STYRENE INTERACTION WITH EMBRYONIC DEVELOPMENT

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PREFACE

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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ABBREVIATIONS

AIA	:	2-allyl-2-isopropylacetamide
ALA	:	Delta Amino levulinic acid
CoA	:	Coenzyme A
COPRO III	:	Coproporphyrinogen III
Cyto. C	:	Cytochrome C
Cyto. P-450	:	Cytochrome P-450
DDC	:	3,5-diethoxycarbonyl, 1,4-dihydrocollidine.
DTT	:	Dithiothretol.
PBG	:	Porphobilinogen
PROTOR III	:	Protoporphyrinogen
UROPOR III	:	Uroporphyrinogen III

AIM AND SCOPE

Recent case report indicates that mothers living in communities with polyvinyl chloride (PVC) production facilities give birth to an excess numbers of children with congenital malformations (1). Embryotoxicity of styrene is reported recently at the level of 20-100 µmole/egg (2). This level is quite high to expect it as an environmental contaminant. However, the possibility of contaminations with styrene, as an occupational and environmental agent can not be ruled out. Infact, the recent report warns that general population may also be exposed to styrene because of its usage in various household plastic items, like polystyrene foam cups and food containers, as styrene is found to be leaching into water and alcohol (3).

Our speculation is that, styrene may produce toxicity even at sublethal dose during embryonic development, since embryos are more susceptible due to lack of proper homeostatic mechanism. Very little attention has been paid to study the effect of environmental pollutants during embryonic development. In the present investigation, chick embryo is used as a model system to study the effect of known quantity of styrene (5-100 nmole/kg.egg wt.) as it can be injected directly into the incubating eggs, to eliminate the variables of maternal influence. Moreover, the relatively easy access

of pollutants to the chick embryo in comparison to mammals has encouraged us to use the chick embryo as a model system. Furthermore, the toxicity due to styrene treatment can be assessed at progressive stages of morphological and biochemical differentiations.

It is reported already that blood formation in embryo proceeds through several stages of development (4, 5), and the relative activities of various enzymes of heme biosynthesis are also changed during the course of development (6). Furthermore, amino levulinic acid synthetase, and amino levulinic acid dehydratase are reported to be the rate limiting enzymes of heme biosynthesis pathway (7-9). Certain chemicals (7, 9-11) and specific environmental stresses (12) are known to alter the activities of these enzymes. Earlier studies have also shown that there is a relationship between ALA synthetase activity and an alteration in cytochrome P-450 linked hepatic drug metabolizing enzymes (13-17). On the other hand, the binding of styrene oxide with hepatic cytochrome P-450, a heme protein, in _in vitro_ condition is reported (18). Thus, the possibility of interactions of styrene with the enzymes involved in heme biosynthesis process cannot be excluded. Therefore, it is of our interest to study if any harmful effect of styrene can be detected in chick embryo

at sublethal doses and to correlate the biochemical basis of styrene toxicity in relation to heme biosynthesis, specially at the level of ALA metabolism. Our aim is to understand whether styrene exposure has any effect on ALA metabolism which ultimately may control the heme biosynthesis. As an integral part of the programme, we have studied the hepatic level, the activity of ALA synthetase, responsible for the formation of ALA as well as the enzyme ALA dehydratase which utilizes ALA.

Using the observations in this study, we propose to use chick embryo as a model system for evaluating the teratogenecity of unknown compounds. Our study can be extrapolated to humans also as there is already a report on common teratogenic response of avian and rodent embryos (19).

The present study reveals some interesting facts concerning the control system operating in the pathway of heme biosynthesis. In future, we propose to study in the following directions:

1. Further determination of heme and cytochrome P-450 content from styrene treated chick embryo.
2. Various other enzymes of heme biosynthesis pathway; namely, uroporphyrinogen synthetase, uroporphyrinogen decarboxylase and heme synthetase. Attempts will be made to specify the role of heme biosynthesis in the control of cytochrome P-450 formation.

