INTRODUCTION

There are variety of proteins and enzymes taking part in various physiological functions of living organisms. These functions include (1) carriage and transfer of oxygen within and among the cells e.g., hemoglobin and myoglobin; (2) the protection of cells against toxic oxidizing agents (catalase, peroxidase); (3) the orderly and efficient transfer of electron with attendant synthesis of ATP (mitochondrial cytochromes), and (4) microsomal metabolism of fatty acids, steroids and xenobiotics (microsomal cytochromes). All these proteins or enzymes are collectively called heme proteins because of their common possession of heme as the functional prosthetic group, without it all of them will be rendered nonfunctional. The functions in which these heme-proteins involve themselves are a few of the essential functions for the survival of an organism. Thus, the coordination of heme and apoprotein synthesis is essential in the formation of biologically active hemeprotein.
Heme is produced in mammals by a multienzyme catalysed reaction called 'Heme Biosynthetic Pathway'.

HEME BIOSYNTHETIC PATHWAY: A BIRD’S EYE VIEW

In bacteria, yeast and higher vertebrates, the first precursor of heme, i.e., δ-aminolevulinate (ALA) is synthesized by ALA synthetase which condenses a molecule of succinyl-CoA with one molecule of glycine. ALA thus formed, now leaves mitochondria and upon coming out in the cytoplasm, two molecules of ALA are condensed to form porphobilinogen (PBG), the monopyrrole precursor of the porphyrins. This reaction is carried out by an enzyme ALA dehydratase. The next step in the conversion of PBG into the next metabolite is executed by two enzymes acting in concert. These two enzymes are uroporphyrinogen I synthetase and Uroporphyrinogen III cosynthetase. The former enzyme acting alone catalyzes a head-to-tail polymerization of four molecules of PBG to form uroporphyrinogen I which is converted finally to uroporphyrinogen III by the enzyme uroporphyrinogen III cosynthetase. Then uroporphyrinogen III is converted into coproporphyrinogen III by the enzyme uroporphyrinogen decarboxylase which decarboxylates the four acetic acid-side chains of uroporphyrinogen III in stepwise manner with the seven to five carboxyl containing intermediates appearing in the reaction. The conversion of coproporphyrinogen III into protoporphyrinogen IX involves the
oxidative decarboxylation of 2 propionic side chains. The oxidative removal of six hydrogen atoms from protoporphyrinogen IX results in the formation of protoporphyrin IX, the immediate precursor of heme. This step is carried out by coproporphyrinogen oxidase which is a mitochondrial enzyme. Into this protoporphyrin IX, now, an enzyme heme synthetase $^{+2}$ incorporates a Fe thus giving rise to heme. Heme, thus formed in the mitochondria, gets distributed within the cell among various cellular proteins depending on the concentrations and affinities of these proteins for heme.

In mammals and other vertebrates in the case of excess synthesis of heme, an endoplasmic membrane bound enzyme - heme oxygenase is induced which degrades heme into biliverdin. Biliverdin so formed is then finally degraded to bilirubin by the action of pyridine nucleotide dependent biliverdin reductase.

FORMATION OF AMINOLEVULINIC ACID BY DIFFERENT ROUTES

In many multienzyme systems, generally, the end product of the pathway act as a specific inhibitor of an enzyme at or near beginning of the sequence of reaction. This is determined by the steady state concentration of the end product. Since the synthesis of 6-aminolevulinic acid is the first step of heme biosynthesis, the ALA formation has been considered as crucial factor.
(a) **ALA formation by ALA synthetase:**

Labelling experiments of Shemin and his associates (1,2) during mid-1950s have led to the recognition that ALA is the first precursor of heme biosynthetic pathway and that glycine and succinate are the substrates for this reaction. ALA is formed by the condensation of succinyl CoA and glycine.

Subsequent studies (3,4) have supported the original suggestion of Shemin and others (5-7) that Succinyl CoA is used in the synthesis of ALA. And later work of Granick (8) and Kikuchi et al. (9) have demonstrated conclusively that succinyl CoA is the substrate that condenses with glycine.

ALA synthetase activity has now been reported in various species (9-13) and has also been purified from various sources (14-17).

(b) **Alanine: DOYA transaminase as an alternate route for ALA synthesis:**

The unitary nature of the biosynthetic pathway of a variety of tetrapyrroles in plants and animals was first suggested by Hoppe-Seyler's comparison of light absorption by heme and chlorophyll and also by early finding that some degradative products of chlorophyll resemble heme derivatives. But it was in 1950, that the biosynthetic unity of porphyrins and chlorophylls was shown by Granick (18) who found in a Chlorella mutant with
defective chlorophyll synthesis, a high accumulation of protoporphyrin. Aminolevulinate is shown as the first committed precursor of the biosynthesis of heme and chlorophyll.

