STUDY OF THE DEVELOPMENTAL PATTERN OF HEME CATABOLISM IN LIVER AND THE EFFECTS OF COBALT ON CYTOCHROME P-450 AND THE RATE OF HEME OXIDATION DURING THE NEONATAL PERIOD*

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The present experiments were undertaken to study heme oxidation in neonatal liver cells with particular reference to the comparative development of this enzymic activity and of cytochrome P-450 mediated drug oxidation; and to examine the responses of both P-450 and microsomal heme oxidation activity to the trace metal cobalt.

Catabolism of heme to biliverdin involves the oxidative cleavage of the ring tetrapyrrole of heme by a microsomal enzyme system, to form the linear tetrapyrrole of the green pigment, biliverdin. The latter is subsequently reduced by a cytosol enzyme to form bilirubin, which then undergoes conjugation and excretion. It has recently been proposed (1) that in the microsomal oxidation of heme—a process known to require NADPH and molecular oxygen—cytochrome P-450 plays an essential role (2), comparable to that which this heme-protein plays in the mixed function oxidation of drugs and other foreign chemicals (3). However, apart from their mutual requirements for NADPH and molecular O_2, drug and heme oxidations differ significantly in a number of characteristics (1, 4) and these differences have raised doubts about the involvement in the latter process of cytochrome P-450 (5, 6). In recent studies from this laboratory, the striking ability of the trace metal, cobalt, to induce microsomal heme oxidation in liver cells was demonstrated, and in the course of this work, it proved possible to completely dissociate heme oxidation from cytochrome P-450 and P-450 dependent drug metabolism (7, 8). Thus, heme catabolism in liver cells is mediated by a non-P-450 dependent enzymic system distinct from that involved in drug transformation.

The dissociation of oxidative processes for heme and for drugs has been further investigated, in this report, in terms of the comparative developmental patterns of both activities during the postnatal period and the responses of P-450 and heme oxidation activity to cobalt. There are some data available concerning the pattern of development of hepatic drug metabolizing enzymes in neonates (9, 10), and information concerning the comparative development of heme catabolizing activity of liver cells and of the response of this enzymic activity to cobalt would be of considerable interest, in view of the inhibitory effect of the metal on heme synthesis (11) and the marked restriction on chemical induction of δ-aminolevulinate synthetase (ALAS),[1] the rate limiting enzyme of heme synthesis, which characterizes late gestation and early postnatal life (10, 12).

[1] Abbreviation used in this paper: ALAS, δ-aminolevulinate synthetase.

The results of these studies indicate that there are clearly separate developmental patterns of hepatic cell capacity to effect heme and drug oxidations in the postnatal period, thus emphasizing the distinctiveness of these microsomal enzyme activities and their regulatory factors. The response of the heme oxidation system in neonatal liver to induction by cobalt is also shown to be substantially different from that of the adult liver. Finally, data are provided on the rate of heme oxidation in neonates which supports the idea that postnatal jaundice results not only from immaturity of bilirubin conjugation mechanisms, but from hepatic overproduction of the pigment as well.

Materials and Methods

Materials. Pregnant Sprague-Dawley rats were purchased from Holtzman Co., Madison, Wis. at the 7th day of gestation. All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Heme solutions (hematin, iron protoporphyrin-IX) were prepared as described earlier (6). Cobalt-(ous) chloride was injected subcutaneously at 25 μmol/100 g body wt 24 h before sacrifice. In the case of embryos (-8 days) and fetuses (0 days, or 21 days gestation), only the mothers were injected with the metal.

