ALTERED REGULATION OF HEME BIOSYNTHESIS BY A CYTOCHROME P-450 INDUCER

Dissertation submitted to the Jawaharlal Nehru University
in partial fulfilment of the requirements for
the award of the Degree of
MASTER OF PHILOSOPHY

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1988

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CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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ACKNOWLEDGEMENTS

I am deeply indebted to Dr.(Mrs) Kasturi Datta and Dr. R. Subramanian for their excellent guidance and constructive suggestions during the course of my investigation. I am also grateful to them for critical comments in writing the manuscript and the affection shown to me.

I would like to thank Prof. V. Asthana and Prof. L.K. Pande, Dean, School of Environmental Sciences for providing me with all the facilities. I take this opportunity to thank Prof. H.K. Das, Prof. Asis Datta and their research scholars for allowing me to use their laboratory facilities. I would like to express my gratefulness to Prof. Anjali Mookerjee for her kind help and encouragement.

I express my sincere thanks to my senior colleagues Dr. Nishi K. Singh, Mr. K. Hoare and Rashmi for their suggestions and enthusiastic cooperation. Thanks are due to my friends Ramesh, Sanjay, Subrat, Jaiswal, Pervez, Venkatesh and Raina for their constant help and encouragement.

Thanks are due to Dr. V.C. Tyagi, Mr. Sharma and the staff of Animal House for their help in maintenance of experimental animals. In addition, I am thankful to Biswajeet, Sanjay and Manohar for their assistance in the laboratory.

I am deeply indebted to my parents, brothers and sister for their everlasting source of encouragement.

Financial assistance from University Grants Commission is thankfully acknowledged.

Lastly, I am thankful to Mr. Ravinder Kumar for expert typing and Mr. R.N. Saini and Mr. Chinmoy Nath for making excellent line diagrams and photographs.

Rakesh

ABBREVIATIONS

A	Absorbance
AIA	Allyl isopropyl acetamide
ALA	δ-aminolevulinic acid
CoA	Coenzyme A
DOVA	4, 5-dioxovaleric acid
g	gram
mg	milligram
mM	millimolar
µg	microgram
µl	microlitre
µM	micromolar
M	Molarity
n mole	nanomole
nm	nanometre
N	Normality
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
RBC	Red blood corpuscles
SDS	Sodium dodecyl sulphate
rpm	Revolution per minute
TEMED	N,N,N',N', -Tetramethylenediamine
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminoethane
U	Unit

AIM AND SCOPE

Heme, an iron-containing porphyrin (protoporphyrin IX) is the most ubiquitous metalloporphyrin chelate of the living system. It constitutes the prosthetic moiety of different biologically active hemeproteins such as hemoglobin, myoglobin and many cytochromes. Thus, heme is vital to maintain the structural and functional integrity of hemeproteins.

A major fraction of heme synthesized specially serves as a prosthetic group in cytochrome P-450, which in turn plays an important role in the detoxification and activation of foreign chemicals. The heme biosynthetic pathway has been clearly elucidated and δ-aminolevulinate (ALA) is considered to be the first committed precursor of porphyrin and heme in both plants and animals. The biosynthesis of ALA in mammalian cells has been thought to occur exclusively through a condensation reaction involving glycine and succinyl CoA, which is mediated by a mitochondrial matrix enzyme ALA synthetase (1,2). It is known that altered regulation at the level of ALA synthetase is associated with various genetic defects which are biochemically characterized with the overproduction of ALA and porphyrins (3-6). It is also well established that certain drugs, e.g., phenobarbital and 3-methyl-cholanthrene, which stimulate two distinct forms of cytochrome P-450, also induce ALA synthetase (7-8). Induction of ALA synthetase by porphyrogenic drugs such as Allylisopropylacetamide (AIA), 3,5-Dicarbethoxy 1,4-dihydrocollidine (DDC) have been reported to be coupled with the depletion of intracellular heme content (9,10). Thus, it is well established from

various facts that the formation of ALA is an important regulatory step in heme biosynthesis.

Though, ALA has been established as an obligatory precursor in green plants for the synthesis of chlorophyll, the absence of ALA synthetase in plants (11) raises a question on the sole responsibility of ALA synthetase for the synthesis of ALA and the regulation of heme bibsynthesis. This led to the discovery of a new pathway of ALA formation in plants. Ultimately evidences continue to accumulate that in plants, ALA is synthesized exclusively via a transaminase reaction between L-alanine and 4,5-dioxovalerate (DOVA) (12,13). The discovery of a new pathway in the plants gave a new dimension to the problem of heme biosynthesis in mammals also. The work from others (14,15) as well as from our laboratory (16-19) confirmed that this alternate pathway of ALA formation in plants by the enzyme alanine:dioxovalerate transaminase is also operative in mammalian systems.

With the discovery of this enzyme in different mammalian tissues (16,18) there is a scope of research into its possible role, mechanism of action and overall physiological significance in heme biosynthetic pathway. When compared, it was observed that alanine:DOVA transaminase has greater efficacy to synthesize ALA than the ALA synthetase (16). This observation suggests that alanine:DOVA transminase might have physiological significance in mammalian tissues to synthesize heme, necessary for the formation of hemeproteins. The presence of high level of this enzyme in kidney apart from liver also led us to examine the role of kidney alanine:DOVA transaminase in heme biosynthesis.

As a part of our programme, we are interested to examine the

effect of phenobarbitone, known to induce cytochrome P-450, the biologically active hemeprotein, on the activity of alanine:DOVA transaminase for further elucidation of the role of this enzyme on heme biosynthesis. For such a purpose, we have chosen phenobarbitone to study the regulation of alanine:DOVA transaminase in liver and kidney. Our study here reveals that phenobarbitone has a differential effect on the liver and kidney enzyme. While it significantly stimulates hepatic alanine: DOVA transaminase, it has little or no effect on the kidney enzyme. Since our laboratory has already reported the regulatory role of exogenous hemin on the activity of alanine DOVA transaminase (20), we propose to utilize this model (phenobarbitone induced alanine:DOVA transaminase) in future to understand the role of this enzyme.

In a multienzyme pathway, the first enzyme usually serves as the key regulator which is determined by the steady state concentration of the end product. Alanine:DOVA transaminase, besides ALA synthetase, also has the potential to be a key regulator by virtue of its being the first enzyme of the pathway. Some studies from our laboratory have already observed that the enzyme alanine: DOVA transaminase is a regulatory enzyme since hemin has the inhibitory effect on its activity as seen from *in vivo* and *in vitro* experiments (19,20). To study the physiological role of alanine: DOVA transaminase on heme biosynthesis, pure and homogeneous preparation of the enzyme is needed. This initiated us to purify this enzyme from kidney mitochondria by a new procedure. Antibody against the pure protein will be raised and used in future in

unravelling its physiological significance as a regulator of heme biosynthesis.

As a part of our future programme we would like to study the structural relationship of the cytosolic and mitochondrial form of alanine:DOVA transaminase which have been found to differ in their molecular weights. In kidney, the cytosolic form of the enzyme has a higher molecular weight than the mitochondrial form. So as a continuation of the project, we are planning to isolate the alanine:DOVA transaminase specific mRNA which will be used for elucidating the structural relationship of the two forms of the enzyme by means of <u>in vitro</u> translation.

CHAPTER I

ALTERED REGULATION OF HEME BIOSYNTHESIS BY PHENOBARBITONE - A CYTOCHROME P-450 INDUCER

INTRODUCTION

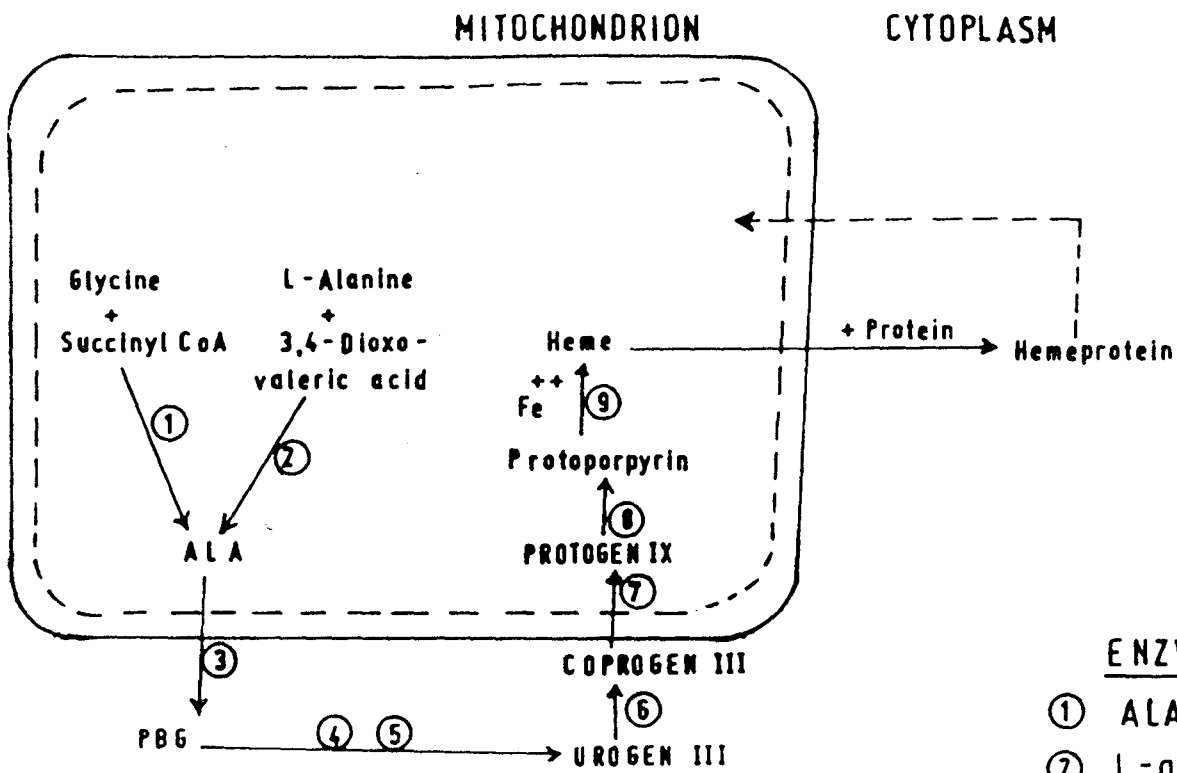
Heme, structurally one of the most stable compounds, is produced in virtually all mammalian tissues. Its synthesis is most pronounced in the bone marrow and liver because of the requirements for incorporation into hemoglobin and the cytochromes, respectively. Heme, largely a planar molecule consists of one ferrous ion and a tetrapyrrole ring, protoporphyrin IX (21). It should be pointed out that heme as a prosthetic group represents one of the only prosthetic groups that is almost entirely synthesized by the animal organism (22).

Heme serves as the prosthetic group in the biologically active hemeproteins which in turn have different cellular function e.g. i) the carriage and transfer of oxygen (hemoglobin, myoglobin), ii) the orderly and efficient transfer of electrons in ATP synthesis (mitochondrial cytochromes), iii) microsomal metabolism of fatty acids, steroids, and xenobiotic compounds (microsomal cytochromes) (23). Thus, heme as a prosthetic group is vital in maintaining the structural and functional integrity of hemeproteins.

Major advances have been made in the chemistry and biology of the porphyrin-heme synthetic pathway in recent years. In addition to defining certain of the factors regulating porphyrin, and heme formation, these advances have shed light on the pathological processes that underlie the porphyric disorders of man. Several excellent reviews have considered the biochemistry of the porphyrin-heme pathway and the clinical and biochemical manifestations of the porphyric diseases (6, 24-28).

Table I. Derangement in Porphyrin Metabolism

Disease State	Tissue/ organ	Enzyme	Activity	Organ Pathology
Acute intermittent Porphyria	Liver	i) ALA synthetase ii) Uroporphyrinogen I synthetase	Increase Decrease	Nervous System
Hereditary Coproporphyria	Liver	i) ALA synthetase ii) Coproporphyrinogen oxidase	Increase Decrease	Nervous System, skin
Variegate porphyria	Liver	i) ALA synthetase ii) Protoporphyrinogen oxidase	Increase Decrease	Nervous System, skin
Porphyria Cutanea tarda	Liver	i) Uroporphyrinogen decarboxylase	Decrease	Skin (induced by liver disease)
Hereditary protoporphyria	Marrow	i) Ferrochelatase	Decrease	Gallstones, liver disease, skin
Erythropoietic porphyria	Marrow	i) Uroporphyrinogen III cosynthase	Decrease	Skin and appendages, reticuloendothelial system



ENZYMES

① ALA Synthetase
② L-alanine:DOVA transaminase
③ ALA dehydratase
④ UROGEN I Synthetase
⑤ UROGEN III cosynthetase
⑥ UROGEN decarboxylase
⑦ COPROGEN oxidase
⑧ PROTOGEN oxidase
⑨ Ferrochelatase (Heme synthetase)

Fig. 1 THE HEME BIOSYNTHETIC PATHWAY

Derangement of porphyrin metabolism are known clinically as the porphyrias. This family of disease is of great interest because it has revealed how complicated the regulation of heme biosynthesis is. The clinical presentations of the different porphyrias provide a fascinating exposition of biochemical regulatory abnormalities and their relationship to pathophysiological processes. Table I lists the details of the different porphyrias.

