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# Heme Regulation of Cytochrome Oxidase Synthesis in Fetal Rat Liver

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SUMMARY

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The role of heme in regulation of the synthesis of cytochrome oxidase, a mitochondrial hemoprotein, was studied in fetal rat liver. A functional association between heme levels and the synthesis of cytochrome oxidase *in vivo* is suggested by the rapid decline in the rate of incorporation of [$^3$H]δ-aminolevulinic acid and, subsequently, [$^{14}$C]leucine into cytochrome oxidase following selective inhibition of heme biosynthesis with $CoCl_2$. Both the functional activity and the rate of [$^{14}$C]leucine incorporation into fetal cytochrome oxidase are stimulated when heme is administered 30 min after $CoCl_2$ is given. In contrast, heme does not stimulate [$^{14}$C]leucine incorporation or enhance the functional activity of cytochrome oxidase following selective inhibition of cytoribosomal protein synthesis with cycloheximide. Thus it is suggested that heme stimulates the synthesis of apocytochrome oxidase *de novo* and mediates the formation of the functional cytochrome in fetal rat liver.

## INTRODUCTION

A functional association between heme biosynthesis and the biogenesis of various proteins in mammalian tissues has been suggested by numerous investigators in recent years. Heme is postulated to act as a physiological regulator of globin synthesis in mammalian erythrocytes (1), and to be essential for initiation of the synthesis of other proteins in reticulocytes (2-4), tumor cells (5), and other tissues (6, 7). A direct relationship between heme synthesis and mitochondrial biogenesis in adult mammalian liver is suggested by the studies of Beattie (8), which demonstrated that induction of δ-aminolevulinic acid synthetase, the rate-limiting enzyme in the heme biosynthetic pathway (9), leads to a substantial elevation of the levels of cytochromes and other protein components of these organelles.

Little is known of the role of heme as a regulator of physiological processes in fetal mammalian liver. However, previous studies from this laboratory (10-12) have demonstrated that both heme biosynthesis, as measured by the levels of heme biosynthetic pathway enzymes, and the biogenesis of mitochondrial proteins proceed at substantially higher rates in fetal than in adult liver. In addition, heme may facilitate the incorporation of ALA[1] synthetase into the mitochondrial structure in fetal liver (13). These findings suggest that a functional association between heme biosynthesis and mitochondrial biogenesis may exist. Further indication of such an association is suggested by the rapid decline in the rate of mitochondrial biogenesis, as measured by the rate of [$^{14}$C]leucine

[1] The abbreviation used is: ALA, δ-aminolevulinic acid.

incorporation into mitochondrial proteins, which is observed following selective inhibition of heme biosynthesis in fetal liver by treatment with specific inhibitors of heme biosynthetic pathway enzymes (11). These observations suggest that heme may play a physiological role in the regulation of the biosynthesis of mitochondrial proteins during fetal hepatic development, and it is interesting to speculate that this function may be mediated through regulation of the synthesis of hemoproteins, which are essential components of the mitochondrial structure.

In the present studies the role of heme in regulation of the synthesis and functional activation of cytochrome oxidase, a hemoprotein which constitutes an integral portion of the mitochondrial inner membrane, was studied in fetal rat liver. The effects of heme on the stimulation of the synthesis of apocytochrome oxidase *de novo* were assessed in pulse-labeling studies following inhibition of fetal heme biosynthesis *in vivo*, and mediation by heme of the functional activation of cytochrome oxidase was investigated in the fetus after treatment with agents which specifically alter heme or protein synthesis in mammalian cells. In addition, the effects of prenatal exposure to hematotoxic agents on the postnatal development of mitochondrial hemoprotein function is discussed in light of the current findings.

## MATERIALS AND METHODS

*Materials.* Cytochrome *c*, L-ascorbic acid, 3-amino-1,2,4-triazole, cycloheximide, and Triton X-114 were obtained from Sigma Chemical Company. Glutathione and heme (hemin) were purchased from Calbiochem. Cobaltous chloride and *N*,*N*,*N*′,*N*′ - tetramethyl - *p* - phenylenediamine dihydrochloride were obtained from K & K Laboratories. [$^{14}$C]Leucine (0.1 mCi/ml) and [$^{3}$H]ALA (1 mCi/ml) were purchased from New England Nuclear Corporation. Other chemicals were of reagent grade and were obtained from standard commercial sources.