Tissue Preparation. It was not possible to isolate sufficient amounts of hepatic microsomal fractions from embryos on the 13th day of gestation (-8 day, Fig. 1) so that at this stage of
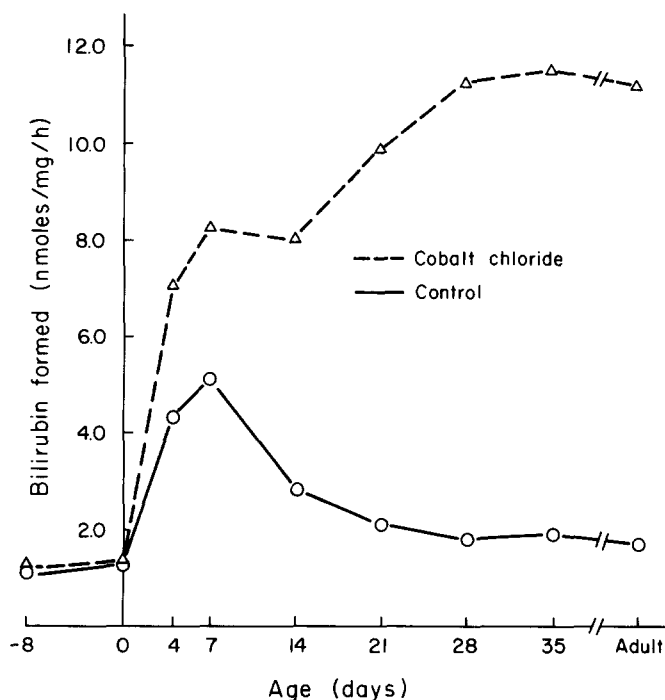

FIG. 1. Developmental pattern of hepatic heme oxidation in the rat and the effect of cobalt on this enzyme activity. Postnatal rats were injected subcutaneously with cobalt chloride (60 mg/kg) or saline (control), and the liver microsomal fractions were prepared 24 h after treatment by pooling livers from 4-8 rats. Heme oxidation activity was measured as described previously (8). The effect of cobalt on the prenatal rats was investigated by injecting pregnant females with cobalt as described above. The oxidative activity is expressed as the nanomoles of bilirubin formed per milligram of protein per hour. Each point represents the mean value of three experiments.

development "microsomal fractions" from whole embryos were prepared. However, in all other instances microsomal fractions from individual organs were used for the assays. Animals were decapitated, livers and spleens were removed, pooled and homogenized in potassium phosphate buffer 0.1 M, pH 7.4 and centrifuged at 9,000 g for 20 min. Thereafter, the microsomal fractions were prepared as described earlier (6, 13). The hepatic microsomal pellets were resuspended in buffer to a concentration of 9 mg/ml which was used in the heme oxygenase assay; and were further diluted to 4.5 mg/ml for the ethylmorphine N-demethylation assay. Splenic microsomal pellets were resuspended in buffer to a protein concentration of 4.5 mg/ml which was used in the heme oxidation assays. The 105,000 g supernatant fraction from adult rat livers was used as the source of the cytosol enzyme, biliverdin reductase (14). In this case the livers were perfused with 0.9% NaCl in situ and the microsomal fractions were prepared as before. The protein concentration of this fraction was adjusted to 10 mg/ml.

Spectral Studies. The hepatic microsomal content of cytochrome P-450 was measured as described by Omura and Sato (15) from the reduced minus CO-difference spectrum using sodium dithionite as the reducing agent. NADPH-cytochrome c reductase activity was measured as described by Williams and Kamin (16). The microsomal content of heme was determined by the pyridine hemochromogen method of Paul et al. (17). All spectral studies were carried out using an Aminco Chance DW-2 spectrophotometer (American Instrument Co., Inc., Silver Springs, Md.).

Protein was determined using the method of Lowry et al. (18). All experiments were conducted at least three times and the results are expressed as the average of these experiments $\pm$SD. The data were analyzed using the Student's t test and the value of $P < 0.05$ was considered as denoting significance.

Enzyme Assays. Hepatic and splenic heme oxidation activities were measured as described in detail previously (8) by incubating (37°C, 15 min) heme at a final concentration of 17 μM in a 3 ml incubation mixture containing 6-9 mg of hepatic microsomal protein or 4.5 mg splenic microsomal protein, 5 mg liver 105,000 g supernatant fraction (biliverdin reductase), MgCl$_2$ (2 mM), an NADPH generating system consisting of glucose-6-phosphate (0.85 mM), glucose-6-phosphate dehydrogenase (1.5 U), NADP (0.8 mM), and phosphate buffer, 0.09 M. The amount of bilirubin formed was measured using an extinction coefficient of 40 mM^{-1}cm^{-1} between 464-530 nm.