Despite the fact that ALA is the precursor of chlorophyll, the presence of ALA synthetase has not so far been demonstrated in any greening plant tissue. While the absence of ALA synthetase in plant was posing an intriguing question to the workers of this field, there was another line of experiment going on in Shemin's laboratory back in 1950s. They demonstrated that ALA could be converted into a product other than porphyrins, namely, purine (19-21). They postulated that α-aminolevulinic acid might be deaminated to 4,5-dioxovalerate which upon losing the terminal carbonyl group would regenerate succinate and one-carbon fragment capable of undergoing further reactions in the biosynthesis of purines. The proposition of the succinate-glycine cycle and the confirmation of it (22) set the stage for a new way of thinking towards the formation of ALA in plants. It was Gibson et al. (23) and Neuberger and Turner (24) in the early 1960s who first showed, while working with a partially purified system from *Rhodopseudomonas spheroides*, the 'reverse reaction' of the 'Succinate-Glycine Cycle', where DOVA was transaminated giving rise to ALA. Feeble support came from the work of Beale and Castelfranco (25) who showed that in greening etiolated leaves and cotyledons, glutamate, α-ketoglutarate can serve as
the precursor of ALA. Later on Schlossberg et al. (26) have shown that α-ketoglutarate and glyoxalate reacts through a step-wise decarboxylation reaction yielding 4-oxo-5-hydroxyvalerate which can be finally oxidised to 4,5-dioxovalerate by the enzyme 4-oxo-5-hydroxyvalerate dehydrogenase. However, even before the actual confirmation of the enzymatic synthesis of 4,5-dioxo- valerate, Lohr and Friedmann (27) have reported the formation of ALA from α-ketoglutarate via 4,5-dioxovalerate in maize extracts by an enzyme alanine: DOVA transaminase. Alanine: DOVA transaminase which catalyses the reaction between alanine and DOVA yielding ALA and pyruvic acid has also been identified in extracts of Zea mays (7) and Chlorella (28).

The principle of biological conservancy combined with the experimental observations has raised a question whether ALA is synthesized from 5-C precursor in the organisms other than plants.

Recently, an additional biosynthetic pathway of ALA has been reported in bovine liver by Varticovski et al. (29) and Noguchi and Mori (30) and in rat liver (31) suggesting the possibility that this enzyme may play a role in the biosynthesis of ALA in vivo. Demonstration of the incorporation of radioactive 4,5-dioxovalerate in the heme in respiring rat hepatocytes (32) on one hand and discovery of the enzyme 4-oxo-5-hydroxyvalerate dehydrogenase in mammalian liver and kidney (33) on the other,
have provided convincing evidence of the physiological importance of alanine:DOVA transaminase in hemebiosynthesis in mammals. However, the regulatory function of this enzyme in hemebiosynthesis still remains to be clarified.

(c) **RNA involves in ALA formation:**

It is an established fact that in plants and algae, most and possibly all of ALA is synthesized directly from glutamate via 5-carbon pathway. Recent reports (34) have indicated the involvement of RNA as a direct participant in the reaction sequence forming ALA from glutarate since the formation of ALA from glutarate catalyzed by a soluble extract from the unicellular algae *Chlorella vulgaris* was abolished after incubation of cell extract with bovine pancreatin RNase. Activity could be reconstituted with *C. vulgaris* fraction containing tRNA, but not tRNA from various other sources. Successful reconstitution was also reported in the systems from barley (35) and Chlamydomonas (36). Though the involvement of low molecular weight RNA in an enzymatic reaction other than the peptide bond formation is unusual, it is not unique. A 2.5s RNA containing 31 nucleotides has been reported to be associated covalently with the 1,4-glucan branching enzyme isolated from rabbit muscle (37). Other example of reported RNA influence on enzyme catalyzed reactions include rabbit skeletal muscle phosphofructokinase (38), potato O-diophenol oxidase (39) and a
sea urchin egg-enzyme catalysing thiol disulfide exchange (40). The mechanistic role of the RNA in the reactions carried out by these enzyme have not been elucidated. Weinstein and Beale (34) suggested the function of RNA in ALA forming system as an intermediate acceptor of α-carboxyl activated glutamate in a manner analogous to its role in peptide-bound formation. The mechanistic roles of RNA involvement in reactions carried out by these enzymes is still to be worked out.