*Treatment of animals.* Sprague-Dawley rats (CD strain) were date-bred by Charles River Laboratories and were utilized on the 18th day after breeding date unless otherwise indicated. Pregnant animals were housed in individual cages and were allowed food and water ad libitum. Anhydrous $CoCl_2$ was dissolved in distilled water to a final concentration of 50 mg/ml and was administered by subcutaneous injection to pregnant rats at a dose of 50 mg/kg. Crystalline hemin was dissolved in a small volume of 0.01 M NaOH and was adjusted to pH 7.0 with 0.1 M potassium phosphate buffer to a final concentration of 20 mg/ml; hemin was then administered to pregnant rats by intravenous injection at a dose of 20 mg/kg. Other drugs were administered intraperitoneally. Cycloheximide was dissolved in 50% ethanol and given at 50 mg/kg. Aminotriazole was dissolved in distilled water and administered at 3 g/kg. All drugs were given within 6 hr after preparation. In pulse-labeling studies isotopes were diluted to the appropriate specific activities with 0.9% NaCl and were administered to pregnant rats by intraperitoneal injection. At least four adult animals or litters were utilized for each experimental point.

*Preparation of tissues.* All animals were killed by decapitation. Livers of adult and fetal rats were rapidly exised, washed, weighed, and homogenized at 4° in 9 volumes of 0.25 M sucrose containing 0.05 M Tris-HCl buffer, pH 7.5, using a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Mitochondria were prepared as previously described (13). The final washed pellet was suspended in 0.2 M potassium phosphate buffer, pH 7.0, at a final protein concentration of 25 mg/ml.

In pulse-labeling studies, amino acid incorporation into mitochondrial proteins was determined after dissolving an aliquot of the mitochondrial protein in a minimum volume of 0.4 M NaOH. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (model 3375) with appropriate corrections for quenching. Measurements were made in glass counting vials containing 20 ml of 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene counting solution. Protein concentrations were determined by the method of Lowry *et al.* (14), using bovine

serum albumin (fraction V) as a standard.

ALA, a much more specific precursor of heme biosynthesis than is glycine, was utilized in these studies in order to ensure maximal incorporation of radioactivity into mitochondrial hemes and to permit differentiation between radioactivity incorporated into hemes and into proteins in double-labeling studies. Since the activities of other enzymes of the heme biosynthetic pathway are highly elevated in comparison with that of ALA synthetase (12, 13) in fetal liver, it was assumed that little advantage would be gained in utilizing labeled glycine in lieu of ALA as a heme presursor.

*Incorporation of [$^3$H]ALA into mitochondrial hemes in vivo.* The incorporation of [$^3$H]ALA into hemes of mitochondria and cytochrome oxidase was determined by a modification of the method of Rieske (15). In this case the mitochondrial pellet or the membranous preparation of cytochrome oxidase was homogenized successively with acetone, chloroform–methanol (2:1, v/v), and acetone. The supernatant solution remaining after centrifugation at 9000 rpm for 15 min was discarded, and the pellet was extracted three times, 15 min each, with 10 volumes of acetone–HCl (0.9 ml of concentrated HCl per 100 ml of acetone). The combined acetone–HCl extracts were then evaporated to dryness under vacuum. The residue was immediately dissolved in 1 ml of pyridine, and an aliquot was removed for counting in a liquid scintillation spectrometer using 10 ml of Biofluor (New England Nuclear) as counting solution. The heme content was determined by the pyridine-hemochromogen method as described by Rieske (15). The entire procedure was performed in the dark at 0–4°.

*Isolation of cytochrome oxidase.* A membranous preparation of cytochrome oxidase was prepared from mitochondrial fractions by a modification of the method described by Jacobs *et al.* (16). Mitochondrial suspensions containing 25 mg of protein per milliliter and 0.003 ml of 20% Triton X-114 per milligram of mitochondrial protein were stirred at 0° for 1 hr. The solution was then centrifuged at 39,000 rpm for 1 hr, and the resulting red supernatant solution was decanted. The greenish pellet, containing all of the cytochrome oxidase activity, was carefully removed from the remaining sedimented material, resuspended in 0.2 M phosphate buffer, pH 7.0, and recentrifuged for 1 hr. The final pellet was then dissolved in phosphate buffer so as to contain approximately 30 mg of protein per milliliter and was assayed for cytochrome *c*-oxidizing activity. An aliquot was dissolved in a small volume of 0.4 M NaOH and counted in a liquid scintillation spectrometer for determination of radioactivity resulting from incorporation of labeled precursors *in vivo,* as described for mitochondrial proteins.