The activity of the hepatic mixed function oxidase system was monitored by measuring N-demethylation of the prototype drug, ethylmorphine, as described earlier (19); formaldehyde formed was determined by the method of Nash (20).

Results

The developmental pattern of heme oxidation, and the inducing effects of cobalt on this process, in embryos (-8 days); in fetuses at term (0 days), and at various times in postnatal life are depicted in Fig. 1. As noted, the data for embryos (-8 days) reflect whole body heme oxidation activity; all other data reflect this enzyme activity in liver alone. At the time periods (-8 days) and (0 days) heme oxidation activity following cobalt, represents enzyme activity in embryos or fetuses after the metal was administered to the maternal host. At all other points, the metal was administered directly to the neonate.

Heme oxidation in fetal liver (0 days) was maintained at a level approximately equivalent to that found in normal adult livers (8) and the same level of enzyme activity was found in the tissue preparations from whole embryos (-8 days). However, in the latter, there was a large contribution of whole embryo protein to the calculated levels of enzyme activity; this would thus be consistent with the findings (21) that at this stage of development the rate of heme oxidation in embryonic liver may be somewhat greater than in adult liver. In the immediate postnatal period, heme oxidation activity in liver increased rapidly, reaching levels between 4-7 days, which were 3-5 times higher than those in adults. This increase in the rate of hepatic heme catabolism returned towards normal within about 2 wk.

The effect of cobalt on this process was of interest. In the adult liver, cobalt exerts potent inducing action on heme oxidation increasing the level of this enzymatic activity between 7-10 times normal (7, 8); in the neonate this inducing activity of the metal was substantially less. When cobalt was administered to the maternal host at 21 days gestation the metal exerted no inducing action on heme oxidation in the fetus at all (0 days, Fig. 1); within the first postnatal week, metal inducibility of the enzyme commenced and reached levels 150-200% higher than control activity, while at 4-5 wk of age cobalt induced hepatic heme oxidation to the same extent (7-10 times control levels) as it did in adult animals.

The reason for the rapid and substantial increase in the rate of hepatic heme oxidation during the first week of postnatal life is not known with certainty. This enhanced enzyme activity could result from postpartum breakdown of erythroid elements in the liver and/or in the peripheral circulation with release of free hemoglobin which itself is known to be an inducer of hepatic heme oxygenase activity (22). However, while it is known that in the neonatal rat most of the circulating erythroid cells are reticulocytes, the specific kinetics of red cell turnover at this time of life have not been studied in detail; thus it remains unclear whether the high levels of hepatic heme oxidation found in neonatal livers do in fact reflect the inducing action of free hemoglobin derived from lysed erythrocytes or their precursors. Induction of hepatic heme oxidation in this period could also reflect (a) the action of circulating humoral agents which are known to alter heme metabolism (23); (b) the release of this enzyme system from repressed synthesis of enzyme components during gestation as occurs with ALAS (12); (c) nutritional changes associated with parturition (21) or combined effects of these or other (24) regulating processes on the system.

Fig. 2 depicts the comparative developmental patterns of the two major oxidative enzyme complexes localized in hepatic microsomes; the system for heme and the system for drugs such as the prototype substrate, ethylmorphine. As this figure makes clear, these enzyme systems exhibited dissimilar patterns of development, with the rate of drug metabolism being very low during the first 2-3 wk of postnatal life while the rate of heme oxidation at this time increased rapidly to levels which were substantially higher than those characteristic of the adult. Conversely, drug metabolism began to increase rapidly to adult levels after the 3rd week of life while heme oxidation activity diminished sharply during this period. It is of interest that the pattern of cytochrome P-450 development was also reciprocal to that of heme oxidation activity, with hepatic content of this heme-protein being low when the rate of heme catabolism is high in liver cells; and increasing (as does the rate of mixed function oxidation of drugs) in late postnatal life when the rate of heme catabolism diminishes. The distinctiveness of the microsomal oxidative systems for heme and for drugs is also reflected in the observation that the rate of heme catabolism in embryonic-fetal life is quite high, whereas in contrast drug oxidizing activity at this time is low.

