SUMMARY AND CONCLUSIONS
Virtually all cells in higher animals are capable of synthesizing heme to meet their obligatory needs for cellular respiration and other biological functions. It is well established that the production of universal precursor δ-aminolevulinic acid is the first committed step of porphyrin biosynthesis, leading to the formation of heme and other tetrapyrroles. It has long been known that the conventional pathway of heme synthesis in animal system is initiated by the rate-limiting enzyme, ALA synthetase which is under tight regulation by the end-product heme. Until recently, most of the investigations on ALA production in animal tissues have focused attention on the regulation of heme biosynthesis by ALA synthetase. However, in higher plants, the absence of ALA synthetase and the presence of ALA as the obligatory precursor in biosynthesis of tetrapyrroles required the alternatives to be considered. Consequently, in a number of other studies on ALA generation in bacterial, plants and animal system, involvement of L-alanine: DOVA transaminase has been suggested to be physiologically relevant. Though some effort has gone towards determining the function of L-alanine: DOVA transaminase and the potential of the reaction it catalyses in ALA production, its several avenues still remain unexplored. Moreover, there are now reports which show that in mammalian system only hemin among all the heme biosynthetic pathway intermediates appear to inhibit L-alanine: DOVA transaminase both in vivo and in vitro. This led to the assumption that the enzyme is under feedback
regulation by the intracellular heme. However, the data in the literature is insufficient to establish conclusively the hypothesis that L-alanine: DOVA transaminase has a regulatory function in heme biosynthesis. Therefore, for a thorough treatment of this topic it was of interest to identify and explore other site(s) of regulation and establish conclusively the physiological significance of this alternate pathway of heme/ALA biosynthesis. It is important to mention here that the process of mitochondrial translocation of ALA synthetase has been found to be inhibited by exogenous hemin added to the in vitro transfer system and this is now believed to be a primary site of regulation of heme biosynthesis at the level of ALA synthetase. Therefore, in order to broaden our understanding of heme biosynthesis and its overall regulation, a similar study has been conducted to elucidate if there is a control mechanism at the level of mitochondrial translocation of L-alanine: DOVA transaminase as well. Accordingly, the present investigation has been divided into three chapters and the results and conclusions obtained for each chapter are summarised below:

I. Subcellular localization of L-alanine: DOVA transaminase by several investigators has already confirmed that the enzyme is present in the mitochondria of mammalian system. For our purpose experiments were undertaken to identify the exact intramitochondrial location of this enzyme. The bulk
of the enzyme L-alanine: DOVA transaminase which catalyses the transamination reaction between L-alanine and 4,5-dioxovalerate to synthesize ALA was predominantly recovered as a soluble component of the mitochondrial matrix. Subfractionation procedure of the mitochondria involved the use of digitonin and lubrol followed by differential centrifugation to separate soluble and particulate enzymes. Lubrol, which is reported to inhibit some of the mitochondrial enzymes including succinate dehydrogenase, does not inhibit L-alanine: DOVA transaminase. Presence of this enzyme in the mitochondrial matrix has been further confirmed by western blot analysis of the minimally intercontaminated mitochondrial sub-fractions. The digitonin and lubrol concentration of 1.6 mg/10 mg mitochondrial protein was found to be optimum for isolating all the four components of the rat kidney mitochondria. The observations made support the conclusion that the mammalian enzyme L-alanine: DOVA transaminase is localized and functions in the mitochondrial matrix.

The enzymes involved in the biosynthesis of heme are distributed in several sub-cellular compartments, with ALA being produced in the mitochondrial matrix. Therefore, the location of L-alanine: DOVA transaminase in the mitochondrial matrix along with the ALA synthetase is consistent with the suggested role of this enzyme in ALA production.
The enzyme L-alanine: DOVA transaminase when assayed at different temperatures showed an optimal activity at 60°C retaining more than 90% of the maximal activity through a temperature range of 55-65°C. When compared the enzyme showed a nearly 3-fold increase in its activity at 60°C than at 37°C. We confirmed that the reaction product ALA was a result of the enzymatic reaction and not a consequence of high temperature reaction. Modifications in the assay procedure have been made to eliminate the conversion of DOVA and ALA into other known reaction products. This amplified assay method has proved effective not only with other mammalian tissues but also with protozoan Entamoeba histolytica where we could stimulate the enzyme activity by 8-9 fold. We have made use of this assay system during the purification of L-alanine: DOVA transaminase from rat kidney mitochondria achieving a significant lowering of the enzyme loss (resulting in better enzyme yield) during the chromatographic fraction assays.