*Assay of cytochrome oxidase activity.* Cytochrome oxidase activity was assessed polarographically, as described by Schnaitman *et al.* (17), by measuring oxygen consumption with a Clark electrode. Reaction mixtures contained 75 mM potassium phosphate buffer, pH 7.2, 0.03 mM cytochrome *c*, 3.75 mM sodium ascorbate, 0.3 mM tetramethyl-*p*-phenylenediamine, and 0.3 mg of protein in a total volume of 1 ml. Reactions were conducted at 30°.

*Assay of ALA synthetase activity.* Mitochondrial ALA synthetase activity was measured by a modification of the method of Scholnick *et al.* (18), as previously described (13). Reaction mixtures contained approximately 4 mg of mitochondrial protein per milliliter.

## RESULTS

In previous studies from this laboratory (12, 19), it was demonstrated that levels of heme biosynthetic pathway enzymes in fetal liver are substantially elevated in comparison with those seen in the adult. These elevated enzyme activities appear, moreover, to be associated with fetal hepatic parenchymal cells rather than with declining erythropoietic cell populations, and therefore are considered to characterize hepatic rather than erythropoietic cell development (13). Thus the activity of ALA synthetase is 6–8 times that of the adult, whereas the level of ferrochelatase, the final enzyme in this pathway, is at least 4

times adult levels. The activities of ALA dehydratase and uroporphyrinogen I synthetase are also substantially elevated in fetal liver. These observations suggest that heme may be synthesized more rapidly in the fetus than in the adult, perhaps in response to greater physiological demands for heme during fetal development. To substantiate this probability, the time course of incorporation of a single pulse dose of [$^3$H]ALA, a heme precursor, into mitochondrial hemes was measured in adult and fetal liver (Fig. 1). The maximal rate of incorporation of [$^3$H]ALA into mitochondrial hemes in the fetus occurred within 10 min following pulse-labeling of the mother. Subsequently a rapid decrease in the rate of labeling of mitochondrial hemes was observed, suggesting a rapid turnover rate of heme in fetal liver. A second labeled peak appeared within 1 hr after pulse-labeling; the origin of this peak is currently unknown, but may represent the synthesis of a second, distinct heme pool in fetal liver. In contrast, the incorporation of [$^3$H]ALA into mitochondrial hemes in the adult liver progressed much more gradually, reaching a maximum 25–30 min following injection of the labeled substrate.

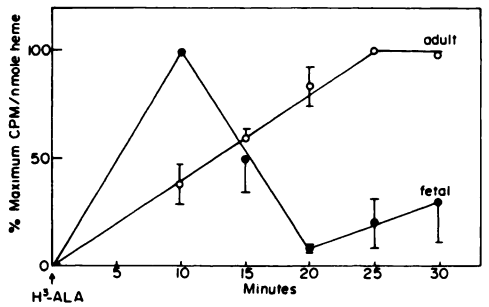


FIG. 1. *Time course of incorporation of [$^3$H]ALA into mitochondrial hemes of adult and fetal rat liver*

Pregnant rats (five per time point) were injected with [$^3$H]ALA (60 $\mu$Ci) at zero time. Animals were killed at various time intervals thereafter, and the radioactivity present in extracted mitochondrial hemes was determined. Maximal specific activities were 3359 and 3214 cpm/nmole of heme in adults and fetuses, respectively. Values in this and subsequent figures represent the means ± standard errors (where given) of at least six experiments. Livers of four adults or four litters of fetuses were pooled for each experimental value.

The rapid turnover rate of heme observed in fetal liver suggests that the heme pool is highly labile in this organ during gestation and indicates further that heme levels may be capable of rapid fluctuations in response to changing demands for heme during development.