HEME BIOSYNTHESIS AND DEGRADATION

Fig.1 is a diagrammatic representation of heme biosynthetic pathway. Some of the important aspects of heme biosynthesis are:

i) the first and the last three enzymatic steps are catalysed by enzymes that are in the mitochondrion, whereas the intermediate steps take place in the cytoplasm.

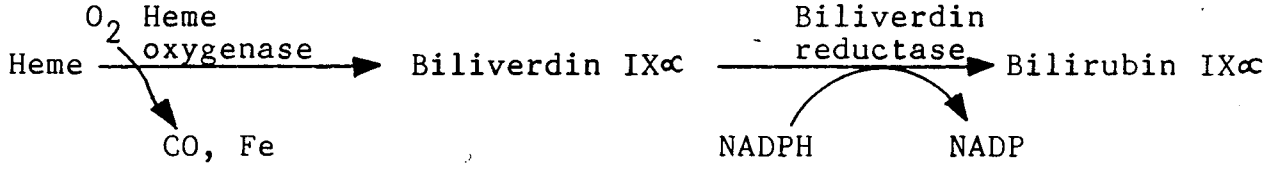
ii) the organic portion of heme is derived totally from eight residues each of glycine and succinyl CoA.

iii) the reactions occuring on the side groups attached to the tetrapyrrole ring involve the colourless intermediates known as porphyrinogens.

Shemin and Rittenberg (1, 29) were the first to demonstrate that glycine was incorporated into the heme moiety of hemoglobin in rat and man. Subsequent studies have shown that all four nitrogen atoms (30,31) and eight of the carbon atoms of the heme molecule are derived from glycine (32, 33), the remaining carbon atoms are derived via kreb's cycle. In the first enzymatic step in heme formation, glycine and succinate are combined to form δ-amino-levulinic acid (ALA). In the next enzymatic reaction two ALA molecules are condensed to give the monopyrrole porphobilinogen

(PBG). The enzymatic condensation of four PBG molecules leads to the formation of uroporphyrinogen (UROGEN), the first tetrapyrrole in the heme pathway. Enzymatic decarboxylation of the four acetic acid side chains of UROGEN yields coproporphyrinogen (COPROGEN) and further side-chain modification by oxidative decarboxylation transforms COPROGEN into protoporphyrinogen IX (PROTOGEN). The oxidative conversion of PROTOGEN to protoporphyrin (PROTO) probably occurs by an enzymatic mechanism that proceeeds the insertion of ferrous ion into the porphyrin ring by the enzyme Ferrochelatase, resulting in the formation of heme (ferrous PROTO). Heme then serves as the prosthetic group of a variety of hemeproteins, such as myoglobin, hemoglobin, the cytochromes, peroxidase, catalase, and tryptophan pyrrolase.

The catabolism of heme-containing proteins present two requirements to the mammalian host: i) the development of a means of processing of hydrophobic products of porphyrin ring cleavage and ii) the retention and mobilization of the contained iron so that it may be reutilized. The sequence of events of heme catabolism are shown below:



In mammals and in other vertebrates, heme is oxidatively degraded to form the open-chain tetrapyrrole, biliverdin, in the course of which three molecules of O_2 are utilized (34, 35). A mole of carbonmonooxide is generated in this reaction and the

central metal ion of heme is released. The enzyme heme oxygenase is substrate inducible and is an endoplasmic membrane bound enzyme (36). The biliverdin formed from heme oxidation is universally of the IX isomer type that is, the oxidation of the heme molecule taking place at the α mesocarbon bridge. In mammals, biliverdin is reduced to bilirubin by the action of pyridine nucleotide dependent (NADPH or NADH) cytosol enzyme, biliverdin reductase (37). The latter is an isomer specific enzyme which is reactive almost exclusively towards the biliverdin IX isomer.

REGULATION OF HEME BIOSYNTHESIS

The activity, presence, and quantity of an enzyme in a cell can be regulated in various ways. Enzyme can be inhibited both irreversibly and reversibly. Irreversible inhibition is caused by substances reacting with groups at the active site in such a way as to destroy it or modify it permanently. In many multienzyme system, generally, the end product or the pathway acts as a specific inhibitor of an enzyme at or near the beginning of the sequence of reactions. Thus, a steady state concentration of the end product is achieved. Heme, because it is the end product of the biosynthetic sequence, may be expected to exert a regulatory role at the level of ALA formation, the first step in the heme biosynthesis.

(1) Regulation at the level of ALA synthetase

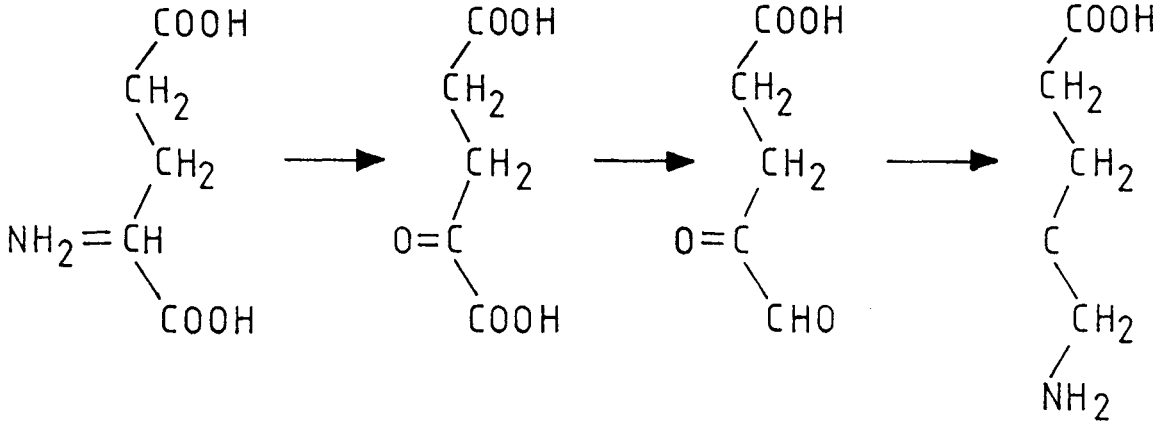
It is well established now that the primary site for regulation of the overall activity of heme biosynthesis in animals is at the level of ALA formation (38). There are several lines of evidence suggesting that heme serves as a feed back regulator of heme bio-

synthesis, acting primarily at the level of ALA synthetase (39,40). Hemin is known to inhibit ALA synthetase (EC 2.3. 1.37) both in vitro (4,41) and in vivo (42,43). Since hematin or heme resembles neither the substrate nor the product of the enzyme's action, it is possible that the latter inhibition occurs at an allosteric site.

Under normal conditions liver mitochondrial ALA synthetase is present at a very low level (44). But in various derangements in porphyrin metabolism (Table I) an increase in ALA synthetase activity has been observed (21). These genetic defects are biochemically characterised by excess excretion of porphyrins and its precursors. This helped in understanding the key regulatory role of ALA synthetase in heme biosynthesis in mammalian (4,40) and chick embryo liver (44). Almost 100 different drugs and metabolites are known to induce ALA synthetase (6,21). The experiments of Granick and Urata first showed that administration of 3,5-Dicarbethoxy 1,4-dihydrocollidine (DDC) to guinea pigs results in 40 fold increase in ALA synthetase activity of liver mitochondria (10). Similar increases in ALA synthetase were reported following administration of allylisopropylacetamide (AIA) (4,45). Kappas and his associates have observed that perinatal rats are refractory to the ALA synthetase inducing properties of certain chemicals (46,47). For example, neonatal rats are not responsive to the action of either AIA or phenobarbital until they are aproximately 5-6 weeks old. It is of interest, however, that the hepatic hemeprotein cytochrome P-450 is inducible by phenobarbital at a time when ALA synthetase is not (47), suggesting that inducibility of the hemeprotein is not directly related to inducibility of ALA synthetase.

Discovery of an alternate pathway of ALA formation

Although ALA is normally synthesized by ALA synthetase in animal systems but its absence in plants raised a question on the sole responsibility of this enzyme for the synthesis of ALA and its regulation on heme biosynthesis. In 1975, Beale et al (11) showed that plants follow an alternative pathway from glutamate through dioxovalerate (DOVA). The reaction shown below is in the manner in which the five-carbon skeleton of glutamate may be used in the synthesis of ALA. This system was thought to be predominant in plants only.



This reaction is not restricted to plants since Varticovski and co-workers (14,15,49) have shown it to be present in bovine liver, and labeled DOVA has been incorporated into ALA and heme in rats (48). Several more studies on rat liver and kidney (16-20) suggested the existence of an alternate pathway for biosynthesis of ALA in mammals.

(II) Regulation at the level of L-alanine; 4,5-dioxovalerate transminase

The formation of ALA, the first committed precursor of heme,

is reported to be mediated by L-alanine:4,5-dioxovalerate transaminase (EC 2.6.1.43), which catalyses a transamination reaction between L-alanine and 4,5-dioxovalerate to yield ALA, in addition to the conventional pathway by ALA synthetase. This enzyme is found to be remarkably heat stable and its capacity to synthesize ALA exceeds by more than a factor of ten as compared to the capacity of ALA synthetase from the same mitochondrial source (15,18,19).

Since L-alanine:DOVA transaminase is also the first enzyme of heme biosynthetic pathway (15,50) a potential was thought to exist, for an end product inhibition. So, a study was undertaken to determine the effects of the intermediate compounds of heme metabolism on purified alanine:DOVA transaminase by Shanker and Datta (17). Their findings clearly indicated that only hemin amongst the intermediates of heme metabolism has a significant effect. The inhibition of enzyme activity was found to be proportional to hemin concentrations. Hemin in lower concentration of 5μM showed 18 percent inhibition while hemin concentration as high as 200μM showed about 66 percent inhibition. Thus, it was evident that hemin was a potential regulatory factor for alanine:DOVA transaminase by means of feed back regulation and thus, in turn regulates heme biosynthesis.

A comparision of all the tissues tested show that alanine:DOVA transaminase activity is always higher than ALA synthetase in the same tissues (18). Therefore, it is quite evident that alanine:DOVA transaminase contributes more in terms of heme synthesis than ALA synthetase under normal physiological conditions. Moreover, the occurance of alanine:DOVA transaminase in different tissues at

high level (like kidney and liver) suggests that it is a component of mitochondria, even in tissues generally not associated with hemopoiesis. The high activity of this enzyme in kidney and liver can be explained on the ground that these tissues have high content of cytochrome P-450, a major hemeprotein with rapid turnover number (38,51) and would require more active heme biosynthesis for the maintenance of normal heme concentration. Similarly, a low enzyme activity in brain is in agreement with the observation that brain heme and cytochrome P-450 levels are much lower than that of hepatic level (52).

Cytochrome P-450 and its inducers

During the 1950s, a number of investigators became involved in the study of the oxidative metabolism of certain endogenous and exogenous compounds (53,54). Various compounds ranging from steroids, aromatic amines, and polycyclic aromatic hydrocarbons to numerous drugs were found to be metabolized by NADPH and O_2 dependent enzyme system localized largely in the endoplasmic reticulum fraction of most animal tissues. Early studies by Klingenberg and Gartinkel demonstrated that a unique cytochrome exists in mammalian liver (55,56) and when characterized by optical spectroscopy these microsomal cytochrome absorbed light maximally around 450 nm (57). Subsequent reports showed that there are many distinct forms of cytochrome P-450 which are now together designated as xenobiotic monooxygenase (EC 1.14.19.1). These have been purified from both rat and rabbit (58,59) and nine distinct forms of cytochrome P-450 from uninduced rabbit liver

microsomes have been purified by Aoyama et al (60,61). The relative concentrations of these forms can be dramatically altered by in vivo exposure to inducers such as phenobarbital, stilbene oxide, polycyclic aromatic compounds etc., which also increase total cytochrome P-450 levels.

Cytochrome P-450 is present in nearly all cells. The forms present in a tissue are characteristic of that tissue, as is the response to each type of inducer. Phenobarbital induces cytochrome P-450 only in liver and intestine, and this tissue specificity has also been shown in the mouse for the far more potent inducers of the same class, 1,4-bis [2-(3,5-dichlorophyridyloxy)]-benzene (62). However, some fetal liver completely lack the competence to respond to certain xenobiotics. For example, rat liver cytochrome P-450 dependent monooxygenase do not respond to phenobarbital induction prenatally, whereas they are readily induced by 3, methylcholantherene (63).

By means of a microspectrophotometric (microdensitometric) technique cytochrome P-450 can be directly measured within hepatic parenchymal cells in unfixed, crystat tissue sections. Livers of rats pretreated with phenobarbital were found to contain more than twice as much cytochrome P-450 than did livers of untreated rats (64).

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 possibly alanine:DOVA transaminase. But from all points of view, the

formation of ALA is considered as 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. With this aim in mind a study on the regulatory role of alanine:DOVA transaminase in heme biosynthesis was made here.

MATERIALS AND METHODS

MATERIALS

FOR ENZYME PURIFICATION: DEAE Cellulose DE-52 was bought from Whatman Biochemicals Ltd., Maidstone, England. AH-Sepharose 4B and Phenyl Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Sweden. Protamine Sulphate was obtained from Sigma (St. Louis, MO). L-Alanine was from Ferak, Berlin. Columns used were of glass manufactured locally.