INTRODUCTION

Now a days, the growing contamination of the environment with different hazardous pollutants like styrene and styrene oxide is a matter of increasing awareness. Recently, there is a report that mothers, living in communities with PVC (polyvinyl chloride) production facilities, give birth to an excess number of children with congential defects (1). Infact, there is a possibility of contamination with styrene, a precursor of many thermoplastic polymers (poly-styrene), as it is widely used in household items. Large number of industrial workers are exposed during the synthesis and polymerization phase. Structurally styrene has a resemblance with vinyl chloride, which has recently been shown to be carcinogenic (20, 21), clastogenic (22) and mutagenic (23, 24).

In mammalian system styrene is converted into styrene oxide (phenyl ethylene oxide) by microsomal monooxygenase system (25, 26). Styrene oxide has been detected as a volatile component in tobacco concentrate (27), as a by-product in commercial samples of styrene chlorohydrin (28), and in effluent water from various latex manufacturing plants (29). It is used as an intermediate in the preparation of agricultural, cosmetics, coatings and the treatment of textile and fibres. Both styrene and styrene oxide have mutagenic activity in bacterial system (24, 30). Styrene can be characterized as a potent mutagen in production of forward mutation in mammalian

somatic cells in culture (31). It is more active than the well known mutagenic agent ethyl methane sulfonate (32). Styrene oxide is an epoxide and is potentially an alkylating agent. Alkene and arene oxides have been suggested to be ultimate toxicants which covalently bind to cellular macromolecules, leading to carcinogenesis (33), mutagenesis (34), and cytotoxicity (35).

Recently, it has been reported that the exposure of styrene and styrene oxide to Drosophila melanogaster induces recessive-lethals frequency (36). The use of phenobarbitone as a pretreatment results in a further increase in the recessive-lethal frequency after exposure to styrene and styrene oxide. Transient neurological impairments as well as eye and nasal irritation in human volunteers are reported when they are exposed to 376 ppm of styrene upto 7 hrs (37). The threshold limit value (TLV) for styrene is considered as 50-100 ppm (38). The distribution of styrene (577 µmole) and styrene oxide (46 µmole) was studied in different organs and was found to be more concentrated in liver, brain, kidney and deodenum than that in blood, lungs and spinal cord (39).

It has been reported recently (40) that the acute exposure of styrene at higher concentrations may produce irritation of the mucous membranes of the upper respiratory tract, nose and mouth, followed by symptoms of nausia cramps

and death due to respiratory center paralysis. The repeated contact may produce a dry, scaly and dermatitis. Electrophysiological investigations of a larger number of workers with low level of styrene exposure describe subtle electroencephalographic changes as well as decreased conduction velocity in the peripheral nerves (41, 42).

All these above studies on styrene toxicity have been made in adult animals at a high level of styrene exposure. Surprisingly little attention has been given to investigate the toxicity of styrene at sublethal doses to the embryo. However, there is one recent report (2), indicating that styrene and styrene oxide can produce malformation and 60% death of the embryos, if they are injected at 50 µmole and 25 µmole/egg respectively. Zlobina _et al_. (43) reported a high incidence of pathological births among pregnant women working in polystyrene plants in Russia. To evaluate the embryotoxicity of inhaled styrene. Regule (44) exposed pregnant rats for 4 hr daily at the level of 0.35, 1.2 or 12 ppm throughout gestation and an increased rate of absorption, was reported at all levels of exposure. Clinical manifestation of hypermenustrual syndrome is also reported in the female workers of polystyrene plants (45).