That an elevated rate of heme biosynthesis may be associated with the development of mitochondrial function in the fetus is suggested by several experimental observations. A close correlation is seen between temporal changes in the rates of heme biosynthesis, as reflected in ALA synthetase activity, and mitochondrial biogenesis, as measured by [$^{14}$C]leucine incorporation, during perinatal development (Fig. 2). The most rapid decrease in the rate of mitochondrial protein synthesis follows the beginning of the decline in ALA synthetase activity by approximately 1 day, suggesting that declining heme levels may trigger a decrease in the rate of mitochondrial protein synthesis during perinatal development. A functional association between heme levels and mitochondrial protein synthesis in the fetus is even more

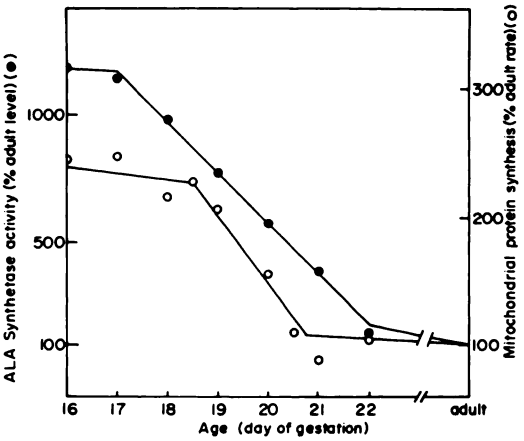


FIG. 2. *Decline in ALA synthetase activity compared with rate of mitochondrial protein synthesis in perinatal rat liver*

Pregnant rats were given pulse doses of [$^{14}$C]leucine (20 $\mu$Ci) 15 min prior to sacrifice. Hepatic ALA synthetase and mitochondrial protein synthesis were assayed as described in MATERIALS AND METHODS. Adult levels of ALA synthetase and mitochondrial protein synthesis were 0.50 nmol of ALA per milligram of protein per hour and 750 cpm/mg of protein, respectively.

strongly supported by the rapid decline in the rate of synthesis of mitochondrial proteins which is observed when heme biosynthesis is inhibited (Table 1). In these experiments the rate of [$^{14}$C]leucine incorporation into mitochondrial proteins in the fetus was inhibited by more than 75% of control levels 30 min following selective inhibition of heme synthesis with either aminotriazole, a selective inhibitor of ALA dehydratase (13, 20), or $CoCl_2$, an inhibitor of ferrochelatase (21), in mammalian liver. As indicated in the case of $CoCl_2$, however, these effects were partially reversible by concomitant or subsequent administration of heme. In contrast, cycloheximide, an inhibitor of the synthesis of those mitochondrial proteins which are synthesized in the cytoplasmic ribosomes in mammalian cells (22), also produced profound inhibition of the incorporation of [$^{14}$C]leucine into fetal mitochondrial proteins. This effect, however, was not prevented by concomitant heme administration.

In adults, on the other hand, less than 20% decrease in the rate of mitochondrial protein synthesis was observed following inhibition of heme synthesis, and this effect was not reversed by concomitant heme treatment. Cycloheximide produced effects in the adult similar to those observed in the fetus. In preliminary experiments aminotriazole was shown to produce 40–50% inhibition of ALA dehydratase in both adult and fetal liver. $CoCl_2$ produced 30% and 60% inhibition of ferrochelatase in the adult and fetus, respectively, within 2 hr after injection. No general inhibitory effect of these agents on protein synthesis per se was observed. Hemin given alone to untreated pregnant animals did not significantly alter the incorporation of [$^{14}$C]leucine into either adult or fetal mitochondrial proteins.

TABLE 1

*Effects of specific inhibitors of heme and protein synthesis on [$^{14}$C]leucine incorporation into mitochondrial proteins of adult and fetal rat liver*

Mitochondria were prepared as described under MATERIALS AND METHODS. Animals were killed 30 min after treatment with the specified drug and 15 min after a pulse dose of [$^{14}$C]leucine (20 $\mu$Ci). Values represent the means ± standard errors of at least four experiments.

| Treatment | [$^{14}$C]Leucine incorporation | |
|---|---|---|
| | Adult | Fetus |
| | *cpm/mg protein* | |
| None (control) | 746 ± 99 | 2131 ± 160 |
| Aminotriazole (3 g/kg) | 596 ± 160 | 473 ± 62 |
| $CoCl_2$ (50 mg/kg) | 619 ± 28 | 319 ± 65 |
| $CoCl_2$ (50 mg/kg) + hemin (20 mg/kg) | 607 ± 42 | 639 ± 30 |
| Cycloheximide (50 mg/kg) | 75 ± 15 | 297 ± 86 |
| Cycloheximide (50 mg/kg) + hemin (20 mg/kg) | 86 ± 31 | 290 ± 72 |