ELECTROPHORESIS MATERIAL: Acrylamide, N,N'-methylene-bis-acrylamide and bromophenol blue were purchased from Bio-Rad Laboratories, U.S.A. Sodium dodecyl sulphate, 2-mercapto-ethanol, Trizma, ammonium persulphate and TEMED were obtained from Sigma Co. (St. Louis, MO). Marker proteins of sodium dodecyl sulphate polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals, Sweden. Silver nitrate was bought from Qualigens Fine Chemicals, Bombay. Other chemicals used were obtained from commercial sources in highest purity available.

OTHER CHEMICALS USED: L-Alanine was purchased from Ferak, Berlin; bovine serum albumin from Sigma; and 3,5-dibromolevulinic acid from Porphyrin product, U.S.A. 4-dimethylaminobenzaldehyde was obtained from Merck (India) Ltd., and Folin Ciocalteu's reagent from SRL Bombay, Ethyl acetoacetate was a product of Ranbaxy Laboratories Ltd., and sodium phenobarbitone of Loba chemicals. All other chemicals were obtained from commercial sources in highest purity available.

Maintenance of rats: Healthy male, young and adult Sprague Dawley rats were obtained from Animal House of Jawaharlal Nehru University, New Delhi. The rats were maintained with proper facility and were fed ad libitum with water and rat feed from Hindustan Lever Ltd. Rats were fasted for 24 hrs. prior to sacrifice.

METHODS

Treatment of rats to study in vivo effects of sodium phenobarbitone: The effect of chronic treatment by phenobarbitone on alanine:dioxovalerate transaminase, in liver and kidney was studied with rats weighing 40 g while acute effects were studied on rats weighing 90 g. For studying the effects of phenobarbitone on the activity of alanine:DOVA transaminase in blood (RBC), adult rats weighing 180±5 g were used. Any group under study had same date of birth and the rats of the same weight were obtained from larger groups available in our Animal House.

Sodium phenobarbitone was dissolved in double distilled water. A dose of 40 mg/kg body weight for acute and 35 mg/kg body weight for chronic study was given in freshly prepared 0.2 ml of injection. The animals were given intraperitioneal injetions. The rats were sacrificed at different hours in case of acute and after 20 days in case of chronic treatment.

Preparation of homogenate from liver and kidney: Rats were killed by cervical dislocation and organs were removed rapidly into 10 mM potassium phosphate buffer, pH 7.6 at $4^{o}C$. Organs were cleaned and washed in the same buffer. The blood clots

were removed to avoid contamination due to hemoglobin. Organs were cut into small pieces and 20% (w/v) homogenate was prepared in 10 mM potassium phosphate buffer pH 7.6, containing 0.25 M sucrose.

The homogenate thus prepared were centrifuged at 5000 rpm for 10 minutes in Hitachi 18PR-52 refrigerated centrifuge. The supernatant were collected and preserved in ice for enzyme assay.

Isolation and hemolysis of blood R.B.C.: Blood was taken from the heart of male rats weighing 180±5 g. Blood was drawn in sterilized syringes having 0.15 M of sodium citrate (pH 7.2) as anti-coagulant. Blood thus collected was transferred into graduated centrifuge tubes containing 0.9%NaCl solution as isotonic medium for blood cells. RBC were washed thrice in the same isotonic solution by centrifuging the tubes at 3000 rpm for 10 minutes and discarding the supernatant. During the last washing centrifugation was done at 4000 rpm for 15 minutes and volume of the packed settled RBC was recorded from the graduated centrifuge tubes. After removing the supernatant chilled water in the ratio of 1:5 (RBC:Water) was added and tubes were kept at $4^{\circ}C$ for 2 hrs. This dilution and incubation period is thought to be good enough for giving sufficient osmotic shock, resulting in hemolysis of all the R.B.C. present.

In the next step, the tubes were centrifuged at 5000 rpm for 15 minutes. The supernatant were collected in another set of tubes for the protein and enzyme assay. The assay was performed

soon after the collection of the final supernatant since some precipitation in the samples is observed if kept for long hours.

Assay of L-Alanine:4,5-dioxovalerate transaminase:

Enzyme L-Alanine, 4,5-dioxovalerate transaminase (Alanine: DOVA transaminase) catalyses an irreversible transamination reaction as shown in the following equation:

$$\text{L-alanine} + \text{4,5-dioxovalerate} \xrightarrow{\text{Alanine:DOVA transaminase}} \delta\text{-Aminolevulinate} + \text{Pyruvic acid}$$

Alanine:DOVA transaminase was assayed according to the method of Varticovski et al(15) by measuring aminolevulinic acid formed in the reaction. The method is based on the conversion of ALA into 2-methyl 3-carbethoxy-4- (3-propionic acid) by the method of Mauzeral and Granick (65) with minor modification and the Ehrlich chromophore was measured spectrophotometrically at 553 nm in Beckman DU-20 spectrophotometer.

Reagents used:

i) Synthesis of 4,5-dioxovalerate: 4,5-dioxovalerate was synthesized following the method of Varticovski et al (15). 1.37 g of 3,5-dibromolevulinic acid was added to 100 ml of distilled water and kept for boiling for 3 hours. Total volume was maintained at 100 ml by periodic addition of water. With boiling time the solution progressively turned yellow. After boiling, the solution was cooled to room temperature. The yellow coloured product was extracted at least thrice with 30 ml of ethylacetate and discarded

carefully. The aqueous layer was washed thrice with 30 ml of diethylether and this top organic layer was discarded again. To evaporate the residual ether, the nearly colourless acidic aqueous phase, was warmed gently in a beaker.

Finally, 4,5-dioxovalerate solution was adjusted to pH 4.2 with 1 N sodium hydroxide solution. This procedure gave a yield of 40 to 50%, which is same as reported earlier.

ii) Preparation of modified Ehrlich Reagent: One gram of 4-dimethyl-aminobenzaldehyde was dissolved in 30 ml of glacial acetic acid to which 8 ml of 70% perchloric acid was added. The final solution was made upto 50 ml with distilled water. Since the reagent is unstable it must be used on the same day.

iii) 0.2 M L-Alanine: 0.3564 g of L-Alanine was dissolved in 20 ml of distilled water. The solution was stored in deep freezer to prevent fungal growth.

iv) 0.1 M Phosphate buffer, pH 7.0

v) 25% TCA: 25 g of Trichloroacetic acid was dissolved in 100 ml of distilled water.

vi) 2 M Acetate buffer, pH 4.6

vii) Ethyl acetoacetate: It is commercially available.

Assay procedure: The system contained 1 mM 4,5-dioxovalerate, 20 mM L-alanine, 50 mM potassium phosphate at pH 7.0, enzyme preparation and distilled water in a final volume of 1 ml.

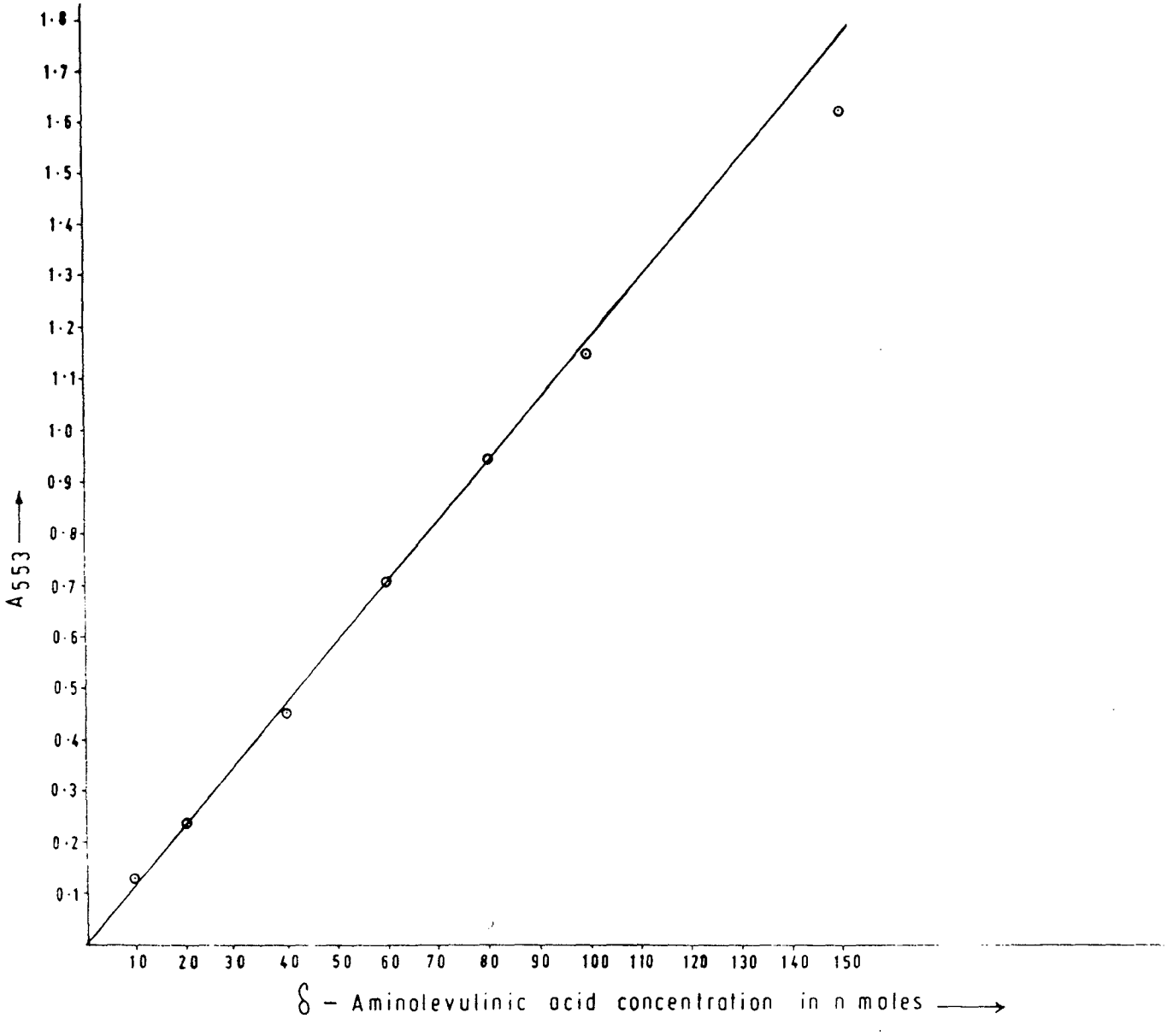


Fig. 2 STANDARD CURVE FOR δ-AMINOLEVULINIC ACID

Incubation was carried out at 60°C for 30 min in case of liver and kidney homogenate, and at 65°C for 60 min in case of blood RBC. After the incubation period the reaction was terminated by addition of 0.1 ml of 25% Trichloroacetic acid. After centrifugation at 5000 rpm for 10 minutes the supernatant was assayed for ALA formation.

Determination of Aminolevulinate: 0.5 ml of deproteinized reaction mixture was collected from the supernatant with the help of a glass pipette. To the mixture 0.5 ml of 2 M sodium acetate buffer (pH 4.6) and 0.1 ml of ethylacetoacetate were added and allowed to react in a boiling water bath for 10 minutes. After cooling to room temperature, 1 ml of Ehrlich reagent was added and the absorbance was measured at 553 nm after 15 minutes against enzyme blank mixture. The colour produced in this reaction is proportional to the ALA content of the solution assayed as shown in Fig.2.

Defination of unit and specific activity: One unit of alanine dioxovalerate transaminase is defined as the amount of enzyme that catalyzes the formation of 1 µmol of aminolevulinic acid at a specific temperature and incubation period. Specific activity in case of liver and kidney homogenate is expressed in nmoles of ALA formed/mg protein/30 minutes at 60°C, while in case of blood it is expressed in nmoles of ALA formed/mg protein/60 min at 65°C.

Estimation of Total Heme content in liver and Kidney: Total heme content may be expressed as the redox spectrum of dithionate-reduced against fully oxidised pyridine haemochromogen in the presence of 0.1 M NaOH (57,66).

Reagents used:

i) 0.5 M NaOH.

ii) 0.154 M Potassium chloride.

iii) 12.5 mM Potassium ferricyanide. Prepared fresh for each set of assay.

iv) Pyridine (analar grade). Its fumes are hazardous to health.

v) Sodium dithionite.

Assay Procedure:

i) Pipetted a suitable volume of homogenate or suspension into two matched stoppered tubes or spectrophotometer cuvettes and then added 0.154 M KCl to each to a total volume of 1.5 ml.

ii) Added 0.5 ml of 0.5 M NaOH to each tube.

iii) Added 0.5 ml of pyridine.

iv) To the 'test' (reduced) tube added a small quantity of solid sodium dithionite.

v) To the 'reference' (oxidised) tube added 10 µl of 12.5 mM freshly prepared $K_3Fe(CN)_6$ solution.

vi) Stoppered both, the 'test' and the 'reference' tube and gently mixed the contents.

vii) Recorded the difference spectrum in spectrophotometer.