The effects of cobalt on hepatic cytochrome P-450 and on total content of microsomal heme during postnatal development are depicted in Fig. 3. As shown, hepatic content of cytochrome P-450 increased after birth, somewhat more rapidly during the first postnatal week, and more gradually thereafter, until adult contents of the heme-protein in liver cells were reached. Cobalt exerted differential effects on the heme-protein during early and late postnatal stages of development.
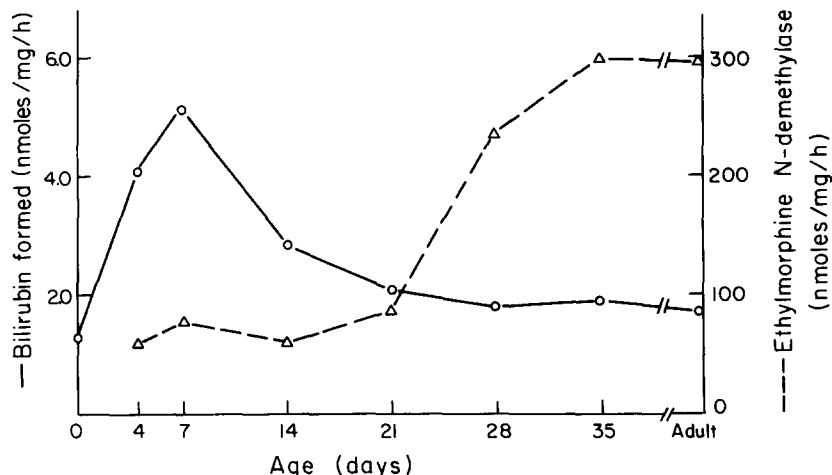
FIG. 2.   The comparative developmental patterns of the hepatic microsomal enzyme systems which oxidize drugs and which oxidize heme. Hepatic microsomal fractions were prepared by pooling livers from 4–8 rats. The developmental pattern of the drug metabolizing system was studied by measuring oxidative N-demethylation activity using ethylmorphine as the prototype substrate and the results are expressed as the nanomoles of product (p-amino-phenol) formed per milligram of protein per hour. The developmental pattern of the heme oxidation was assayed using hematin as the substrate and the enzyme activity is expressed as nanomoles of bilirubin formed per milligram of protein per hour. The procedures described in the Materials and Methods section were used for the analysis of these activities. Each point represents the mean value of three experiments.
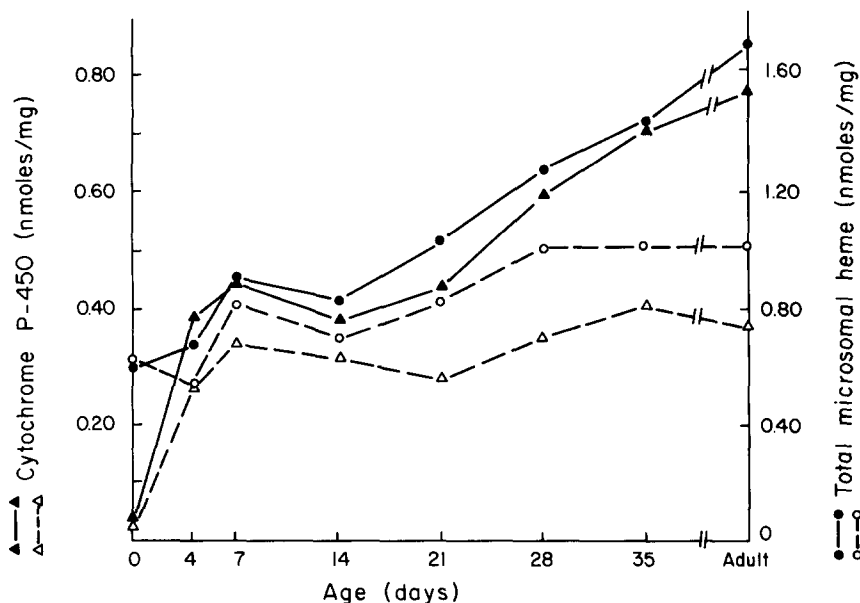

FIG. 3.   The effects of cobalt treatment on the microsomal contents of heme and cytochrome P-450 in the livers of developing neonates. Hepatic microsomal fractions were prepared from cobalt chloride (60 mg/kg) or saline treated postnatal rats and cytochrome P-450 was determined by the method of Omura and Sato (15); total microsomal heme was measured by the method of Paul el al. (17).