We also report for the first time, the occurrence of L-alanine: DOVA transaminase in mitochondria lacking protozoan Entamoeba histolytica. The occurrence of L-alanine: DOVA transaminase in all the green plants, animals and micro-organisms tested so far, and the absence of ALA synthetase in green plants, and most micro-organisms appears to reflect the evolutionary
significance of this pathway. It is probable that the evolutionary antecedents of modern day plants and animals produced ALA mainly via L-alanine: DOVA transaminase until an urgency for another pathway (primarily by animals) to meet the excessive requirement for ALA was necessitated.

II. Antibodies are an indispensable tool to study the translocation of proteins across biological membranes. Therefore, the enzyme L-alanine: DOVA transaminase was purified from rat kidney mitochondria by an entirely new procedure and antibody raised against the purified enzyme preparation. Thus, the results of purification and partial characterization of L-alanine: DOVA transaminase have been presented here.

Among the organs examined, the specific activity of the enzyme L-alanine: DOVA transaminase was highest in kidney followed by liver. Therefore, the enzyme was purified from the kidney mitochondria of adult Wistar rats by a new three-step procedure. The three-step procedure involved the use of digitonin and lubrol for the mitochondrial matrix preparation and L-alanine-sepharose affinity chromatography followed by gel filtration step on sepharose 6B. The procedure employed is relatively much rapid than the ones reported earlier from other animal sources. The enzyme has been purified 23-fold from the mitochondrial matrix to an apparent homogeneity with a high yield of 37.5%.
Some improvement in the enzyme yield was also attained by utilizing the amplified assay procedure for L-alanine: DOVA transaminase. Since it is now known that L-alanine: DOVA transaminase is a soluble component of the mitochondrial matrix, and affinity chromatography as well as amplified enzyme assay method have proved effective, the present procedure may also be applied to other mammalian tissues, for these properties are commonly shared by other enzyme sources as well.

The apparent native molecular weight of the enzyme was found to be 210 kDa by 4-30% gradient polyacrylamide gel electrophoresis. This was in close agreement to our estimation by gel filtration where it was estimated to be 225 kDa. The subunit molecular weight of the enzyme determined by SDS-PAGE under denaturing condition was found to be 50 kDa. Thus, the enzyme is a homotetramer as in the case of bovine and rat liver.

SDS-polyacrylamide gel electrophoresis of L-alanine: DOVA transaminase under reduced and non-reduced conditions invariably exhibited differences in electrophoretic mobilities. In relation to reduced state, in non-reduced state the enzyme band position shows up below 50 kDa position along the 43 kDa position. Such increases in mobilities of the protein in non-reduced form manifests the presence of intrachain disulphide bond(s). Presence of cysteine
residues (by amino acid analysis) in this protein also indicated this possibility.

The results of two-dimensional gel electrophoresis analysis showed that the enzyme L-alanine: DOVA transaminase is an acidic protein having an isoelectric point (pI) of 5.0. The pI values of L-alanine: DOVA transaminase reported earlier from various plant and animal sources have also suggested it to be acidic in nature.

Attempts undertaken to sequence the N-terminal end of L-alanine: DOVA transaminase suggested that this protein cannot be sequenced by Edman degradation method because of a blocked N-terminal amino acid residue.

The enzyme L-alanine: DOVA transaminase was found to be a glycoprotein by its reactivity towards concanavalin A. Recently, a general survey of mitochondrial proteins has already demonstrated that about 14% of the mitochondrial matrix proteins are glycosylated and bind to concanavalin A (232). To our knowledge L-alanine: DOVA transaminase is perhaps the first protein to be identified as a glycoprotein from the mitochondrial matrix.

The thermal stability of the enzyme at 65°C over a time period of 60 min was determined in absence of its substrates. After the various incubation periods at 65°C the enzyme activity was measured under optimal
conditions. We observed that when the enzyme was incubated for 40 min at 65°C, it lost 80% of its activity exhibiting that the presence of both the substrates is essential for its activity at higher temperatures. The enzyme might be an interesting model in order to investigate the still unclear molecular basis for protein thermal stability.

Finally, polyclonal antibody against the homogeneous preparation of L-alanine: DOVA transaminase were raised in New Zealand white rabbit. Immunological comparison of liver and kidney enzyme were made by ouchterlony double diffusion analysis. The antibody raised against the rat kidney mitochondrial enzyme was found to cross-react with the rat liver mitochondrial L-alanine: DOVA transaminase indicating that the enzyme present in the mitochondria of these organs are immunologically identical.