Calculation of results:

i) From the spectrophotometer determined A (557-575) nm, that is the difference between the absorption maximum at 557 nm and 575 nm.

ii) The extinction coefficient of pyridine haemochromogen has been determined to be 32.4 cm^2/n mole (57). Hence the total heme concentration (n mole/ml) of the sample is calculated from the formula:

$$\frac{A(557\text{-}575)\ nm}{1} \times \frac{1000}{32.4} \times \frac{2.5}{\text{Volume of the sample (ml)}}$$

iii) The total heme content of the sample is expressed as n mole/ mg protein.

Estimation of Protein:

Protein was assayed by the method of Lowry et al (67).

Reagent used:

Reagent A : 2% Na_2CO_3 in 0.1 N NaOH

Reagent B : 0.5% $CuSO_4$. $4H_2O$ in 1% sodium potassium tartrate. 1 ml of reagent B was mixed in 50 ml of reagent A. This reagent was prepared fresh for each assay.

Folin Ciocalteu's phenol reagent:

The stock commercial reagent is stored at 4^oC and any unused reagent discarded after 24 weeks. Great care should be taken to avoid contamination of the stock reagent (2N). It was diluted with one volume of distilled water before use.

Procedure: For standard graph : To 0.5 ml of a solution containing 10-100 mg protein, 2.5 ml of protein reagent was added and after, at least 10 minutes, 0.25 ml of 1N Folin reagent was added and mixed thoroughly. After 30 minutes of incubation at room temperature, the contents were mixed again and absorbance at 660 nm was recorded.

For estimation of protein in unknown samples, a suitable dilution of the sample was made and assayed in duplicates by the same procedure as given above.

PREPARATION OF ALANINE SEPHAROSE-4B COLUMN

4 g of freeze dried AH-Sepharose4B powder was suspended in 0.5M NaCl. To remove additives it was washed with 0.5M NaCl (200 ml/g freeze dried powder) on a sintered glass (G3). The ligand L-alanine was dissolved in distilled water (pH 4.5) and added to the gel in a final concentration of 0.2M Similarly, carbodiimide was dissolved in water (pH 4.5) and added to a final concentration of 0.1M to the swollen gel and gently stirred for 24 hrs at room temperature. A final gel concentration 2:1 (liquid:gel) makes an acceptable slurry for stirring. The pH was maintained between 4.5 and 6.0 After the reaction period the gel was thoroughly washed alternatively with high and low pH buffer solutions. Sodium acetate buffer (0.1M, pH 4.0) and bicarbonate buffer (0.1M, pH 8.3), each containing 0.5M NaCl were used. This procedure ensures that no free ligand remains ionically bound

to the immobilized ligand. The gel was finally washed with distilled water and equilibrated with 10mM potassium phosphate buffer, pH 7.6, containing 10% glycerol.

ELECTROPHORESIS

a) Polyacrylamide Gel Electrophoresis (PAGE): The purity of the enzyme was checked by slab gel electrophoresis at pH 8.9 by the method of Davis (68). The separation gel was made of 7.5% acrylamide and 0.27% N,N-methylene bis-acrylamide in 0.375M Tris-HCl buffer, pH 8.9. The stacking gel had 4% acrylamide and 0.106% N,N-methylene bis-acrylamide in 0.067M Tris-HCl buffer, pH 6.7. Then the solutions were deaerated. Chemical polymerization was initiated by adding 0.05% ammonium persulphate and 0.05% TEMED. The solutions prepared were quickly poured between the glass plates. The sample protein with 10% glycerol and 0.001% bromophenol blue were loaded. The running buffer contained 0.05M Tris-HCl and 0.3 M glycine at pH 8.3. The electrophoresis was commenced by applying 80-90 volts for as long the dye was in the stacking gel. Later 150 volts were applied till the marker dye reached about 0.5 cm from the bottom of the slab gel. After electrophoresis the protein bands were developed by silver staining method (70) which is claimed to be upto 100 times more sensitive than Coomassie blue, apparently being able to detect 0.38ng/mm^2 of bovine serum albumin.

b) Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-PAGE was done according to the method of Laemmli

(69). The separating gel was made of 12% acrylamide and 0.2% methylene bis-acrylamide, 0.1% SDS and 0.375M Tris-HCl buffer, pH 8.8. The various components were mixed, deaerated and polymerization was initiated by adding ammonium persulphate and TEMED. The solution was poured between the sealed glass plates, and water layered on the top. After polymerization was over, stacking gel components containing 4% acrylamide, 1.06% N,N-methylene bis-acrylamide, 0.1% SDS and 0.125M Tris-HCl (pH 6.8) were mixed and poured to polymerize. The running buffer (pH 8.3) was composed of Tris (0.02M), glycine (0.192M) and 0.1% SDS.

The sample buffer for SDS-PAGE has the following composition:

Tris-HCl	- 0.0625M (pH 6.8)
SDS	- 2%
Glycerol	- 10%
2-Mercaptoethanol	- 5%

The samples were immersed for 2 minutes in boiling water bath, then cooled and loaded onto the gel slots. Standard proteins were also run along with the samples. The electrophoresis was done at90 volts till the marker dye crosses the stacking gel. Later on a constant voltage of 150 volts was applied till the marker dye reached about 0.5 cm from the bottom of the gel. After electrophoresis the protein bands were developed by the silver staining method.

SILVER STAINING METHOD OF THE GELS

The following steps were used for silver staining of the gel. Precaution was taken not to touch the gel with hands.

(i) 100ml of methanol and 15 ml of acetic acid was made upto 200 ml with distilled water. Soaked the gel in the solution for 20 min.

(ii) 60 ml ethanol and 30 ml of acetic acid made upto 600 ml with distilled water. The gel was soaked in 200 ml of the solution thrice for 10 minutes each.

(iii) 0.2 g of $K_2Cr_2O_7$ was dissolved in 200 ml of distilled water and 57 µl of concentrated HNO_3 was added and mixed. Soaked the gel in the solution for 5 minutes.

(iv) The gel was washed in 200 ml of distilled water 4 times for two minutes each.

(v) Dissolved 0.4 g of silver nitrate in 200 ml of distilled water. Soaked the gel for 25 minutes, followed by rinsing the gel with distilled water.

(vi) 8.88 g of Na_2CO_3 was dissolved in 300 ml of distilled water and 150 µl of formaldehyde added to it and mixed. The gel was first soaked in 100 ml of the above solution with gentle shaking. The solution turned blackish and was discarded. The remaining solution was then added and gently shaken till bands developed.

(vii) The gel was preserved in 1% acetic acid.

RESULTS

Effect of acute treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in liver and kidney

In order to study the regulation of heme biosynthesis at the level of alanine:DOVA transaminase, we treated the rats with phenobarbitone - a drug known to stimulate two different forms of cytochrome P-450. Attempt has also been made to determine the total heme level in the livers and kidneys of the same rats so that interrelationship between the heme level and the enzyme alanine:DOVA transaminase activity may be clarified.

Fig.3 represents the changes in the activity of alanine: DOVA transaminase and the total heme level in liver at different time intervals after phenobarbitone treatment. It is observed that after two hours of phenobarbitone injection, highest level of heme is attained (24%) whereas a maximum decrease (-65%) in the activity of alanine:DOVA transaminase is observed after 4 hours of phenobarbitone injection. After 6 hours of giving injection there is little or no change in the heme level and the activity of alanine:DOVA transaminase. Thus, after giving acute dose of phenobarbitone an inverse relationship of heme level and the activity of alanine:DOVA transaminase in liver has been observed upto 4 hours.

Fig.4 shows the percent changes in the total heme and the

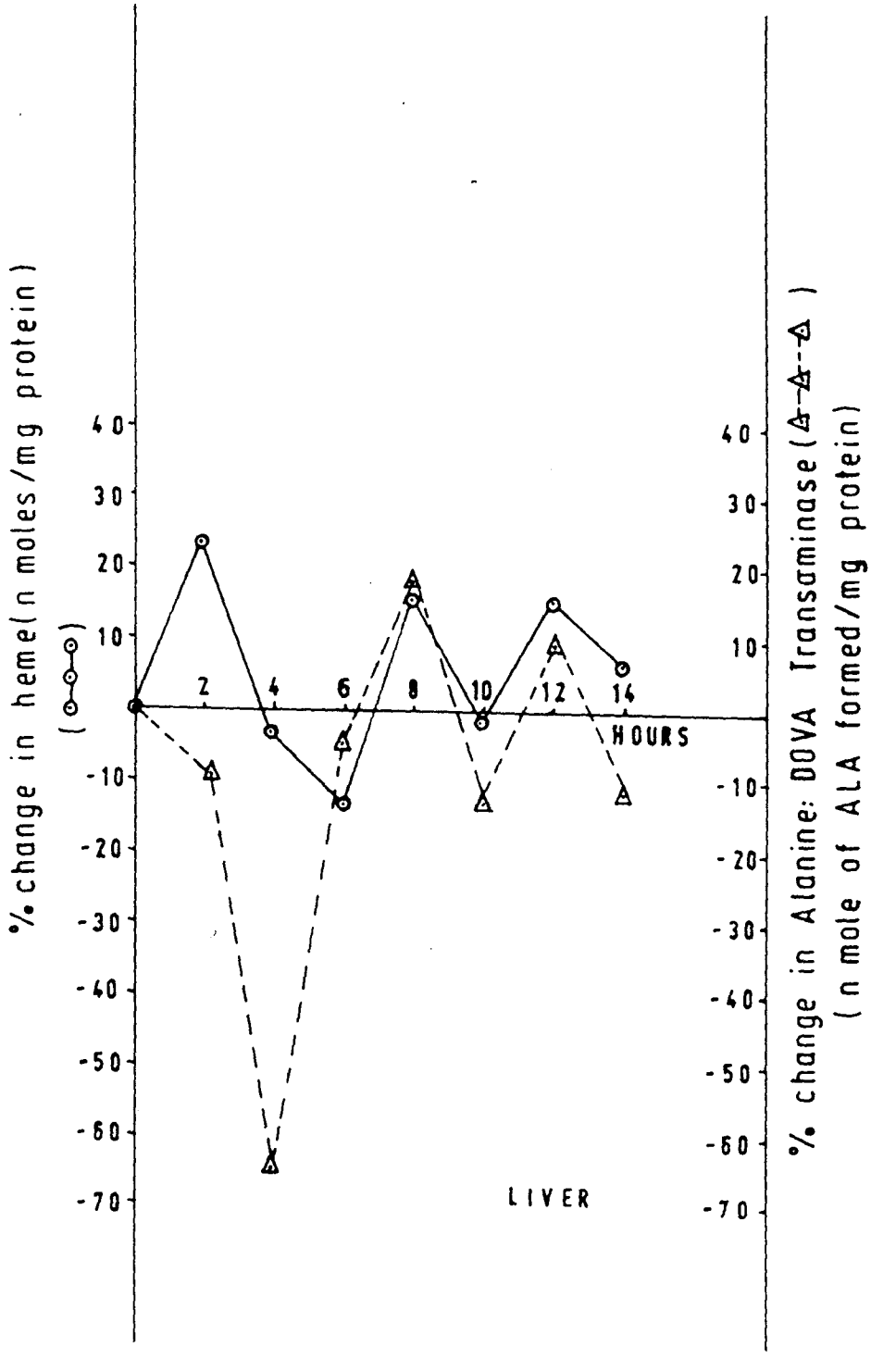


Fig.3 Effect of acute treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in liver.

Each group consists of at least 5 animals.