During the first 7–14 days of neonatal life cytochrome P-450 appeared somewhat refractory to the metal, although cobalt is known to substantially diminish hepatic concentrations of the heme-protein in adult liver (7, 8, 25). There is clear evidence that there is more than one species of the heme-protein collectively termed "P-450" and it is possible therefore that there is in the neonate a specific P-450 moiety having special resistance towards degradation by the activated heme oxidation system. The degradative effects exerted by cobalt on P-450 increased as maturation proceeded; and the total microsomal content of heme exhibited responses to the metal in early and late neonatal life similar to those of cytochrome P-450 (Fig. 3).

Table I depicts the effects of cobalt on hepatic heme oxidation and other microsomal enzyme activity in 4-day old control neonates; in 4-day old neonates

TABLE I

Microsomal Enzyme Activities in Neonatal Animals after Direct Cobalt Injection or Injection of the Metal into the Nursing Mother

Group	Treatment	Bilirubin formed	Ethylmorphine N-demethylase	Cytochrome P-450	Microsomal heme	TPNH-cytochrome c reductase
		$nmol/mg/h$	$nmol/mg/h$	$nmol/mg$	$nmol/mg$	$nmol/mg/min$
1	4-day old neonate; mothers injected with cobalt	4.48	83.5	0.354	0.614	69.4
2	4-day old neonates; cobalt injected	7.10*	42.5*	0.265*	0.519*	39.0
3	4-day old neonates; controls	4.31	59.2	0.386	0.673	54.1

The nursing mothers (group 1) or the suckling neonates (group 2) were treated with cobalt (60 mg/kg, subcutaneously). The hepatic microsomal fractions from the neonates were prepared from pooled livers (4–5) and various microsomal enzyme activities and components were measured as described in the Materials and Methods. All experiments were carried out three times and mean values are shown. Values in group 2 animals marked by an asterisk are significantly different ($P < 0.05$) from those of the group 1 and 3 animals.

exposed to the metal via maternal milk; and in 4-day old neonates receiving the metal by direct injection. In control neonates hepatic heme oxygenase activity was elevated above normal adult levels as expected; the ethylmorphine demethylation rate was low as was NADPH cytochrome c reductase activity and microsomal contents of P-450 and total heme. Direct injection of the neonate with cobalt produced a small increase in the rate of heme oxidation; a reciprocal change in drug metabolism; and decreases in P-450 and microsomal heme content as earlier observed in adults (7, 8). The change in NADPH-cytochrome c reductase activity was not statistically significant. In contrast, 4-day old neonates, suckling from mothers who had received comparable doses of cobalt, but who had not themselves been injected with the metal, displayed no significant alterations in the parameters measured, the data for the enzyme activities determined for the group 3 neonates not being significantly different from those for the group 1 neonates. These findings indicate that in nursing mothers cobalt is

not transported to the suckling neonate and supports the idea (8) that the active (i.e. heme oxygenase inducing) species of cobalt is in the ionic form rather than the metal in a complexed form as would be the case if cobalt were secreted into maternal milk.

Heme oxidation in the microsomal fraction from the spleen of neonates immediately after birth was somewhat lower than that found in adults but reached the adult range within the first 2 wk of postnatal life. Cobalt had no inducing effect on this enzyme activity in the spleen in neonates or in adults. Heme oxidation activity in the livers of pregnant animals at days 13 and 21 of gestation was not significantly different from this enzyme activity in the livers of nonpregnant animals. Cobalt induced heme oxidation activity in the livers of pregnant animals to approximately the same extent (7–10 times) as it did in controls; and comparable changes after cobalt treatment occurred in both groups with respect to the other enzymic parameters measured as well. Gonadectomy and adrenalectomy in nonpregnant animals and steroid hormone replacements, had no significant effects on heme oxidation activity of liver microsomes. NADPH-cytochrome c reductase activity increased gradually during the first 4 wk of postnatal life to adult levels. Moreover, the activity of this enzyme was not significantly altered at any time by cobalt treatment. The co-factor requirements of the heme oxidation system in the maternal host during gestation and in neonates were similar to those of control animals.