CELLULAR CONTROLS ON THE HEME BIOSYNTHETIC PATHWAY

Several studies in recent years have brought to light the fact that heme is one of the major factors involved in several regulatory mechanisms in the cell. Thus, the regulation of heme biosynthesis at different levels has been worked out with great interests. In a multienzyme pathway it will not be too far from the truth to speculate that any form of disorder in the gene coding for any of the enzymes involved in pathway will drastically alter it. However, it does not necessarily mean that under normal physiological conditions all the enzymes of the pathway will be regulatory in nature. Usually, only a few enzymes of the pathway possesses the key regulatory functions.

Heme biosynthetic pathway has some of its enzymes in the mitochondrial matrix and others in the cytoplasm. This suggests that besides end product inhibition or repression, typical of
multienzyme pathways, there can be other regulatory mechanisms operating such as: by compartmentation or by differential permeability of mitochondrial membranes for key intermediates of the sequence.

(a) ALA synthetase as the key regulatory enzyme:

The primary site of control of heme biosynthesis has been shown to be at the level of formation of ALA, the first committed precursor of heme (41,42). It is reported that heme biosynthesis is regulated by ALA metabolism in different systems, e.g., plant cells (43), erythrocytes (44) and liver (45).

The enzyme ALA synthetase is present in liver mitochondria under normal conditions at very low level (46). But various genetic defects known as ‘acute intermittent porphyria’, characterized biochemically with excess excretion of porphyrins and its precursors, is associated with increase in ALA synthetase activity. Over the past few decades a host of information on the inducibility of ALA synthetase by variety of compounds has been accumulating. It helped in delineating the key regulatory role of ALA synthetase in heme biosynthesis in mammalian (12,47) and chick embryo liver (46). The well known inducers of ALA synthetase are the porphyrogenic drugs, e.g., allylisopropylacetamide (AlA), 3,5-dicarbethoxy 1,4-dihydrocollidine (DDC), stimulator of cytochrome P-450, e.g., phenobarbitone (48,49) and 20 methylcholanthrene (50,51), steroids (12,46) and ethanol (52)
and other xenobiotic compounds (53). Granick in 1966 (47) suggested that the mechanism by which these drugs increased the activity of ALA synthetase may be by interfering in some way with the feedback control of heme. Another important observation that indicated the regulatory role of ALA synthetase was that an increased supply of ALA increases, both \textit{in vitro} and \textit{in vivo} the liver synthesis of porphyrins and heme (47, 54-57), while an increased supply of the precursors of ALA does not. This proved that ALA synthetase is a rate limiting enzyme of the pathway. Basing on these information, the regulation of heme biosynthesis by ALA synthetase in conjunction with heme, the end product of the pathway has been extensively studied. Burnham and Lascelles (58) have provided the evidence of feedback inhibition of ALA synthetase by heme in \textit{R. sphaeroides}. In this prokaryote, inhibition of ALA synthetase by hemin is affected by a concentration as low as \(10^{-7}\) M. This inhibition is due to the formation of a hemichrome with the enzyme (59). Heme at the concentration of \(5 \times 10^{-4}\) M can inhibit the activity of partially purified rat liver ALA synthetase (60). Granick and Sassa (12) pointed out that the concentrations of hemin employed are abnormally high and the ability of these concentrations of heme to be reached by the mitochondria has not been demonstrated. Endogenous generation of heme at a level of which is 75 times the physiological concentration also could not bring about any substantial inhibition of ALA synthetase in intact rat liver
mitochondria (61), thus raising questions about its relevance in the regulation of heme biosynthesis. However, this cannot exclude the possibility of a short term regulation of ALA synthetase by means of feedback inhibition by heme.