All these above findings suggest the possible embryotoxicity of styrene, although the actual mechanism

is still to be clarfied. In avian, especially in chick embryos, teratogens act in a specific manners leading to malformations (46) from the interference with localized metabolic needs. Cell division and differentiation in the developing embryos involve an increase of enzyme activity and protein synthesis. The enzymes participating in the embryonic development may be more susceptible to toxic levels of those agents which are able to permeate the placental barrier. The rate of permeability of agents depend on the physical and chemical properties, as well as lipid solubility and degree of ionization to reach the developing embryo. In the present investigation, chick embryo is used as a model system to study the interaction of styrene with embryonic development. It seems from the different observations that chick embryo is used extensively for the studies on normal embryonic development. This system has some advantages since known quantities of substances can be injected directly into incubating eggs avoiding troublesome variables of maternal influence.

On the other hand, it is reported that cytochrome P-450 P-450 linked monooxygenase, known as drug metabolizing enzymes are absent or very low in normal chick embryo liver (47), but can be induced by xenobiotics at an early stages of fetal development (48). In support of this view, styrene

has been found to bind predominantly to phenobarbital induced cytochrome P-450 in rat liver microsomes (49). Moreover, intraperitonial administrations of styrene oxide, causes a loss of liver microsomal cytochrome P-450 and decreased the ability of liver microsomes to metabolize drugs (50). This indicates that styrene may interact with a heme protein, cytochrome P-450. It is also known that a major fraction of heme synthesized in the liver is utilized for the formation of cytochrome P-450 (51). These observations suggest that an altered cytochrome P-450 level due to styrene interaction might be a reflection of impaired heme biosynthesis. Furthermore, the cellular heme levels are largely controlled by the relative activities of ALA synthetase and ALA dehydratase (7-9). A number of groups have postulated that ALA synthetase may be a significant factor in the regulation of heme containing enzymes(85, 86). Thus, our interest is to study the effect of styrene on chick embryo liver ALA synthetase and the next enzyme ALA dehydratase.

ALA synthetase is the first step in heme biosynthesis pathway which allows the condensation of glycine with succinyl-CoA (52-54) to form ALA. This mitochondrial enzyme is of particular interest since it has an inducible nature in mammalian liver (10, 55-60). A variety of substances have been shown to induce ALA

synthetase including drugs (84, 11) and steroids (7, 10). The increased synthetase activity is in correspondence with increasing level of microsomal oxidase activity (80). Such increase in hepatic activity of ALA synthetase lead to porphyrin accumulation and excretion. This is first observed in 1963 in guinea pigs treated orally with (DDC) 3,5-diethoxycarbonyl, 1,4-dihydrocollidine (10). The amount of ALA synthetase in both the cytosol and the mitochondrial fraction of cock liver is greatly increased by the administration of 2-allyl-2-isopropylacetamide (11), but this can be altered by cobalt injection, which produces biphasic changes in ALA synthetase activity with an initial depression of ALA synthetase followed in several hours by moderate increase in formation of enzyme (87, 88).

Studies in cultured chick embryo liver cells (7) have shown that the induction of ALA synthetase by drugs is sensitive to inhibitors of RNA and protein synthesis. It has been, therefore, postulated that alternation in enzyme activity with different drugs results due to changes in the rate of enzyme synthesis (7). However, other workers claim that the effect of chemical as inducer is to activate pre-existing ALA synthetase (90).

ALA synthetase has also shown to be inhibited by it's end product, heme (61, 89, 70, 71). According to them, heme

may regulate hepatic ALA synthetase with the help of (i) repression of the synthesis of enzyme or (ii) prevention of the transfer of newly synthesized enzyme from cytosol to mitochondria; or (iii) end-product inhibition of ALA synthetase. Experiments involving the use of protein synthesis inhibitors in chick embryo liver cell culture (62-64) suggest that the most important mechanisms is repression of ALA synthetase by heme at the translational stage of protein synthesis. However, a direct inhibition of the ALA synthetase by heme has also been reported with partially purified preparations of the liver enzyme (65, 66). Since heme synthetase, the last enzyme in the pathway is in close association with the ALA synthetase at or near the mitochondrial inner membrane (67, 68, 69), the possibility should be considered that end-product inhibition may also play a part in the regulation of ALA metabolism (70).