Discussion

The present study demonstrates that the developmental patterns of the microsomal oxidative systems for heme and for drug substrates in liver are significantly different. Microsomal heme oxidation proceeds at near-adult rates in late fetal life and undergoes, in the immediate postnatal period, an abrupt and substantial increase in activity before subsiding, after 14–21 days, to the levels of enzymic activity characterizing adult animals. In contrast, drug oxidation in hepatic microsomes, exemplified by the prototype substrate ethylmorphine, proceeds at a very low rate in late fetal and early neonatal life and only reaches adult levels of activity 4–5 wk after birth. Thus, at the time when drug oxidation in microsomes has reached its maximum level of activity, the rate of heme oxidation in liver has diminished to the low levels characteristic of mature animals. Microsomal contents of total heme and of cytochrome P-450 parallel more closely the developmental pattern of the drug oxidation, than the heme oxidation system in liver.

The reasons for the low levels of hepatic cytochrome P-450 in neonates are not known, although it is suggested that the refractoriness of hepatic ALAS to chemical induction during this period may account in part for this phenomenon. In the absence of increased ALAS activity, heme synthesis would be limited to some extent in liver cells and if heme catabolism were to proceed at higher than normal rates during the neonatal period, the contents of hepatic heme-proteins would be expected to be lower than those found in adults. The data presented in Figs. 1 and 3 are keeping with this view since they demonstrate a reciprocal relationship between the rate of heme oxidation activity and the microsomal

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contents of cytochrome P-450 and total heme. As development progresses, these parameters change in reverse fashion with a shift to a high level of cytochrome P-450 and heme occurring as the capacity of hepatic microsomes to oxidize heme diminishes. These reciprocal alterations suggest, despite earlier uncertainty concerning this question, that endogenous microsomal heme derived from the turnover of membrane bound heme-proteins does in fact serve as a substrate for microsomal heme oxygenase in the liver.

It should be noted that although ALAS is completely refractory to chemical induction in the immediate postnatal period its basal level of activity shortly after birth is relatively higher than that in adults; conversely later in development (i.e. 2–4 wk after birth) when basal activity of this liver enzyme is low, it becomes highly responsive to induction. Thus in the immediate postnatal period the higher than normal basal activity of hepatic ALAS appears to provide sufficient heme synthesis such that some increase in heme and heme-protein (cytochrome P-450) content in liver can take place despite the exaggerated rate of hepatic heme oxidation activity characterizing this period. In later postnatal life (i.e. 4–5 wk after birth) the newly developed capacity of ALAS to respond to chemical or "feed-back" induction can also provide enough heme to sustain P-450 dependent drug oxidation and to replace heme which is degraded normally or through the action of exogenous substances such as environmental chemicals, trace metals etc. In very early, as well as in late postnatal life therefore, spontaneously occurring or inducibly high-levels of ALAS activity can contribute to the equilibrium—reflected in the cytochrome P-450 content of microsomes—which is determined by the rate of heme degradation by heme oxidation, the turnover rates of microsomal heme-proteins and the rate of heme synthesis in liver cells.

The disparate effects of cobalt on the rates of oxidation of heme and of drug substances in hepatic microsomes suggested to us earlier (7, 8) that these reactions are catalyzed by separate microsomal enzymes. Differences in the characteristics of these enzymes and in their responses to chemical inducing agents had been noted by other investigators (26); and the dissociation of cytochrome P-450 from heme catabolism in both the liver (7, 8) and in the spleen systems (27) provided strong evidence that P-450 is not essential for the oxidation of heme, as it is for the mixed function oxidation of drugs and other foreign chemicals. The demonstration in this study that the microsomal enzymes which catalyze heme degradation and drug oxidation also undergo significantly different patterns of development in neonatal life supports our earlier conclusion concerning the distinctiveness of these enzymes and indicates that the components of the microsomal electron transport chain which mediate the oxidation of drugs are not identical to those involved in the catabolism of heme nor are they subject to the same control mechanisms.