Although the physiological role of heme in the end product inhibition of ALA synthetase is yet to be clarified, its role as the end product repressor of ALA synthetase is very clear. Metalloporphyrins at concentrations of 0.5 - 5 μg/ml of medium inhibit porphyrin formation (47) in hepatocyte cultures through a mechanism that does not involve inhibition of preformed ALA synthetase activity. Lascelles (62) has also suggested that ALA synthetase formation in R. spheroides is repressed by exogenous ALA consequent to the formation of heme. Sassa and Granick (63) have shown that 5 x 10^-6 M hemin does not affect the total protein or total RNA synthesis in chick embryo liver cells nor does it alter the rate of DNA synthesis. They concluded that hemin inhibits the synthesis of ALA synthetase at the translational level. These observations are supported by the report of Strand et al (64). Besides, the end product inhibition and translational inhibition of ALA synthetase by hemin, a possibility of a regulation at the transcriptional level was suggested by Whiting (65) and Srivastava (66). Later, Yamamoto et al (67) demonstrated that hemin, indeed, inhibits the transcription of the gene coding for ALA synthetase. Kikuchi and
co-workers (68-70) and Masayaki et al. (71) have shown that there is yet another mechanism of regulation of ALA synthetase by heme. Hemin at low concentration appears to act at the transcriptional level to prevent induction of ALA synthetase, while at higher concentration it acts at the post-transcriptional level. ALA synthetase is shown to be synthesized on cytoplasmic ribosomes as a precursor in both rat (70,72) and chick (73,74) liver and subsequently processed to a mature mitochondrial matrix enzyme. It has been shown that hemin inhibits the drug-mediated induction of mitochondrial ALA synthetase. Later, Kikuchi and co-workers (70,72) in rat liver and Srivastava et al. (75) in chick embryo liver showed that hemin injection caused an accumulation in the liver cytosol of precursor form of ALA synthetase thus leading to the decrease in the activity of the mitochondrial ALA synthetase. Considering all the above reports, it can be concluded that the activity of heme biosynthesis appears to be subject to feedback regulation by heme at the step of ALA synthetase possibly at three sites: inhibition of synthesis of the enzyme at both transcriptional and post-transcriptional steps, and inhibition of the enzyme transfer from cytosol into mitochondria. Recently, Yamamoto et al. (71) demonstrated the inhibitory role of hemin of in vitro translocation of chicken liver ALA synthetase into mitochondria. They were able to demonstrate that ALA synthetase, synthesized in reticulocyte lysate cell free translational system, was imported into mitochondria and concomitantly
converted to the size of mature enzyme when incubated with isolated mitochondria. Furthermore, import of the precursor enzyme was blocked by the addition of hemin to the in vitro transfer system, and failure to bring about inhibition of transfer of another matrix enzyme, pre-pyruvate carboxylase, into mitochondria (75) eliminated the general toxic effect of hemin on mitochondrial import of protein which implies that hemin specifically inhibits the transfer of pre-ALA synthetase.

(b) Regulation of heme biosynthesis: Role of heme oxygenase and porphobilinogen deaminase

Regulation of heme biosynthesis is also governed by the dual action of two key enzymes, heme oxygenase and PBG deaminase in addition to ALA synthetase, which are involved in heme synthesis and degradation. Recent studies have shown the concomitant induction of heme oxygenase not only by its substrate hemin (76,77) but also with numerous endogenous (78-80) and exogenous substrates, e.g., some heavy metals (81,82) and the drugs decreasing cytochrome P-450 content (83). Although ALA synthetase is the main regulator of heme biosynthesis in non-erythroid tissue like liver, the control of heme biosynthesis in erythroid cells appears to be under a different control mechanism. In the prenatal liver which is predominantly erythropoietic, heme has been shown not to repress ALA synthetase (84).
It has been shown that hepatic and erythroid ALA synthetase exhibit different responses to hypoxia, polycythemia, erythropoietin and porphyrogenic chemicals (85). Beru and Goldwasser (86) have shown that differentiating marrow cells in culture under erythropoietin treatment showed no change in the activity of ALA synthetase and alanine:DOVA transaminase. However, they observed that porphobilinogen deaminase activity and the rate of heme synthesis were increased several fold whereas the enzyme heme synthetase remained unaltered. Further, it was observed that induction of heme synthetase as well as porphobilinogen synthetase were dose dependent. From these studies, it was concluded porphobilinogen deaminase may be the primary enzyme that controls heme biosynthesis in erythroid cells in culture.

(c) The concept of 'Regulatory Heme Pool'