III. This part of the investigation deals with the in vitro translocation studies of L-alanine: DOVA transaminase into mitochondria. This exploration was undertaken to examine the possibility of inhibition of L-alanine: DOVA transaminase translocation by hemin. Such a study was conducted not only to examine the proposed regulatory role of this enzyme but to further elucidate the physiological significance as well as the potential of this enzyme in ALA production in animal system. The
results and conclusions derived out of this part of investigation are presented here.

The rabbit reticulocyte lysate system has been used in recent years for the translation of eukaryotic mRNAs and also for in vitro protein translocation studies. Prior to translation of mRNAs in this system it is essential to choose the correct radioactive tracer. For this reason amino acid analysis of the enzyme L-alanine: DOVA transaminase was performed and the protein was found to be significantly rich in methionine containing about 2.8% of these residues. Therefore, for this purpose we utilized $^{35}$S-methionine as the radiolabel.

A knowledge of half-life of a protein is helpful in determining the incubation period of in vitro translated product with the mitochondria. Cycloheximide treatment to rats suggested that the half-life of rat kidney L-alanine: DOVA transaminase is more than 3 hours.

Monospecificity of the polyclonal antibody raised against the highly purified L-alanine: DOVA transaminase was confirmed by western blot analysis, which yielded a single band, both, in pure and crude enzyme preparations.

Since purified IgG gives cleaner immunoprecipitation results with radiolabelled polypeptides, the IgG
fraction was isolated by passing the antisera over a protein A-sepharose column. Immunotitration studies indicated that about 40 µg of protein A purified IgG was sufficient to ensure complete immunoprecipitation of the translocated $^{35}$S-labelled polypeptide for L-alanine: DOVA transaminase and the pre-existing cold enzyme present in 150 µg of mitochondria. About 5 µg of purified IgG/10 µl total translation product was found to be adequate for immunoprecipitation.

Prior to definitive experiments, the optimal concentration of poly(A)$^{+}$ RNA for best translation results was determined. Rat kidney poly(A)$^{+}$ RNA isolated by oligo(dT)-cellulose column chromatography from total RNA preparation was translated in rabbit reticulocyte lysate using $^{35}$S-methionine as the radiolabel. Results indicated that poly(A)$^{+}$ RNA at a concentration of 25 µg/ml gives maximum stimulation (translation) over the background.

Using optimal translation and immunoprecipitation conditions, the polypeptide for L-alanine: DOVA transaminase was seen as a major band on the SDS-PAGE fluorographs.

A quantitative estimate of the L-alanine: DOVA transaminase in the total translated product demonstrated that the enzyme represents about 0.85% of the total radiolabelled proteins synthesized in vitro.
We observed that hemin does not impart any specific inhibition at the nascent polypeptide chain elongation step during the translation of L-alanine: DOVA transaminase.

Mitochondrial proteins synthesized in reticulocyte lysate system could be translocated into the respiring mitochondria under well defined conditions. About 59% of the radiolabelled polypeptides for L-alanine: DOVA transaminase from the total translated product could be successfully translocated into the mitochondria where it was no longer accessible to the protease digestion.

In order to examine whether hemin regulates its own biosynthesis by inhibiting the translocation of L-alanine: DOVA transaminase, studies were undertaken using hemin up to a concentration of 50 μM. Our findings suggest that the relative translocation of L-alanine: DOVA transaminase remains unaffected by hemin. Therefore, we conclude that hemin does not regulate its own biosynthesis by imparting its influence at the translation or translocation level of L-alanine: DOVA transaminase.

Mammalian L-alanine: DOVA transaminase is a 'housekeeping enzyme'

Over the last decade a significant proportion of investigation on L-alanine: DOVA transaminase has convincingly established that the production of ALA in
mammalian tissues takes place through a reaction other than the one catalysed by ALA synthetase. Hemin is a potent modulator of concentration of ALA synthetase in liver, kidney and other tissues. In the case of L-alanine: DOVA transaminase, hemin led to decreases in enzyme activity in rat liver, kidney and murine primary hepatocyte cultures. Hemin inhibition of L-alanine: DOVA transaminase were also reported from *Pennisetum typhoideum* and *Pseudomonas riboflavina*. The observation that only hemin among all the heme biosynthetic pathway intermediates appears to inhibit L-alanine: DOVA transaminase both *in vitro* and *in vivo* led to the suggestion that the enzyme is under feedback regulation by intracellular heme pool. However, the data in the literature is insufficient to establish conclusively the hypothesis that L-alanine: DOVA transaminase has a regulatory function in heme biosynthesis. During the course of this investigation a few reports appeared which were not fully consistent with the earlier observations. Therefore, taking into account the overall observations made here as well as by others, it is reasonable to conclude that the enzyme is not under regulation by intracellular 'heme-pool' but maintains a 'housekeeping' level of ALA in mammalian system. The reasons for the conclusion derived are discussed below:

a. The high catalytic activity and high levels of L-alanine: DOVA transaminase even under normal physiological condition suggest that the enzyme is not
under tight control. On the other hand, ALA synthetase is under a stringent control by hemin.