The quantitative determination of ALA is of importance not only in the measurement of ALA synthetase activity but also in the diagnosis of several clinical disorders including intermittent porpyria (72-75).

The elevation of ALA has been reported both in plasma (76-79) and urine (80) of lead poisoned animals. The

inhibitory effect of lead in ALA synthetase was reported earlier, whereas, recent studies have shown an activation of the enzyme by lead (64, 83).

ALA is further metabolized by the enzyme ALA dehydratase (E.C. 4.2.1.24). Two molecules of ALA are condensed to produce porphobilinogen (PBG). Presence of either reduced glutothione (GSH) or dithiothretol (DTT) is required for the activation of the enzyme system of ALA dehydratase (91). This enzyme plays an important role in the formation of porphyrins, hemes and chlorophylls. It is a sulfydral enzyme, found in the cytoplasm of all aerobic cells. ALA dehydratase has been extensively studied in a number of animals (92, 93) and microorganisms (94-96).

ALA dehydratase is widely accepted as the most sensitive indicator of lead poisoning in man and animals(97-100). It is well known that low concentrations of lead effectively inhibits this enzyme (9, 101-105). The other studies also indicate the inhibitory role of heavy metals like, mercury, cadmium, copper, silver and other thiol agents (106).

In contrary to that, the activity of ALA dehydratase has increased in the liver of animals treated with porphyrogenic drugs, e.g., 3,5-diethoxycarbonyl, 1,4-dihydrocollidine (DDC), 2-allyl-2-isopropylacetamide (AIA) and griseofulvin

(59, 107-109). It has been shown recently that the ALA dehydratase is a zinc-depend enzyme (108-110). Earlier studies show that zinc can activate ALA dehydratase both *in vivo* and *in vitro* (108, 110). However, in contrast to ALA dehydratase from other species, the mouse enzyme is activated by EDTA, Hg^{2+} and Fe^{2+} ions (92). ALA dehydratase is also increased considerably in the blood of rats poisoned by successive injection of phynylhydrazine and reaches a maximum corresponding to the number of reticulocytes (113). It is evident that blood lettings in rats could stimulate erythropoiesis and a subsequent increase in the activity of ALA dehydratase due to a greater number of circulating reticulocytes (8). The third important step in heme biosynthesis is the enzyme heme synthetase which catalyses the incorporation of ferrous ion into the porphyrin ring structure (114).

In the present investigation, the development and mortality of chick embryo, treated with varying sublethal doses of styrene at different developmental stages have been studied. A distinct correlation between toxicity development and biochemical basis of it has yet to be established. Attempt has been made to understand the biochemical basis of styrene toxicity, specially at the

level of ALA metabolism. Furthermore, our interest has been extended to correlate the embryotoxicity of styrene with alteration of ALA metabolism. In future, we propose to study the interaction of styrene with other enzymes involved in heme biosynthesis and also at the level of hemeprotein, cytochrome P-450.

MATERIALS AND METHODS

Amino levulinic acid, p-dimethylaminobenzaldehyde, ethylacetoacetate, trizma base, glycine, bovine serum albumin, dithiothretol, porphobilinogen were purchased from Sigma Chemical Co., U.S.A. and some of the other chemicals were purchased from BDH (AR grade). Folin ciocalteu reagent was purchased from Biochemical Unit, Patel Chest Institute, Delhi. Ethyl acetate (E. Merck), and other chemicals were obtained from commercial sources in highest purity availabile. Styrene was purchased from SISCO. Microsyringe has been purchased from Hamilton & Co., Switzerland.