The precise manner in which the developmental patterns of heme and drug oxidations are regulated in the neonatal period are not known. The microsomal enzymes which catalyze these oxidations belong to a larger category of hepatic enzymes which show increases in their specific activities during certain periods of postnatal life; these include uridine diphosphate glucuronyl transferase; tyrosine aminotransferase; serine dehydratase; acetyl coenzyme A carboxylase; fumarase;

and histidase, among others (see ref. 10). Hormonal factors have been implicated in the development of certain hepatic enzymes after birth, and the P-450 dependent microsomal mixed function oxidase complex represents one system whose pattern of development is dependent in part on sex-steroid influences.

The microsomal heme oxidation system differs in this respect from the enzyme complex mediating drug transformations, and in this study proved unresponsive to any major influences exerted by exogenously administered steroid hormones or those to which the maternal host and developing fetus were exposed during gestation. The sharp increase in heme oxidation activity characterizing the immediate postnatal period thus does not seem attributable to the abrupt alterations in the steroid hormonal milieu of the neonate associated with parturition. Whatever the basis for this large and transient increase in the rate of hepatic heme oxidation in liver it is evident that this process results in a substantial over-production of bilirubin during a period when enzymic conjugation mechanisms for this pigment in hepatic cells have not matured. Studies utilizing endogenous production of carbon monoxide as an index of heme catabolism (28) have also provided data indicating a level of bilirubin production in normal newborns which is substantially higher than that found in adults. Neonatal jaundice can thus be considered a consequence not only of delayed development of the glucuronidation system for bilirubin, but of the excessive production of this pigment by the heme oxidation system in hepatic, and perhaps other cells[2] as well.

It is reasonable to suggest therefore that therapeutic approaches to this problem may include consideration of means for regulating hepatic heme oxidation activity at a low rate in early neonatal life, in addition to those present attempts to induce, prematurely by drugs, the bilirubin glucuronidation system in the liver or to photo-oxidize circulating bilirubin to derivatives which are nontoxic for the nervous system.

Summary

The comparative development patterns of heme oxidation and of cytochrome P-450 dependent drug oxidation in rat liver were examined. High levels of heme oxygenase activity were present in whole embryo preparations at day 13 of gestation. At birth this enzyme activity in liver was approximately equal to that of normal adult liver. In the immediate postnatal period the rate of hepatic heme oxidation increased sharply, reaching levels 3-5 times normal during the first week postpartum. Thereafter, this enzyme activity progressively decreased and returned to normal adult levels by the 28th postpartum day. The development of microsomal heme oxidation and of P-450 dependent drug oxidation exhibited reciprocal patterns, with the latter being at low levels of activity during the immediate postnatal period and reaching adult activity only 4 or more wk after birth.

Cobalt injected into pregnant animals or into nursing mothers did not induce heme oxygenase in the fetus or suckling neonate. However, when treated directly

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[2] Maines, M. D., S. Sassa, and A. Kappas. 1975. Heme oxidation activity of cultured human skin fibroblasts. Manuscript in preparation.

with the metal, 4-day old neonates exhibited a small induction response of this enzyme; and the inducibility of heme oxygenase increased gradually to fully adult levels by the end of the 4th postpartum week. Cobalt at all postnatal developmental stages was capable of diminishing hepatic contents of total microsomal heme and P-450; however this effect of the metal was small in the immediate period after birth and increased progressively with maturation.

These findings demonstrate that the patterns of development of hepatic capacity for carrying out the oxidation of heme and the P-450 dependent oxidation of drugs are different and thus provide further evidence that these microsomal enzyme systems are distinct from each other and under separate regulatory mechanisms. The degree of induction response for hepatic heme oxygenase evoked by the trace metal, cobalt, was also shown to have developmental determinants as did the susceptibility of hepatic cytochrome P-450 to degradation by this metal. The very high levels of hepatic heme oxygenase activity which characterize neonates during the first week of life indicate that over-production of bilirubin contributes significantly to the mechanism of neonatal jaundice.

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