b. Although several investigators have reported the inhibition of L-alanine: DOVA transaminase activity by heme, the concentration required for the inhibition appears to be too high to be physiological. Although quite a large dose of hemin should be administered to observe a significant enzyme inhibition, the results of the present study do not necessarily exclude the possibility that heme may directly inhibit this enzyme. It remains to be determined whether intra-mitochondrial concentrations of heme ever become high enough to affect this enzyme. Even if hemin rises to such high levels in the mitochondrion it will rapidly escape from it and such direct inhibition of the enzyme activity will contribute negligibly at the physiological concentration of hemin in the cell.

c. Several agents have been known to modulate hemeprotein production together with the activity of ALA synthetase. However, unlike ALA synthetase, none of the cytochrome P-450 inducers (DDC, phenobarbitone, AIA etc.) proved to be potent inducers of L-alanine: DOVA transaminase (161, 239, 240).

d. The half-life of the enzyme L-alanine: DOVA transaminase has been estimated to be more than three hours while that of ALA synthetase has been reported to be about 30 minutes. The short half-lives of rate-
limiting enzymes have been viewed as an indication that protein turn-over can play a major role in regulating the activities of multi-enzyme pathways. For instance, when the synthesis of a rate-limiting enzyme ceases, a short half-life for the enzyme ensures a rapid decrease in the pathway’s activity.

e. The regulation of translocation of ALA synthetase into mitochondria by heme has been identified to be an important site of regulation of heme biosynthesis (34,62,66). However, we observed that hemin does not impart its influence either at the translation or translocation level of L-alanine: DOVA transaminase.

It is well known that under normal physiological conditions the level of ALA synthetase is very low or even undetectable. However, when compared the level of L-alanine: DOVA transaminase was found to be many-fold higher than the ALA synthetase from the same mitochondrial source. Also, the heme biosynthetic pathway has been interrelated with glyoxalase system (137,157,166) and succinate-glycine cycle (92,95), thereby suggesting the other metabolic fates of heme biosynthetic precursors. From the observations discussed above it is apparent that the enzyme L-alanine: DOVA transaminase is not under a tight control by hemin, the end-product of the pathway. So from the overall picture that emerges now, it appears that under normal physiological state of animal, the enzyme L-alanine: DOVA transaminase maintains a ‘housekeeping’ level of ALA and this enzymic
product in turn might have several other metabolic fates, both, within and outside the mitochondria. However, when a sudden increase in heme production becomes necessary, induction of ALA synthetase would follow which might be yet another important ALA source to fulfil the excessive need for heme. From the evolutionary point of view, it is probable that the ALA synthetase pathway became prominently operative in animals as an adaptive measure to the changing environment where these mobile animals were consistently being exposed to a number of new xenobiotics and synthetic chemicals.

Though there have been many advances in our understanding of heme biosynthesis there are issues which yet remain to be resolved. If a significant role is to be assigned to L-alanine: DOVA transaminase in ALA/heme biosynthesis, then in vivo availability of L-alanine and DOVA must be considered. L-alanine is, of course, readily available in all the cells and the natural occurrence of DOVA in biological system has been reported. It is not clear yet, however, whether the substrate DOVA is present in the same mitochondrial compartment with the enzyme and at high enough concentration to render the reaction physiologically significant. Therefore, procedures need to be devised for selectively inhibiting in non-disrupted cells the activity either of L-alanine: DOVA transaminase or ALA synthetase and then measuring ALA or tetrapyrrole production rates in the presence of the block. The approach would
allow an assessment of the contributions of each of the two enzymes in ALA production. Gabaculine, an inhibitor of ALA synthesis in plants, is recently reported (160) to be a potent inhibitor of L-alanine: DOVA transaminase activity when present either in the assay reaction mixture or in the medium of primary hepatocyte cultures. The compound should prove useful in future analysis on the contribution of this transaminase in heme biosynthesis.