrum. Indeed, the CO and EtCN spectra were quite similar to those presented in Fig. 6. In addition, the pH-dependent ratio ΔA 430–490/ΔA 455–490 nm was altered only slightly. If hemoglobin was responsible for the 420 nm peak of the CO spectrum, it was not separable from the microsomal fraction by ultracentrifugation. The identity of the hemoprotein interfering with the EtCN spectrum of cytochrome P-450 remains unclear at the present time. The findings do, however, point out the necessity of obtaining microsomal preparations free of hemoglobin or cytochrome P-420 in order to obtain meaningful EtCN data on the whole microsomal fraction.

Figure 6 also depicts shifts in the maxima of the CO and EtCN difference spectra at 450 nm for the former and 455 nm for the latter in fetal and neonatal preparations. The dithionite difference spectra of CO-cytochrome P-450 (sample and reference cuvettes saturated with CO; sodium dithionite added to the sample cuvette), however, produced a maxima at 449–450 nm, indicating that there was no age-related change in the CO-cytochrome P-450 spectral peak.

DISCUSSION

Several investigators[2,5,27] have demonstrated marked postnatal increases in the cytochrome P-450 content of whole liver. Eling *et al.*,[6] however, found much less dramatic increases in the amount of cytochrome per milligram of microsomal protein in the rat from 5 days after birth to 1 month of age. Indeed, Gram *et al.*[7] found that the microsomal concentrations of cytochrome P-450, although tending to rise, changed only slightly in the rat from 1- to 12-weeks postpartum. In contrast to these findings, the specific content of this hemoprotein increased considerably after birth in the pig. This increase occurred in spite of a concomitant rise in protein levels.

The cytochrome b_5 content of the microsomal fraction is often found to be approximately one-half of the cytochrome P-450 levels in untreated adult rats. While this relative proportion was achieved shortly after birth, the ratio was reversed in fetal preparations. The possibility that a NADH-cytochrome b_5 contribution to substrate oxidation might be higher during fetal life than after birth was previously studied.[9] No additional contribution was found in fetal preparations.

The high level of NADPH-cytochrome *c* reductase in fetal pig liver is consistent with high levels of azo-reductase activity previously described,[2,9] as this enzyme has been implicated in the direct reduction of the azo-bond.[28] Dallner *et al.*[27] also found appreciable levels of this enzyme in the rat fetus 1 day before birth; however, adult levels had been reached by the first day postpartum. The finding that the rate of NADPH reduction of cytochrome P-450 per unit of cytochrome was much lower at 1 week prior to birth than at later ages is interesting. NADPH-cytochrome *c* reductase activity certainly does not limit the rate of cytochrome P-450 reduction at this time. There are several possible explanations for this observation which should be considered. The rate of cytochrome P-450 reduction may be rate limited by the presence of endogenous substrates such as steroids or fatty acids. Numerous investigators have demonstrated that substrates for the MFO system can influence the rate of reduction of cytochrome P-450. Secondly, an additional electron-transferring component, such as "microsomal Fe_x," interposed between NADPH-cytochrome *c* reductase and cytochrome P-450, may be deficient in the fetal microsomal preparation. In this connection, it is interesting to note that Dallner *et al.*[27] concluded that an "x" component (microsomal Fe_x?) was rate limiting to aminopyrine demethylation during the first postnatal week in the rat. A similar but more tenable possibility is that there is a deficiency in some lipid component of the endoplasmic reticulum associated with the structure and reducibility of the cytochrome. This contention becomes plausible in consideration of evidence for a permissive role for phospholipids (phosphatidylcholine)[29] in substrate hydroxylation and because the endoplasmic reticulum undergoes considerable structural change at birth.[30] Finally, there may be other alterations in the structure of cytochrome P-450 associated with birth, i.e. fetal porcine cytochrome P-450 may exist in a different iron spin state (or ratio of iron spin states) or may be characterized by other structural modifications which distinguish it from the adult form. Such differences could be reflected in the rates of reduction of the total hemoprotein. Evidence for such a change could not be obtained from CO or EtCN spectra of whole microsomal preparations because of interference from an unidentified hemoprotein. However, the fact that the dithionite difference spectrum of CO-cytochrome P-450 showed that there was no age-related change in the position of the Soret maxima argues against an age-related change in forms of cytochrome.

Gram *et al.*[7] reported marked changes in ethylmorphine *N*-demethylase activity (V_{max}) for rat liver microsomes from 1 to 6 weeks after birth. The pattern of activity was biphasic and did not relate to concentrations of cytochrome P-450. The K_m for the reaction increased and then decreased during this period. The V_{max} for another type 1 spectral substrate, (+)-benzyphetamine, was shown by Eling *et al.*[6] to increase with postnatal age in the rat, while the K_m did not change. Our data indicated that in in the pig the V_{max} for ethylmorphine demethylation increased markedly between the first and sixth postnatal week, while a small but progressive and significant ($P < 0{\cdot}05$) change occurred in the K_m. In addition, the V_{max} for demethylase activity correlated

well with increases in cytochrome P-450 content, NADPH-cytochrome *c* reductase activity and the rate of NADPH reduction of cytochrome P-450. The data suggested that there was little change in the relative amounts of activities of components of the MFO system after 1 week of age in the neonatal pig.