The rapid turnover of fetal mitochondrial heme, coupled with the profound inhibition of incorporation of [$^{14}$C]leucine into mitochondrial protein following inhibition of heme synthesis, suggests that heme is tightly coupled to the regulation of mitochondrial biogenesis in fetal liver. A reasonable mechanism through which heme might act in this process is through regulating the synthesis and/or assembly of hemoproteins, such as cytochrome oxidase, which constitute functional portions of the mitochondrial structure. A schematic representation of the formation of cytochrome oxidase is presented in Fig. 3. Numerous studies regarding the physical and chemical nature of cytochrome oxidase from various sources have appeared in recent years (23–26). A molecule of cytochrome oxidase has been defined as a monomeric, heme $aa_3$-containing enzyme which catalyzes the oxidation of ferrocytochrome *c*. The enzyme complex is considered to contain six or seven distinct polypeptide subunits (25), the catalytic subunits containing 1 heme and 1 copper atom. Estimates of the molecular weights of various preparations of cytochrome oxidase isolated from different sources range from approximately 100,000 to 500,000 (24). In its most basic form cytochrome oxidase may be considered to consist of a prosthetic heme moiety, synthesized in the mitochondria, and an apoprotein moiety, which is synthesized partially within existing mitochondria and partially on cytoplasmic ribosomes (23). In the present studies it was of interest to determine whether heme regulates the synthesis of cytochrome oxidase through stimulation of the synthesis of the

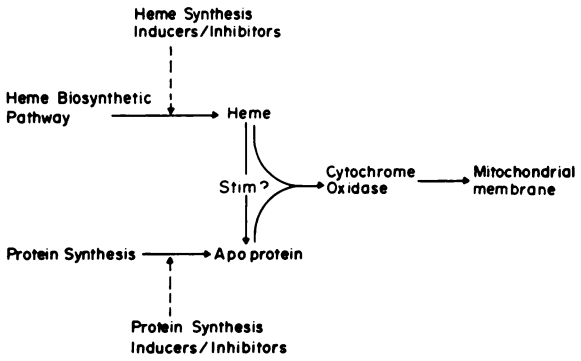


FIG. 3. *Schematic representation of biosynthesis of cytochrome oxidase*

apocytochrome, resulting in the formation of a functional hemoprotein in the mitochondria.

As an initial approach to the assessment of the relationship of heme to the synthesis of cytochrome oxidase in fetal liver, the effect of inhibition of heme biosynthesis on the incorporation of heme and protein precursors into cytochrome oxidase *in vivo* was determined (Fig. 4). A rapid decline in the rate of incorporation of [$^3$H]ALA into the heme moiety of cytochrome oxidase occurred within a few minutes following treatment with $CoCl_2$. Subsequently a delayed decrease in the rate of [$^{14}$C]leucine incorporation into cytochrome protein was observed, dropping to less than 25% of zero-time values within 30 min following inhibition of heme synthesis. These results suggest that synthesis of apocytochrome oxidase is inhibited when heme levels decrease below those observed prior to administration of the inhibitor.

In order to demonstrate more directly the effects of heme on the stimulation of apocytochrome oxidase synthesis in fetal liver, pulse-labeling experiments were performed following selective inhibition of heme synthesis; in these studies the effects of heme on the incorporation of radioactivity into cytochrome oxidase *in vivo* were observed when heme was subsequently administered to the fetus by intravenous injection of the $CoCl_2$-treated mother. These experiments were based on the rationale that if heme is required for synthesis of the apoprotein, inhibition of heme synthesis would terminate synthesis of the apoprotein (as suggested in Fig. 4); subsequent

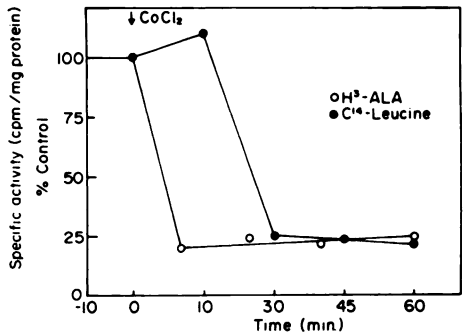


FIG. 4. *Effects of $CoCl_2$ on time course of incorporation of precursors into cytochrome oxidase in fetal rat liver in vivo*