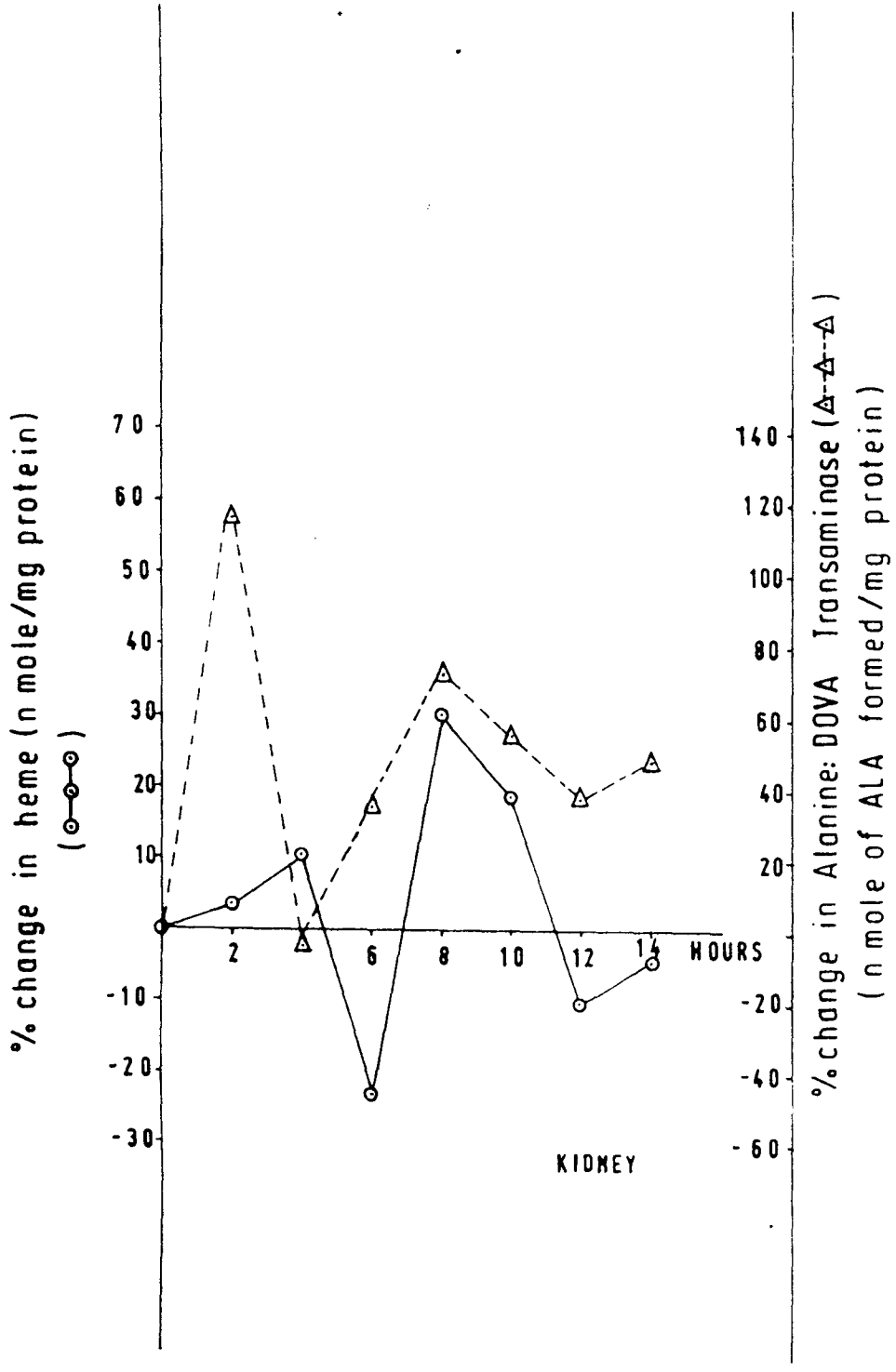


Fig.4 Effect of acute treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in kidney.

Each group consists of at least 5 animals.

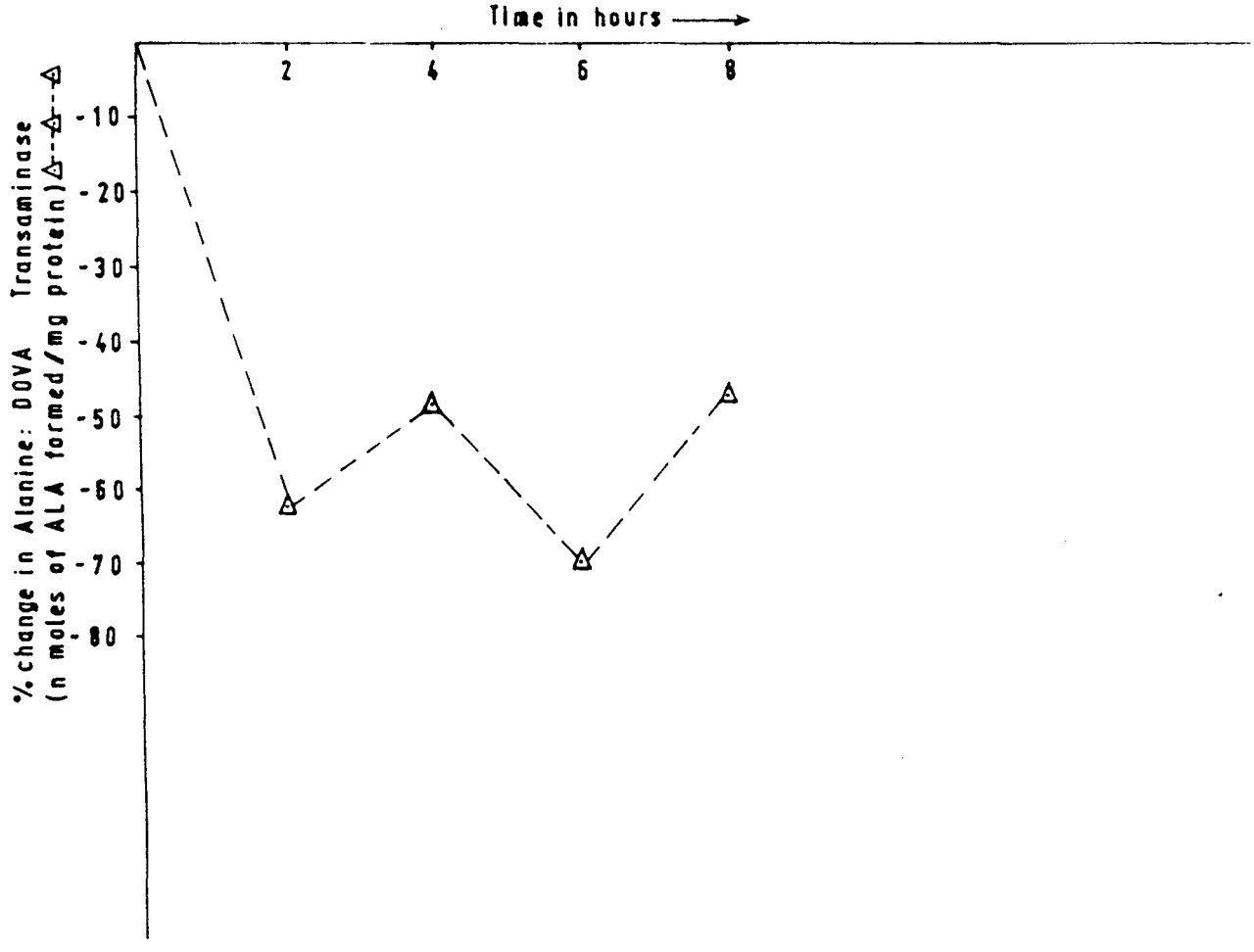


Fig.5 Effect of acute treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase in blood R.B.C.

Table 2. Effect of acute treatment by phenobarbitone on the activity of alanine:DOVA transaminase in blood RBC

Animal	Specific activity in n moles/mg protein after 60 min of incubation at 65°C	% decrease in specific activity
CONTROL	1.47 ± 0.29*	00
TREATED		
2 hours	0.56 ± 0.02	61.91
4 hours	0.77 ± 0.06	47.62
6 hours	0.45 ± 0.14	69.39
8 hours	0.79 ± 0.21	46.26

* Represents mean value ± S.D. Each group consists of at least 4 animals.

activity of alanine:DOVA transaminase in kidney at different time intervals after treatment with phenobarbitone. It is seen that effect of phenobarbitone on the activity of alanine: DOVA transaminase and total heme in kidney differs considerably as compared to the liver. Contrary to liver alanine: DOVA transaminase activity in kidney at 2 hours is seen to go up by 116%. It comes down to control level at the 4th hour when there was hardly any change in heme level. Between the 4th and the 6th hour 'heme-pool' depletion is observed both in kidney and liver; with kidney heme level falling by 33% and liver heme level falling by 37% from their previous highest levels. With the depletion of heme the enzyme level starts rising from 4th hour to 8th hour and it increases till it is 74% higher than the control. As a result total heme also increases. In later hours they tend to assume a steady state.

Fig.5 (Table 2) shows the percent variation in the levels of alanine:DOVA transaminase in hemolysed R.B.C. contents at different hours after treatment with phenobarbitone. In comparison to control it is seen that there is a depression in the activity of alanine:DOVA transaminase in all the treated rats. Thus, it is observed that acute dose of phenobarbitone lowers the activity of alanine:DOVA transaminase in R.B.C. contents.

Effect of chronic treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in liver and kidney

As there is an alteration in the level of total heme as well as alanine :dioxovalerate transaminase in acute treatment experiments, it was of interest to study the effect of chronic treatment by phenobarbitone on the enzymatic activity and total heme level.

Effect of 20 days chronic dose of phenobarbitone was studied on the levels of alanine:DOVA transaminase and total heme in liver and kidney. After the injection of phenobarbitone for continuous 20 days, the treated rats were divided into two different groups. One had the usual dose of phenobarbitone and sacrificed after 4 hours of the last injection and the other group did not have any drug during the last 24 hours. After the sacrifice, livers and kidneys were assayed for the activity of alanine:DOVA transaminase as well as total heme level.

Fig.6 (Table3) shows comparison between control and treated rats with respect to i) increase in body weight, ii) kidney enlargement, and iii) liver enlargement. When compared with control group, the treated group showed an increase in body weight by 8.74%. With respect to control rats, the treated rats exhibited kidney enlargement by 17.51% and liver enlargement by 28.84%. No relationship was observed between kidney and liver enlargement of the treated rats, i.e. kidney enlargement was not in proportion to the liver enlargement of the same rat.

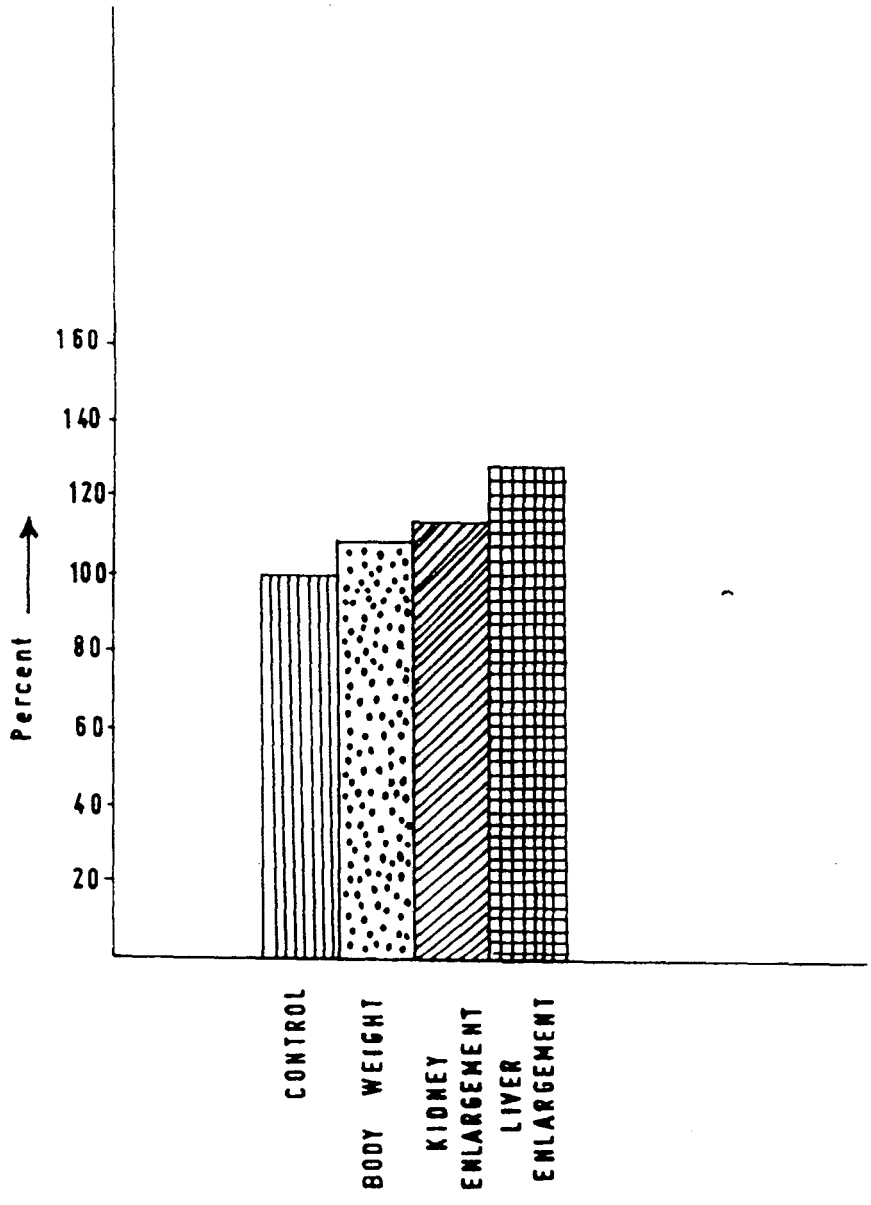


Fig.6 Effect of chronic treatment by phenobarbitone on the body, kidney and liver weights.

Table 3. Effect of chronic treatment by phenobarbitone on the body, kidney and liver weights

	CONTROL	TREATED	% INCREASE
Body weight (in gms)	95 ± 3.3*	103.3 ± 3.5	8.74
Kidney (in gms)	0.868 ± 0.077	1.02 ± 0.057	17.51
Liver (in gms)	3.71 ± 0.25	4.78 ± 0.34	28.84

* Represents mean value ± S.D. Each group consists of at least 10 animals.