The major changes in the relative activities of NADPH-cytochrome *c* reductase and the rate of NADPH reduction of cytochrome P-450 occurred during the first 2 weeks of the study, with the greatest difference occurring between birth and day 107 of gestation. It should be noted, however, that the data do not indicate that the changes in relative activities were progressive during the last week of gestation, i.e. it may have been temporarily related more closely to the event of birth than was implied by data obtained at weekly intervals.

In summary, the results of this study indicated that there was little MFO activity with the exception of NADPH-cytochrome *c* reductase activity prior to birth in the pig. The specific activities of the parameters measured increased to a plateau between 4 and 6 weeks after birth. Except for a small increase in the K_m for ethylmorphine demethylation, there appeared to be only quantitative changes in MFO activity between 1 and 6 weeks of age. The relative rates of activity of the parameters measured were fairly constant during this period, and a rate-limiting step to ethylmorphine demethylation was not discernable. An age-related change in the relative activities of NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase occurred in the immediate perinatal period. The reducibility of cytochrome P-450 by NADPH-cytochrome *c* reductase in the fetal pig is currently under investigation.

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REFERENCES

1. J. R. FOUTS and R. H. ADAMSON, *Science, N.Y.* **129,** 897 (1959).
2. W. R. JONDORF, R. P. MAICKEL and B. B. BRODIE, *Biochem. Pharmac.* **1,** 352 (1959).
3. J. R. FOUTS, in *Perinatal Pharmacology. A Report of the Forty-First Ross Conference on Pediatric Research* (Ed. C. D. MAY), p. 54. Ross Labratories, Columbus, Ohio (1962).
4. R. KATO, P. VASSANELLI, G. FRONTINO and E. CHIESARA, *Biochem. Pharmac.* **13,** 1037 (1964).
5. R. KATO, *J. Biochem. Tokyo* **59,** 574 (1966).
6. T. E. ELING, R. D. HARBISON, B. A. BECKER and J. R. FOUTS, *Eur. J. Pharmac.* **11,** 101 (1970).
7. T. E. GRAM, A. M. GUARINO, D. H. SCHROEDER and J. R. GILLETTE, *Biochem. J.* **113,** 681 (1969).
8. C. R. SHORT and L. E. DAVIS, *J. Pharmac. exp. Ther.* **174,** 185 (1970).
9. C. R. SHORT, M. D. MAINES and B. A. WESTFALL, *Biol. Neonate* **21,** 54 (1972).
10. R. I. GLAZER, J. B. SCHENKMAN and A. C. SARTORELLI, *Molec. Pharmac.* **7,** 683 (1971).
11. B. S. S. MASTERS, J. BARON, W. F. TAYLOR, E. L. ISAACSON and J. LOSPALLUTO, *J. biol. Chem.* **246,** 441 (1971).
12. J. R. GILLETTE and T. E. GRAM, in *Microsomes and Drug Oxidations* (Eds. J. R. GILLETTE, A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS and G. J. MANNERING), p. 133. Academic Press, New York (1969).
13. J. B. SCHENKMAN and D. L. CINTI, *Biochem. Pharmac.* **19,** 2396 (1970).
14. J. B. SCHENKMAN, *Molec. Pharmac.* **8,** 178 (1972).
15. Y. HASHIMOTO, T. YAMANO and H. S. MASON, *J. biol. Chem.* **237,** PC 3843 (1962).
16. R. W. ESTABROOK and B. COHEN, in *Microsomes and Drug Oxidations* (Eds. J. R. GILLETTE, A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS and G. J. MANNERING), p. 95. Academic Press, New York (1969).
17. T. NASH, *Biochem. J.* **55,** 416 (1953).
18. P. L. GIGON, T. E. GRAM and J. R. GILLETTE, *Molec Pharmac.* **5,** 109 (1969).
19. Y. IMAI and R. SATO, *Biochem. biophys. Res. Commun.* **23,** 5 (1966).
20. H. L. JACKSON and B. C. MCKUSICK, *Organic Synthesis* (Ed. T. L. CAIRNS), p. 62. John Wiley, New York (1955).

21. O. H. Lowry, N. H. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
22. G. W. Snedecor and W. G. Cochran, *Statistical Methods* (6th Ed.), p. 147. Iowa State Univ. Press, Ames (1967).
23. N. E. Sladek and G. J. Mannering, *Biochem. biophys. Res. Commun.* **24,** 668 (1966).
24. G. J. Mannering, N. E. Sladek, C. J. Parli and D. W. Shoeman, in *Microsomes and Drug Oxidations* (Eds. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering), p. 303. Academic Press, New York (1969).
25. O. Pelkonen and N. T. Karki, *Proceedings of Fifth Int. Cong. of Pharmacology*, p. 179. International Union of Pharmacology, San Francisco (1972).
26. C. R. E. Jefcoate, R. L. Calabrese and J. L. Gaylor, *Molec. Pharmac.* **6,** 391 (1970).
27. G. Dallner, P. Siekevitz and G. E. Palade, *Biochem. biophys. Res. Commun.* **20,** 135 (1965).
28. P. H. Hermandez, J. R. Gillette and P. Mazel, *Biochem. Pharmac.* **16,** 1859 (1967).
29. A. Y. H. Lu, H. W. Strobel and M. J. Coon, *Molec. Pharmac.* **6,** 213 (1970).
30. G. Dallner, P. Siekevitz and G. E. Palade, *Biochem. biophys. Res. Commun.* **20,** 142 (1965).