Pregnant rats were injected with $CoCl_2$ (50 mg/kg) at zero time. Pulse doses of [$^3$H]ALA (60 $\mu$Ci) and [$^{14}$C]leucine (20 $\mu$Ci) were administered intraperitoneally 15 min prior to sacrifice. Cytochrome oxidase was isolated from fetal liver and was further treated for analysis of radioactivity incorporated into the heme and protein moieties as described in MATERIALS AND METHODS. Control (100%) values for [$^{14}$C]leucine and [$^3$H]ALA were 718 and 3473 cpm/mg of protein, respectively.

administration of heme would then stimulate synthesis *de novo* of new apocytochrome oxidase, detectable by an increase in both the functional activity and the rate of incorporation of [$^{14}$C]leucine into the hemoprotein. The results presented in Fig. 5 tend to substantiate this hypothesis.

In these experiments pregnant rats were treated with $CoCl_2$ at zero time to inhibit heme synthesis, and were killed at 15-min intervals up to 1 hr thereafter. Pulse doses of [$^{14}$C]leucine were administered 15 min prior to death. Cytochrome oxidase was isolated from fetal livers at each time point

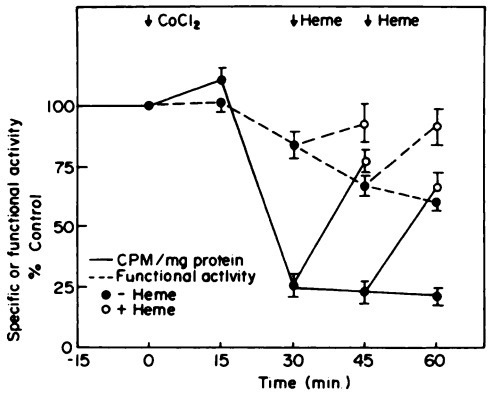


FIG. 5. *Effects of $CoCl_2$ and hemin on rate of synthesis and activity of mitochondrial cytochrome oxidase in fetal rat liver*

Pregnant rats were treated with $CoCl_2$ (50 mg/kg) at zero time and with heme (20 mg/kg) at the times indicated. Pulse doses of [$^{14}$C]leucine (20 $\mu$Ci) were given concomitantly with hemin, by intraperitoneal injection 15 min prior to sacrifice. Cytochrome oxidase was isolated from fetal liver mitochondria at each time point and analyzed for oxidizing activity and specific radioactivity. At zero time cytochrome *c*-oxidizing activity was 1.72 ± 0.22 $\mu$g atoms of $O_2$ per milligram of protein per minute. Radioactivity was 684 ± 68 cpm/mg of protein.

and analyzed for both cytochrome *c*-oxidizing activity and specific radioactivity. As shown in Fig. 5, the rate of incorporation of [$^{14}$C]leucine into the protein of cytochrome oxidase was inhibited by 70–75% of zero-time levels 30 min following inhibition of heme biosynthesis with $CoCl_2$. In addition, the functional activity of cytochrome oxidase declined to 75% of control values. When heme was administered at this point, however, the rate of incorporation of [$^{14}$C]leucine into new apocytochrome oxidase was dramatically stimulated, with specific radioactivity levels increasing by as much as 200% of levels in the absence of heme, within 15 min after the heme was administered. An increase in the functional activity of the isolated cytochrome oxidase was also observed.

Similar results occurred if heme was administered at later time points following inhibition of heme synthesis. If pulse doses of [$^{14}$C]leucine were administered at the time of inhibition of heme synthesis (zero time), however, less than a 10% increase in radioactivity was detected in the cytochrome oxidase which was isolated 15 min after heme was administered. Thus synthesis of the apocytochrome decreases rapidly when heme synthesis is inhibited, and the apoprotein does not accumulate in the absence of heme.