Heme is now known to regulate its own synthesis, exerting control at different stages. As it is already indicated that heme serves as a feedback regulator of heme biosynthesis, acting primarily at the step of ALA synthetase. Usually, under normal physiological conditions the biosynthetic pathway is very efficiently regulated and little waste of the intermediates occur. This regulation is done by a fine balance existing between the rate of ALA synthetase and the level of intracellular heme called 'Regulatory Heme Pool'. The concept of 'Regulatory
heme pool' is a result of several conjugate observations on the relationship between variation of intracellular heme concentration and its effect on ALA synthetase. The concentration of 'regulatory heme' has been indirectly estimated to be $10^{-7} - 10^{-8}$ M under normal conditions. Since direct determination of regulatory pool is technically not feasible, Badaway et al. (87) presented evidence suggesting that tryptophan pyrrolase might be a very sensitive marker to assess delicate changes in liver heme concentration since the degree of saturation of this enzyme varied considerably under various conditions, for instance, it increased significantly after the injection of heme (87) whereas it decreased with administration of porphyrogenic drugs (88). It has been shown in a number of experiments that a single dose of AIA brings about an approximately reciprocal relation between the levels of ALA synthetase activity and cytochrome P-450 (89). DDC, another known inducer of ALA synthetase although is structurally unrelated to AIA, can cause destruction of cellular cytochrome P-450. Lim et al. (90) showed that induction of ALA synthetase is dependent upon the depletion of cellular heme which is brought about by porphyrogenic drugs. Their studies suggest that induction of porphyria is a combination of two distinct processes: (a) induction of ALA synthetase synthesis by destruction of cytochrome P-450 heme and consequent depletion of cellular free heme, and (b) maintenance of continued ALA synthetase by preventing the replenishment of cellular heme.
either by inhibiting heme synthesis or by promoting continuous removal of newly synthesized heme. Further, an indirect proof came from the work of Marver et al. (91) on tryptophan pyrrolase, an enzyme which has heme as the prosthetic group. Upon injection of L-tryptophan in rats, tryptophan pyrrolase is induced and more of heme gets bound to the enzyme which results in less availability of intracellular heme which in turn induced ALA synthetase. In support of the hypothesis of heme mediated controls of ALA synthetase Srivastava et al. (92) showed that incubation of chick hepatocytes with exogenous hemin in order to induce heme oxygenase and deferoxamine methanesulphonate to inhibit heme synthetase, lead to induction of ALA synthetase by approximately 23-fold. This has been further confirmed that the continued presence of chemical inducer is not required after initiation of induction provided that the cellular heme synthesis is prevented. Another line of evidence came from clinical research on porphyrias. It has been frequently seen that, in porphyric patients there is excess production of the intermediates of heme biosynthesis and ALA synthetase is also induced (93,94). But upon therapeutic injection of hemin to these patients there is rapid and dramatic decline of ALA in serum (95) indicating that the hemin administered may exert a negative feedback regulation on the level of ALA synthetase.
(d) Cytochrome P-450 and regulation of heme biosynthesis:

The cytochrome P-450 belongs to a family of heme-containing proteins that are involved in drug metabolism, manifesting inducer specificity. The different species of cytochrome P-450 are induced by a variety of endogenous (96) and exogenous substrates including drugs like phenobarbitone (97,98), polycyclic aromatic hydrocarbon: 20-methylcholanthrene (50,51). The biosynthesis of cytochrome P-450 is coupled to the intracellular concentration of heme and thereby directly involved with the biosynthesis of heme. It is believed that cytochrome P-450 heme perhaps plays an important role in the feedback regulation of ALA synthetase since it has been supported by the observation that destruction of cytochrome P-450 heme brought about by AIA is attendant with the induction of ALA synthetase. In the case of AIA injection, it was observed that heme moiety of cytochrome P-450 was specifically destroyed (99,100), while leaving the apoprotein form of cytochrome P-450 intact. Therefore, under these conditions it can be envisaged that a transitory phase exists during which the level of apocytochrome P-450 is present at a high level which finally elicits an induction of ALA synthetase synthesis. These observations generate the model where heme is considered to be the feedback repressor molecule and it has gained much popularity among the workers of this field. However, Padmanaban and co-workers (101,102) suggested that perhaps regulation of ALA synthetase involves a positive control. The
newly synthesized heme would saturate the apo-cytochrome protein and holoprotein will have no effect on ALA synthetase. There are direct or indirect evidences which support this positive regulation mechanism. Phenobarbitone injection enhances the rate of transcription of cytochrome P-450 coding sequence (103, 104). It has been shown that phenobarbitone increases the efficiency of the translation of cytochrome P-450 mRNA (105).

From the literature available till date, it may be concluded that regulation of heme biosynthesis is at the different enzyme levels, namely ALA synthetase, heme oxygenase and PBG deaminase. From all points of view, the formation of ALA is considered as one of the prime regulatory factor in heme biosynthesis. In the last few years, the contribution of alanine: DOVA transaminase as an alternate route of ALA synthesis is accepted, which poses the intriguing question as to which is the major route for control of heme biosynthesis in mammalian system.

As mentioned earlier the aim of this work is to study the regulatory role of alanine: DOVA transaminase on heme biosynthesis.