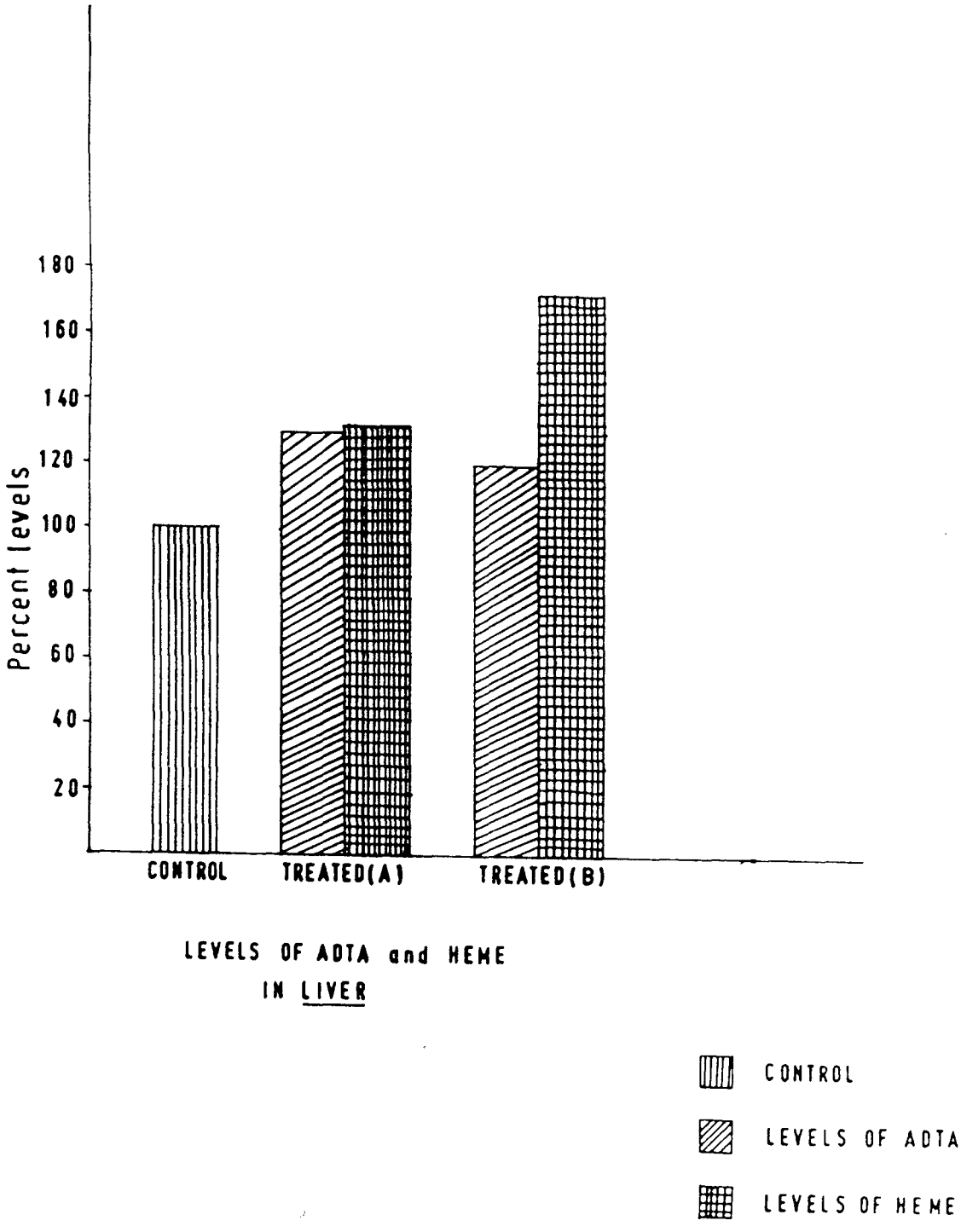


Fig.7 Effect of chronic treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in liver.
Treated (A) group sacrificed after 24 hrs and Treated (B) sacrificed after 4 hrs of giving the last dose of phenobarbitone.

Table 4. Effect of chronic treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in LIVER

Animals	Specific activity in n moles/mg protein after 30 min of incubation at 60°C	Heme content n moles/mg Protein	% change in enzyme level	% change in heme level
CONTROL	64.04 ± 4.7*	3.52 ± 0.28	00	00
TREATED (24 hrs)	83.31 ± 4.3	4.64 ± 0.43	30.09	31.82
TREATED (4 hrs)	76.80 ± 1.3	6.05 ± 0.12	19.93	71.88

* Represents mean value ± S.D. Each group consists of at least 5 animals.

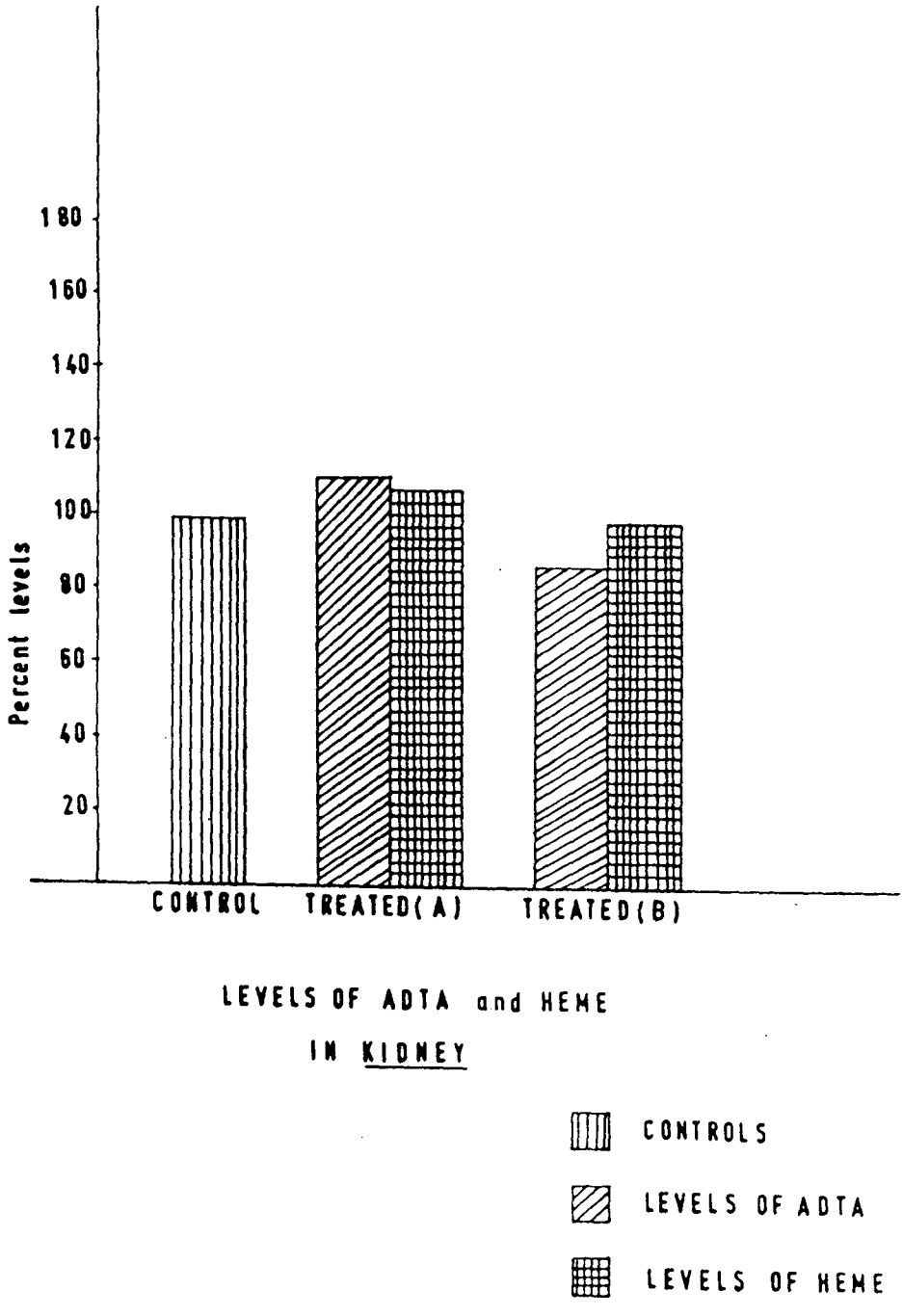


Fig.8 Effect of chronic treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in kidney.

Treated (A) group sacrificed after 24 hrs and Treated (B) sacrificed after 4 hrs of giving the last dose of phenobarbitone.

Table 5. Effect of chronic treatment by phenobarbitone on the activity of alanine:dioxovalerate transaminase and total heme in KIDNEY

Animals	Specific activity in n moles/mg protein after 30 min of incubation at 60°C	Heme content n moles/mg Protein	% change in enzyme level	% change in heme level
CONTROL	144.33 ± 11.9*	6.79 ± 0.32	00	00
TREATED (24 hrs)	160.57 ± 11.2	7.23 ± 0.59	11.25	6.48
TREATED (4 hrs)	124.62 ± 5.8	6.73 ± 0.69	-13.66	-0.88

* Represents mean value ± S.D. Each group consists of at least 5 animals.

Changes in the levels of alanine:DOVA transaminase and total heme in liver are shown in the Fig. 7 (Table 4). In comparison to the control, the treated groups had higher levels of alanine:DOVA transaminase and total heme. Group sacrificed after 24 hours of giving the last dose had enzyme level higher by 30% and heme level higher by 32%. Group sacrificed after 4 hours of giving the last dose of phenobarbitone had enzyme level higher by 20% and heme level as high as 72% in comparison to the control group.

Levels of alanine:DOVA transaminase and total heme in kidney are shown in Fig.8 (Table 5). It is observed that the chronic dose of phenobarbitone had little or no effect on the kidneys of the same rats. Levels of alanine:DOVA transaminase and total heme in treated groups are seen to be nearly the same as control group.

A unique feature observed in this study is that alanine: DOVA transaminase and total heme level in kidney and liver separately are seen to be nearly at the same level when the group did not receive the drug dose for 24 hours. But this steady state of levels are disturbed when sacrificed after 4 hours of giving the last dose of phenobarbitone. It is also observed that chronic effect of phenobarbitone is different from acute dose effect with respect to changes in the levels of alanine:DOVA transaminase and total heme in the liver and kidney.

DISCUSSION

The present study demonstrates that alanine:DOVA transaminase is inducible by treatment with phenobarbitone, suggesting a regulatory role of this enzyme in heme biosynthesis. Furthermore, the induction of this enzyme can be correlated with the depletion of intracellular heme level.

In order to study the regulation of heme biosynthesis at the level of alanine:dioxovalerate transaminase, rats were treated with phenobarbitone - a drug known to induce cytochrome P-450 (64). Phenobarbitone, one of the earliest known anti-epileptic drugs, was found to induce cytochrome P-450 only in liver and intestine (62). Since, heme serves as the prosthetic moiety of hemeproteins including cytochrome P-450, and also regulates its own synthesis by feed back regulation, the use of phenobarbitone would have examined the possibility of a correlation of heme level with the induction of alanine:DOVA transaminase. Keeping this in view the rats were treated with phenobarbitone and levels of alanine:DOVA transaminase and total heme were measured in livers and kidneys of the same rats.

By acute treatment with phenobarbitone only, it was evident that phenobarbitone has differential effect on the liver and kidney. In liver it was observed that after two hours of phenobarbitone injection, highest level of heme is attained (24%) as a result a maximum fall (-65%) in the activity of alanine:DOVA transaminase is observed after 4 hours of phenobarbitone injection.

Thus, after giving acute dose of phenobarbitone an inverse relationship of heme level and the activity of alanine:DOVA transaminase in liver is observed upto 4 hours. Contrary to liver the enzymatic activity in kidney at 2 hours is seen to increase by 116%. Without adding much to the total heme level it comes down to the control level at the 4th hour. Between the 4th and the 6th hour heme depletion is observed both in kidney and liver and this fall may be partially attributed to enzyme heme oxygenase which is substrate (heme) inducible (36). With the depletion of heme the enzyme level was seen to rise again and as a result total heme also increased. Though, enzyme induction and its inhibition is quite clear in liver, the kidney has somewhat a differential response to phenobarbitone. Perhaps heme level at this dose of phenobarbitone could not rise enough to show its inhibitory effect on the kidney form of alanine:DOVA transaminase. Our laboratory work has shown that liver form of the enzyme is a homohexamer composed of identical subunits of Mr 41,000$\pm$ 2000 (16) while kidney form is a tetramer having identical subunits of Mr 50,000.

Presence of alanine:DOVA transaminase in blood R.B.C. contents is also reported here. Acute treatment with phenobarbitone showed a significant lowering of its activity in all the treated rats. Further studies on this enzyme from R.B.C. are in progress in our laboratory.

As there was an indication of inverse relationship between

alanine:DOVA transaminase and heme it was interesting to study the chronic effects of phenobarbitone. Effect of 20 days chronic dose of phenobarbitone also revealed that liver and kidney respond differentially in the same rats.When compared to control, the treated rats showed higher levels of alanine:DOVA transaminase and total heme in liver. Major changes in their levels are seen to occur in the early hours of the drug dose. They seem to assume a steady state in these levels in later hours of the drug injection. After 4 hours of the phenobarbitone injection to the chronic rats the level of total heme in liver was seen to go up by 72%. On the other hand the chronic treatment of phenobarbitone had little or no effect on the kidney levels of alanine:DOVA transaminase and total heme. They were found to be nearly same as the control group. It is also observed that chronic effects of phenobarbitone is different from acute with respect to alteration in the level of alanine:DOVA transaminase and total heme in liver and kidney.