In another experiment heme was ineffective in stimulating synthesis of apocytochrome oxidase *de novo* or in affecting the functional activity of the cytochrome following inhibition of cytoribosomal protein synthesis with cycloheximide (Fig. 6). These results indicate that heme acts primarily at the level of cytoribosomal protein synthesis to effect biosynthesis of apocytochrome oxidase. The increase in specific cytochrome *c*-oxidizing activity observed in this experiment may thus be explained in terms of the greater magnitude of inhibition of protein synthesis induced by cycloheximide, as compared with that resulting indirectly from $CoCl_2$ administration. The more rapid onset of the decline in the functional activity of cytochrome oxidase following $CoCl_2$ treatment (Fig. 5), as compared with the effects seen

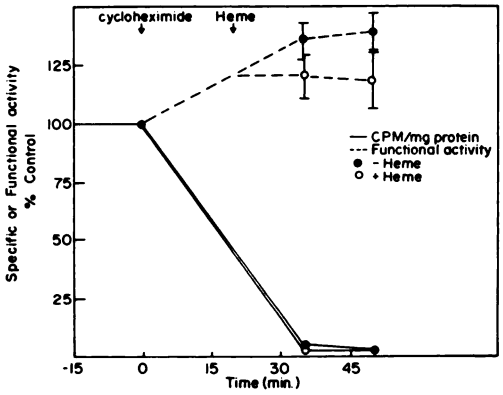


FIG. 6. *Effects of cycloheximide and hemin on rate of synthesis and activity of cytochrome oxidase in fetal rat liver*

Pregnant rats were treated with cycloheximide (50 mg/kg) at zero time and with heme (20 mg/kg) 20 min later. Pulse doses of [$^{14}$C]leucine (20 $\mu$Ci) were given by intraperitoneal injection 15 min prior to sacrifice. Cytochrome oxidase was isolated from fetal liver mitochondria at each time point and analyzed for oxidizing activity and specific radioactivity. At zero time cytochrome *c*-oxidizing activity was 2.13 ± 0.16 $\mu$g atoms of $O_2$ per milligram of protein per minute. Radioactivity was 554 ± 25 cpm/mg of protein.

after cycloheximide injection (Fig. 6), suggests that heme not only stimulates the synthesis of apocytochrome oxidase but mediates the functional activation of the enzyme complex as well.

## DISCUSSION

Although considerable information exists in regard to the regulation of hepatic heme biosynthesis in both adult (27–31) and perinatal (13, 32–35) mammalian liver, little is known of the mechanisms by which heme is utilized in the regulation or synthesis of hemoproteins in this organ at either stage of development. Knowledge of such processes in fetal mammalian liver is of particular importance for understanding the mechanisms by which drugs and other environmental agents affect the postnatal development of hemoprotein function. The results of the present study suggest that heme plays a regulatory role in the synthesis of cytochrome oxidase in fetal rat liver cells, both by stimulating synthesis of the apoprotein and in effecting functional activation of the enzyme complex in the mitochondria.

These results might then explain the observed effects of alteration of heme synthesis on the over-all rate of mitochondrial biogenesis in fetal liver (11). Since cytochrome oxidase forms an integral portion of the mitochondrial inner membrane, alteration in the rate of synthesis of this hemoprotein would be expected to lead to subsequent modification of the biosynthesis of the mitochondrial membrane per se. The present studies suggest that heme levels may control the rate of synthesis of mitochondrial membranous components via regulation of the synthesis of hemoproteins, such as cytochrome oxidase, which make up the mitochondrial structure. Whether this control is exercised primarily over the synthesis of protein components of the enzyme complex which are synthesized within mitochondria or over those components of the apoprotein synthesized on the cytoplasmic ribosomes, or both, is not currently known. However, the previous demonstration (13) that heme may facilitate the incorporation of ALA synthetase into the mitochondrial structure, subsequent to its synthesis in the endoplasmic reticulum in fetal liver, suggests that a similar process may occur in regard to the association of cytochrome oxidase in mitochondria in the fetus. These results thus suggest that the synthesis of cytochrome oxidase occurs as a fundamental process in mitochondrial membrane formation during fetal hepatic development.

The mechanism by which heme exerts regulatory control of the synthesis of proteins such as cytochrome oxidase is not currently understood; it is not known, therefore, whether heme is rate-limiting in the biosynthesis of other hemoproteins in fetal mammalian liver. Although a direct action of heme at the site of apocytochrome oxidase synthesis could be hypothesized from the results of these studies, it may be that heme mediates this effect through the action of a translational repressor which, in turn, controls the rate of protein synthesis (1, 5, 7). This type of mechanism would suggest a rather universal control by heme of protein synthesis in mammalian cells. On the other hand, Correia and Meyer (36) have recently suggested that the apoprotein may be the rate-limiting factor in the formation of microsomal cytochrome P-450 in adult rat liver. This conclusion was partially based on the observation that administration of heme precursors failed to increase the biosynthesis of cytochrome P-450, whereas inducers of protein synthesis increased the amount of available apoprotein under conditions that inhibited heme synthesis. This situation, however, does not appear to be the case with regard to cytochrome oxidase synthesis in the fetus. Administration of neither heme nor heme precursors increases the rate of biosynthesis of cytochrome oxidase or the incorporation of [$^{14}$C]leucine into mitochondrial proteins. However, the elevated levels of heme biosynthetic pathway enzymes observed in the fetus, and the refractoriness of ALA synthetase to induction or to other regulatory mechanisms known to be present in the adult (13, 32–34), suggest that under steady-state conditions heme biosynthesis normally progresses at maximal rates in

fetal liver. Thus the hypothesis that a functional correlation exists between heme synthesis and the synthesis of mitochondrial proteins in fetal liver would dictate that mitochondrial protein synthesis also progresses at a maximal rate in the fetus. Administration of heme precursors, therefore would be expected to be ineffective in increasing the levels of cytochrome oxidase or other proteins in the fetus, and this, in fact, is what is observed. Moreover, the observation that inhibition of heme biosynthesis results in a dramatic decrease in cytochrome oxidase synthesis, and therefore of mitochondrial protein synthesis, and that concomitant administration of heme partially prevents this process, further supports the contention that the biosynthesis of cytochrome oxidase is dependent on the action of heme in the fetal liver. In this context it is interesting that cycloheximide treatment does not inhibit incorporation of [$^3$H]ALA into the heme of cytochrome oxidase in the fetus until substantial inhibition of the synthesis of ALA synthetase occurs.[2] Thus the biosynthesis of heme does not appear to be dependent upon the presence of the apoprotein, at least in the case of cytochrome oxidase.

The rapid turnover of the early peak of labeled heme observed in these studies in fetal but not in adult liver suggests the presence of a unique heme pool which is capable of responding quickly to demands for heme during hemoprotein formation in rapidly developing fetal liver cells. Such a hypothesis is consistent with the concept of a pool of "free" or "unassigned" heme, which acts as the prosthetic group in the synthesis of various hemoproteins or in governing the rate of over-all mitochondrial protein synthesis (37, 38). In this regard, the refractoriness of fetal ALA synthetase to regulation by mechanisms which restrict heme biosynthesis in the adult (13, 34) and the elevated levels of heme biosynthetic pathway enzymes in the fetus may represent a gestational adaptation to the requirement for greater demands for heme during fetal development.

[2] Unpublished observations.

A rate-limiting requirement for heme in the biosynthesis and functional activation of cytochrome oxidase in the fetus has obvious implications in regard to assessment of the affects of prenatal inhibition of heme biosynthesis on the postnatal development of hepatic mitochondrial hemoprotein function. In previous studies (19) it was demonstrated that administration of a single dose of aminotriazole or $CoCl_2$ to pregnant rats during the 18th to 20th days of gestation results in delayed or impaired development of cytochrome oxidase activity during the immediate postnatal period. Although recovery from these effects occurs during the period of perinatal development, such animals may be more susceptible than controls to the adverse effects of drugs or environmental agents such as various trace metals (39), which selectively impair mitochondrial function in mammals. These results may have even greater implications in regard to the susceptibility of individuals who undergo chronic exposure to hematotoxic agents during gestation.

In conclusion, a rate-limiting role for heme in the biosynthesis of cytochrome oxidase in fetal liver cells is indicated by the results of these studies. Whether heme plays a similar role in the stimulation of the synthesis of microsomal cytochromes or other hemoproteins in the fetus remains to be determined. It is apparent, however, that agents which alter hepatic heme synthesis and/or its utilization during gestation may result in delayed or impaired postnatal development of mitochondrial hemoprotein function, and may thereby enhance the susceptibility of the organism to the toxic effects of drugs and other environmental agents during the postnatal period.

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