Chronic treatment by phenobarbitone also showed some significant changes in other parameters of the treated rats. In comparison to control the treated rats exhibited increase in their body weights. With respect to control rats, the treated rats showed significant enlargement of their kidneys and livers.

Recent report from our laboratory has already indicated that intracellular heme content in kidneys, when sharply reduced

by phenylhydrazine or cobalt chloride,has a concomitant rise in alanine:DOVA transaminase activity (85). Similarly, excess generation of endogenuous heme by addition of enzyme (ALA synthetase) substrates was also seen to inhibit alanine: DOVA transaminase in rat liver mitochondria (86). The work presented here with phenobarbitone - a cytochrome P-450 inducer also correlated with our previous reports. Therefore, it is reasonable to conclude that there is an inverse relationship between the activity of enzyme alanine:DOVA transaminase and heme, and also that alanine:DOVA transaminase is under the control of an intracellular 'heme-pool'.

CHAPTER II

AFFINITY PURIFICATION OF ALANINE: 4,5-DIOXOVALERATE TRANSAMINASE FROM RAT KIDNEY MITOCHONDRIA

INTRODUCTION

The initial biosynthesis sequence for synthesis of heme, chlorophylls and corrins, is the same and in each case utilizes porphyrinogens as direct intermediates of the final product (71). The first step, the rate-controlling step of heme biosynthesis, is the condensation of succinate and glycine to form ALA. The enzyme catalyst for this reaction, ALA synthetase, is a mitochondrial protein which requires pyridoxal phosphate as a cofactor (40,72-74). It is a relatively unstable enzyme with a short half-life *in vivo* of about 1 hour in mammalian species with a low basal activity (75). In mammalian systems, ALA synthetase is found principally in the mitochondrion. Low activities found in other subcellular fractions are probably due to the activity of precursors of the enzyme newly synthesized in the cytoplasm and in passage to the mitochondrion. The cytoplasmic protein has a greater molecular weight than the mitochondrial enzyme (76, 77), thus limiting the translocation of cytosolic ALA synthetase to the mitochondrion (78). Studies on the subcellular location of the mitochondrial enzyme show that ALA synthetase is loosely bound to the inner mitochondrial

membrane, although it may also be found free within the mitochondrial matrix (79-81).

Although ALA is normally synthesized by ALA synthetase in mammalian systems, its synthesis by plants follows an alternative pathway from glutamate through dioxovalerate (DOVA) (11). This reaction is not restricted to plants since Varticovski and co-workers (15) have shown it to be present in bovine liver, and labeled DOVA has been incorporated into ALA and heme in rats (48). Moreover, the capacity of ALA synthesis by alanine:DOVA transaminase appears to be far greater than the capacity of ALA synthetase from the same source (18). Though alanine:DOVA transaminase is also the potential candidate for the formation of ALA in mammalian system, little work has been done on the regulatory role of alanine:DOVA transaminase on heme biosynthesis. As seen in the case of ALA synthetase even alanine:DOVA transaminase may be markedly altered by various drugs and foreign compounds, and it is on the basis of such changes that one may define its role as a control point for the biochemical pathway, by negative feedback of the end product of the reaction, heme, upon it.

It is well established that the primary site for the regulation of heme biosynthesis is at the level of ALA formation (38,71). There are several lines of evidences suggesting that heme regulates its own biosynthesis by end product regulation of ALA synthetase (40,82,83). Recent studies with

alanine:DOVA transaminase also showed that only hemin among the tested intermediate metabolites of heme biosynthesis acts as an inhibitor of this enzyme (16-20).

As it is seen from the previous work that there is an alteration of heme biosynthesis by phenobarbitone and it can affect the activity of alanine:DOVA transaminase, the antibodies raised against this purified protein can further elucidate, more specifically, the role of alanine:DOVA transaminase in regulation of heme biosynthesis. Also, a study of this enzyme in our laboratory has already shown that kidney mitochondrial form of alanine:DOVA transaminase has a lower molecular weight than the cytoplasmic form in the native as well as in denatured condition (18). Our future programme includes the study of its probable translocation from kidney cytoplasm to mitochondria by making use of the antibodies raised against the enzyme purified by this method.

RESULTS

All the purification procedures were carried out at 4°C unless stated otherwise. The mitochondria were isolated from the kidneys of male rats weighing 150-200 gms.

STEP 1 Isolation of Rat kidney mitochondria: This method is based on the procedure originally described by Johnson and Lardy (84).

Rats were sacrificed by cervical dislocation and the kidneys were removed rapidly. The capsule was removed by gently pinching the kidneys between thumb and forefinger, causing the tissue to be extruded from the membrane. The kidneys were washed with ice cold buffer (10 mM potassium phosphate buffer,(pH 7.6) before mincing so as to remove all the blood. After the wash kidneys were minced and homogenised at medium speed in polytorn, with 4 volumes of 0.25M sucrose in 10mM potassium phosphate buffer, pH 7.6. The crude homogenate was centrifuged at 2000 r.p.m. for 20 minutes to sediment nucleic acid and debris. The spin was repeated once more. After the second spin, the supernatant was centrifuged at 12,000 r.p.m. for 30 minutes. The pellet from this spin contains the mitochondrial fraction. In appearance the pellet has three distinct regions:

a) lower layer which is white and red, composed of residual cell debris and red blood cells,

b) middle layer which is medium to dark brown in colour contains largely intact mitochondria and some lysosomes,

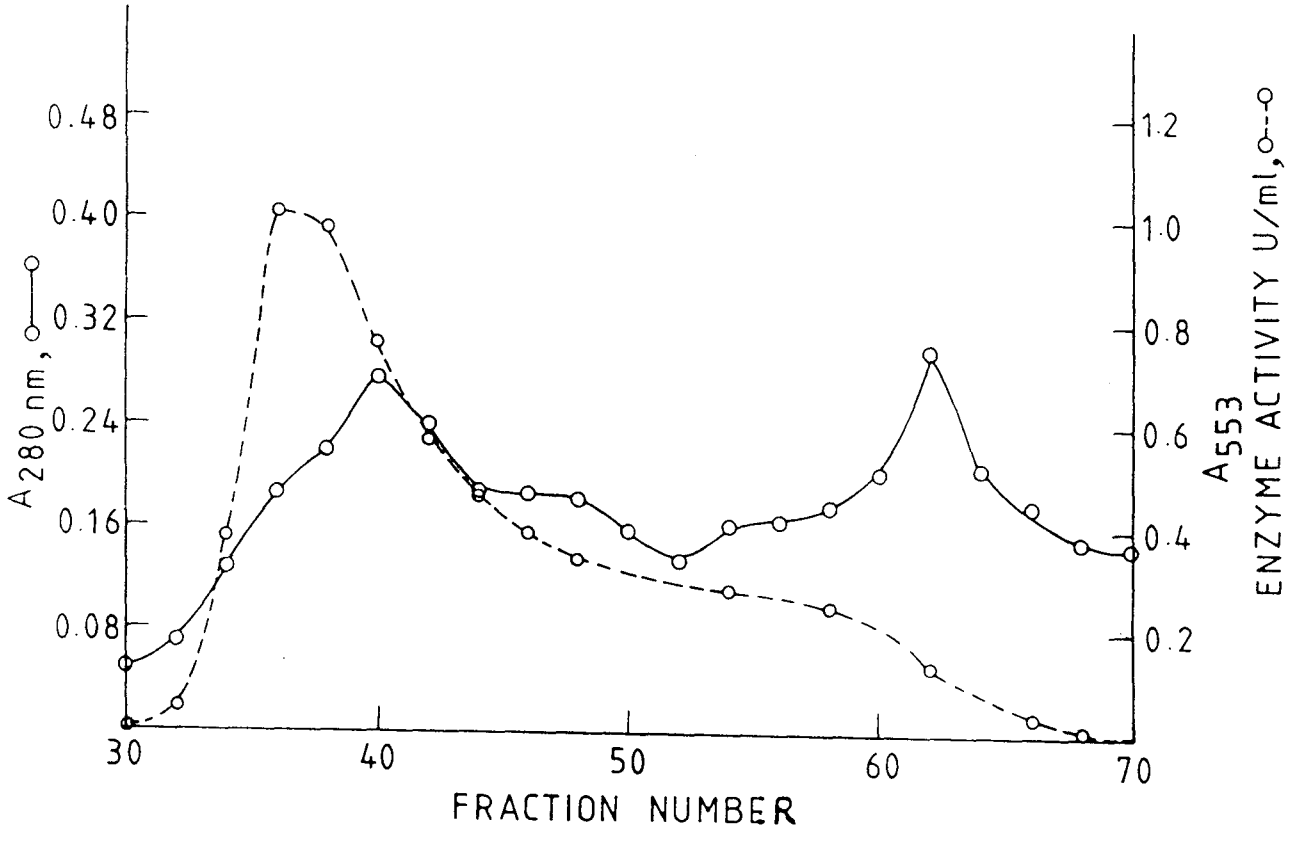


Fig. 9 DEAE cellulose DE-52 chromatography of rat kidney mitochondrial L-alanine:4,5-dioxovalerate transaminase. After extensive washing the enzyme was eluted using a linear gradient of 0-0.3 M KCl (500 ml) in potassium phosphate buffer, pH 7.6, containing 10% glycerol. Flow rate, 18 ml/hr. Fraction size 4.5 ml.

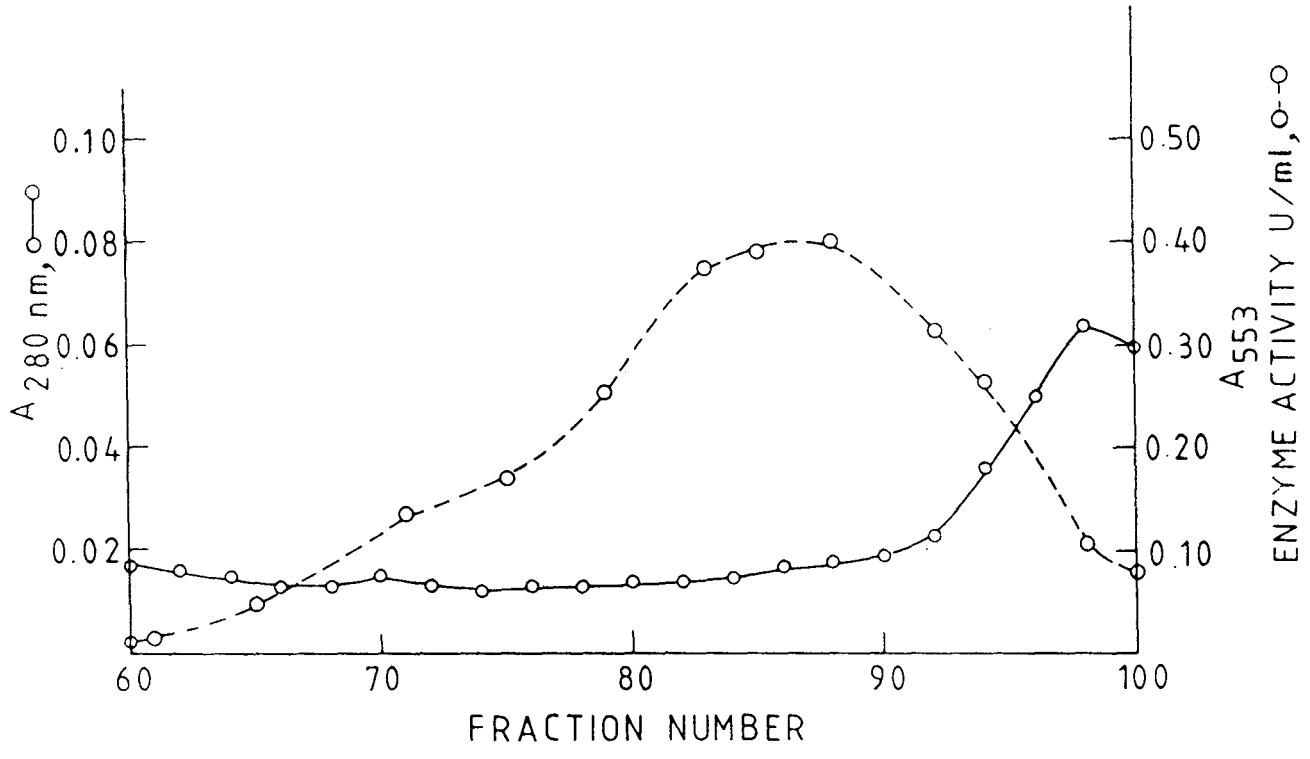


Fig.10 L-Alanine:Sepharose-4B affinity chromatography of rat kidney mitochondrial L-alanine:4,5-dioxovalerate transaminase. After extensive washing, the elution of the enzyme was done using a linear gradient of 0-0.3 M KCl (400 ml) in potassium phosphate buffer, pH 7.6, containing 10% glycerol. Flow rate 30 ml/hr. Fraction size 3 ml.

c) an upper fluffy, light, pinkish brown layer containing broken mitochondria and microsomes.

With gentle swirl the upper layer was dislodged and discarded. Without disturbing the lower layer, the packed mitochondria in middle layer were suspended in 2 volumes of 10mM potassium phosphate buffer containing 0.25M sucrose and 10% glycerol and left overnight at -20°C.

The frozen mitochondrial suspension was then thawed and sonicated at 10 μA for five pulses, each pulse lasting 15 seconds. It was then centrifuged at 12,000 r.p.m. for 30 minutes. The pellet was washed twice with 0.5 volume of the same buffer and the supernatants were pooled for purification.

STEP 2 Precipitation with Protamine sulphate: To the above 50 ml of the supernatant 10 ml of 2% (w/v) protamine sulphate dissolved in 10mM potassium phosphate buffer pH 7.6 was added dropwise with constant stirring at 4°C for 20 minutes. The supernatant was collected after centrifugation at 12,000 r.p.m. for 10 minutes. The enzyme activity was checked in both, pellet and supernatant. Enzyme activity in the pellet was negligible.

Step 3 DEAE-Cellulose DE-52 column: The supernatant collected after precipitation with protamine sulphate was loaded onto DEAE Cellulose DE 52 ion exchanger column (2.75 x 18.5 cm) previously equilibrated with 10mM phosphate buffer (pH 7.6) with 10% glycerol. After the buffer wash, the elution was

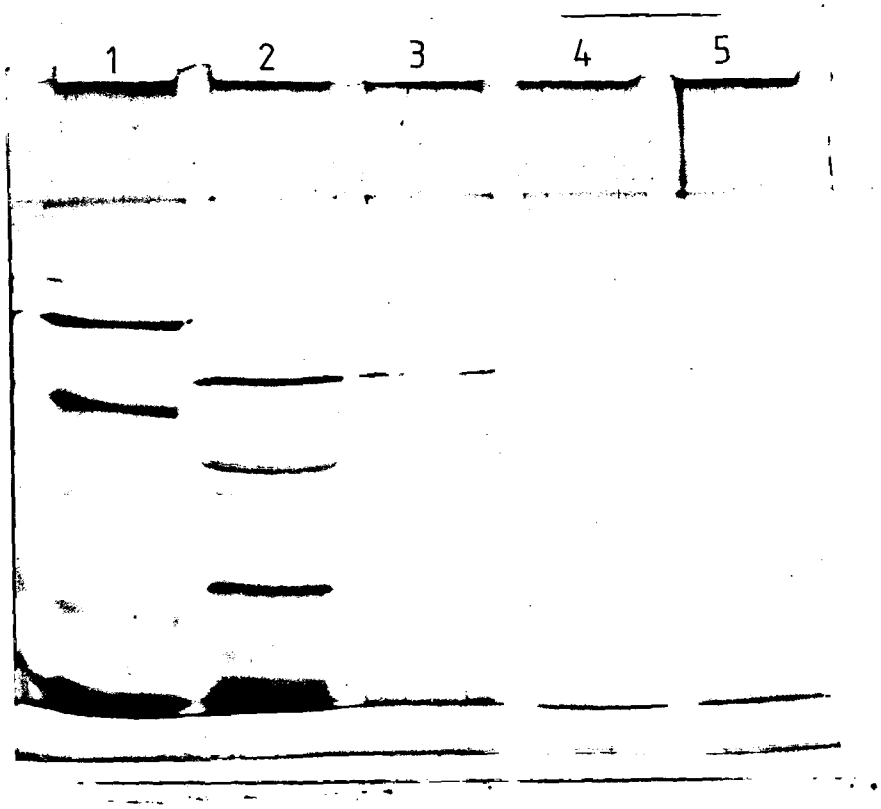
done with a linear KCl gradient (0-0.3M) at a flow rate of 18 ml/hr. The total volume of the gradient applied was 500ml. The enzyme was eluted approximately in the middle of the gradient (Fig. 9). The fractions with high activity were pooled and concentrated with polyethylene glycol 20,000. This procedure resulted in a purification of approximately 9.21 fold with an overall recovery of 67.4% (Table 6).

STEP 4 L-Alanine-Sepharose-4B column chromatography: The pooled sample of DEAE cellulose DE-52 was loaded onto Alanine-Sepharose-4B column (2.0 x 6.5 cm), which was previously equilibrated with 10mM potassium phosphate buffer, pH 7.6, containing 10% glycerol. The column was first washed with 100 ml of the same buffer and then the enzyme was eluted with 0-0.3M KCl linear gradient having a total volume of 400 ml in the same buffer, at a flow rate of 30 ml/hr. The fraction having enzyme activity were pooled and concentrated with polyethylene glycol 20,000. This procedure resulted in a purification of 43.3 fold with an overall recovery of 22.7% (Table 6).

STEP 5 Phenyl sepharose CL-4B column: The sample from alanine sepharose- 4B column was loaded onto the phenyl sepharose CL-4B column (2.75 x 12.5 cm), previously equilibrated with 10mM potassium phosphate buffer pH 7.6. The column was washed with the same buffer containing 10% glycerol. The enzyme did not bind to the hydrophobic support and was eluted in the buffer

Fig. 11 Polyacrylamide gel electrophoresis (PAGE) of the purified kidney mitochondrial alanine:dioxovalerate transaminase. 2 µg of the sample was electrophoresed at pH 8.3 in 7.5% gel.

Fig. 12 Molecular weight determination of alanine:DOVA transaminase by SDS-polyacrylamide gel electrophoresis. 2 µg of each standard proteins and purified enzyme were treated as described in "Materials and Methods". Mobilities of the proteins were expressed with respect to bromophenol dye front (R_f). Samples electrophoresed in different slots were: 1- standard marker proteins, 2- sample after DEAE cellulose chromatography, 3- sample after Alanine Sepharose-4B affinity chromatography, 4- blank, 5- purified protein after Phenyl sepharose CL-4B.


1
2
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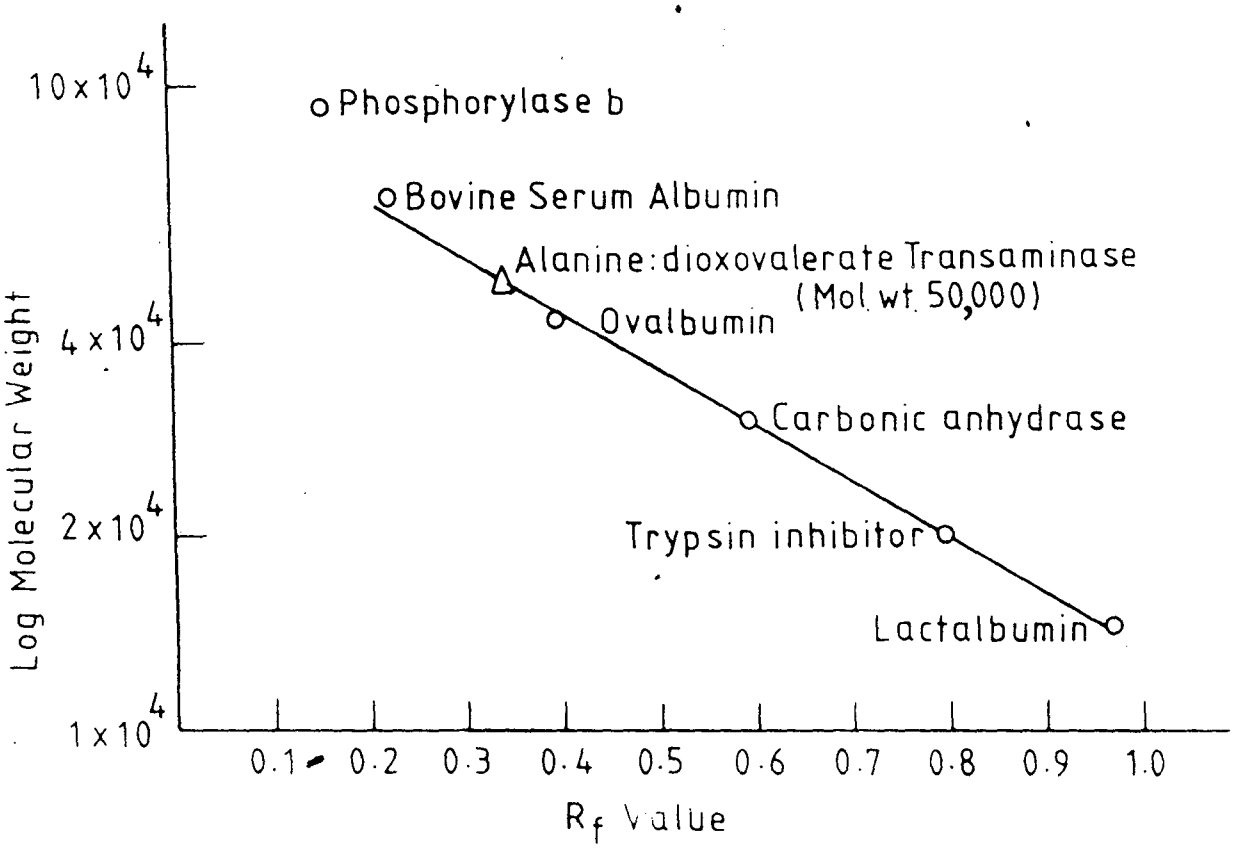
10x10^4
4x10^4
2x10^4
1x10^4
Log Molecular Weight
Phosphorylase b
Bovine Serum Albumin
Alanine:dioxovalerate Transaminase
(Mol. wt. 50,000)
Ovalbumin
Carbonic anhydrase
Trypsin inhibitor
Lactalbumin
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf Value

Table 6. PURIFICATION OF L-ALANINE:4,5-DIOXOVALERATE TRANSAMINASE FROM RAT KIDNEY MITOCHONDRIA

Purification	Protein (mg)	Total Activity (μ moles)	Sp. Activity μ mol/mg Protein	Purification (fold)	Yield (%)
1. Mitochondria extract	304.05	95.95	0.315	1	100
2. Protamine sulphate	208.45	88.24	0.423	1.34	91.9
3. DEAE-Cellulose	22.29	64.68	2.902	9.21	67.4
4. Alanine sepharose	1.60	21.83	13.644	43.3	22.7
5. Phenyl sepharose	0.701	12.60	18.00	57.1	13.1

wash. The fractions with high activity were pooled and concentrated with polyethylene glycol 20,000. The concentrated sample was dialysed against 10mM potassium phosphate buffer, pH 7.6, containing 10% glycerol. This procedure resulted in a purification of approximately 57.1 fold with an overall recovery of 13.1% (Table 6).

Homogeneity: From the above concentrated and dialysed sample 2 µg of protein was used to check the purity of the enzyme by 7.5% polyacrylamide gel electrophoresis (PAGE) at pH 8.3. Fig.11 shows that kidney mitochondrial alanine:DOVA transaminase purified from this procedure was homogeneous as evidenced by a single band in the gel.

Molecular weight determination: Molecular weight of the subunits of alanine:DOVA transaminase was determined by SDS-PAGE under denaturing condition which showed a single band. The R_f values of the alanine:DOVA transaminase with respect to those of the marker proteins electrophoresed simultaneously, showed that the identical subunit of alanine: DOVA transaminase has a molecular weight of 50,000 daltons (Fig.12).

DISCUSSION

This report describes the affinity purification of alanine: DOVA transaminase from rat kidney mitochondria. An improvement in the purification of this enzyme was accomplished by using the L-alanine-sepharose-4B affinity column. Using this modified method the enzyme was purified 57.1 fold with a recovery of 13.1%. The final enzyme preparation has specific activity of 18.0 u/mg protein. Unlike earlier methods here enough protein can be recovered in one purification to raise antibodies against it. Thus, this method may be used when a large quantity of alanine:DOVA transaminase is required in a pure form.

Therefore, after purification of alanine:DOVA transaminase to homogeneity, we believe that it will be a useful system to study the regulatory role of this enzyme in heme biosynthesis and to examine whether drugs or inducers of cytochrome P-450 have any effect on this enzyme activity. Also, further experiment may be performed to elucidate if the cytoplasmic alanine:DOVA transaminase is the precursor of mitochondrial enzyme so that this enzyme can be used as a model system to study the translocation of mitochondrial matrix proteins and also the biogenesis of alanine:DOVA transaminase in relation to regulation of heme biosynthesis.

SUMMARY

The formation of ALA, the first committed precursor of heme is now known to be mediated by alanine:dioxovalerate transaminase, in addition to the conventional pathway by ALA synthetase. The present study examined the effects of phenobarbitone - a potent cytochrome P-450 inducer, on the activity of alanine:dioxovalerate transaminase and total heme in both acute and chronic treated rats. The study revealed that alanine:DOVA transaminase is inducible by phenobarbitone and there is an inverse relationship between the activity of alanine:DOVA transaminase and the heme level. It was observed that enzyme alanine:DOVA transaminase is under the control of an intracellular 'heme-pool' and is a regulatory enzyme of the heme biosynthetic pathway. The response of kidney to the phenobarbitone treatment was found to differ considerably as compared to the liver. This may be partially attributed to the fact that kidney form of alanine:DOVA transaminase differs from the liver form of the enzyme.

Presence of alanine:DOVA transaminase in blood RBC is reported and it was seen that acute treatment with phenobarbitone inhibited the enzyme in all the treated rats.

The chronic treatment by phenobarbitone showed that there was an increase in body, kidney and liver weights of the treated rats.

The enzyme alanine:DOVA transaminase is purified from rat kidney mitochondria by an improved method, making use of L-alanine-sepharose-4B affinity column.

Molecular weight of the subunit of alanine:DOVA transaminase is determined to be 50,000 daltons.

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