Maintanance of the Eggs :

Freshly laid, white leghorn strain zero day old eggs were obtained from Govt. Poultry Farm, Satbari, New Delhi. Immediately after bringing the eggs into laboratory, they were cleaned thoroughly with wet cotton, weighed and marked properly for future experiment. After that, they were placed in BOD incubator, the temperature of which was already maintained at 37°C. The humidity of incubator was maintained at 65% by keeping a tray, full of water in presence of a hygrometer.

Eggs were rotated manually once a day and were examined through the candler every day for their proper growth and viability. Unfertilized and dead eggs were discarded on

3rd day of incubation and the rest were incubated for 21 days unless it is mentioned.

METHODS

Treatment of the chick embryos for development of styrene toxicity :

Injections of styrene, dissolved in ethanol and then diluted with olive oil at varying concentrations of 5.0 nmole to 100 nmole/kg. egg wt. in 50 µl were given on different days of development (3rd, 7th and 14th day). These doses can also be expressed as 0.52 to 10.4 ppm. Control eggs were given the same amount of solvent injection on the same day of incubation. For giving an injection the eggs were wiped with distilled alcohol, dried and placed on the candler. With the help of passing light from the candler, the egg shell was scrapped and made a thinner hole with the scalpel. Injections were made into the yolk sac of embryos with the help of microsyringe. Immediately, after giving injection, scrapped area was covered with surgical tape to prevent contamination. Sterile syringe, scalpel were used throughout the experiment. After giving the injection, eggs were kept in the incubator with minimum disturbance. Then, they were examined through candler for the mortality everyday. Either after hatching on 21st day or the day when mortality was observed, embryos were opened and examined visually for viability, size, growth and malformations.

Treatment of chick embryos for the studies of ALA metabolism under styrene exposure :

For studying the effect of styrene exposure on ALA metabolism, the embryos were injected on 7th or 14th day of incubation with different concentrations of styrene (5, 25, 50 and 100 nmole/kg. egg wt.). Embryos were opened on 18th day of incubation and liver was collected for further experiments.

Measurement of hepatic ALA level :

Hepatic ALA concentrations were measured according to the method of Granick *et al.* (115) modified by Piedra *et al.* (116). After deproteinizing liver homogenate, supernatant was allowed to react with 0.2 ml of ethyl acetoacetate in presence of 1 M acetate buffer pH 4.6 in a boiling water bath for about 10 minutes. After attaining the room temperature, 3 ml of ethylacetate was added and tubes were shaken for 10 secs and then centrifuged at 4,000 x g to extract the amino levulinic acid pyrrole.

2 ml of the upper layer and equal volume of modified Ehrlich reagent was added. The absorbance was measured at 553 nm after 10 minutes.

Estimation of protein :

Protein was assayed by the method of Lowry *et al.* (117).

Reagents used :

Protein reagent:

Reagent A : 2% Na_2CO_3 in 0.1 NaOH.

Reagent B : 0.5% $CuSO_4.4H_2O$ in 1% sodium or potassium tartarate. 50 ml of reagent A was mixed with 1 ml of reagent B.

Folin Ciocalteu reagent : A mixture consisting of 10 gm Na-tungstate ($Na_2WO_4.2H_2O$), 7 ml of water, 5.0 ml of 85% phosphoric acid and 10 ml of concentrated hydrochloric acid was refluxed gently for 10 hours. 15 gm lithium sulfate, 5 ml of water and a few drops of bromine water were added. The mixture was boiled for 15 minutes without condenser to remove excess bromine. Then, it was cooled, diluted to 100 ml and filtered. The reagent should not have a greenish tint.

Procedure :

To 0.25 ml of solution containing bovine serum albumin (25 - 200 ug), 2.5 ml of protein reagent was added and after 10 minutes, a further addition of 0.25 ml of folin's reagent was made. After 10 minutes, the absorbance was measured at 660 nm. The colour produced is proportional to the concentration of protein in the solution assayed. The standard curve is shown in Fig. 1.

Assay of ALA synthetase activity from liver :

Hepatic ALA synthetase activity of chick embryo was

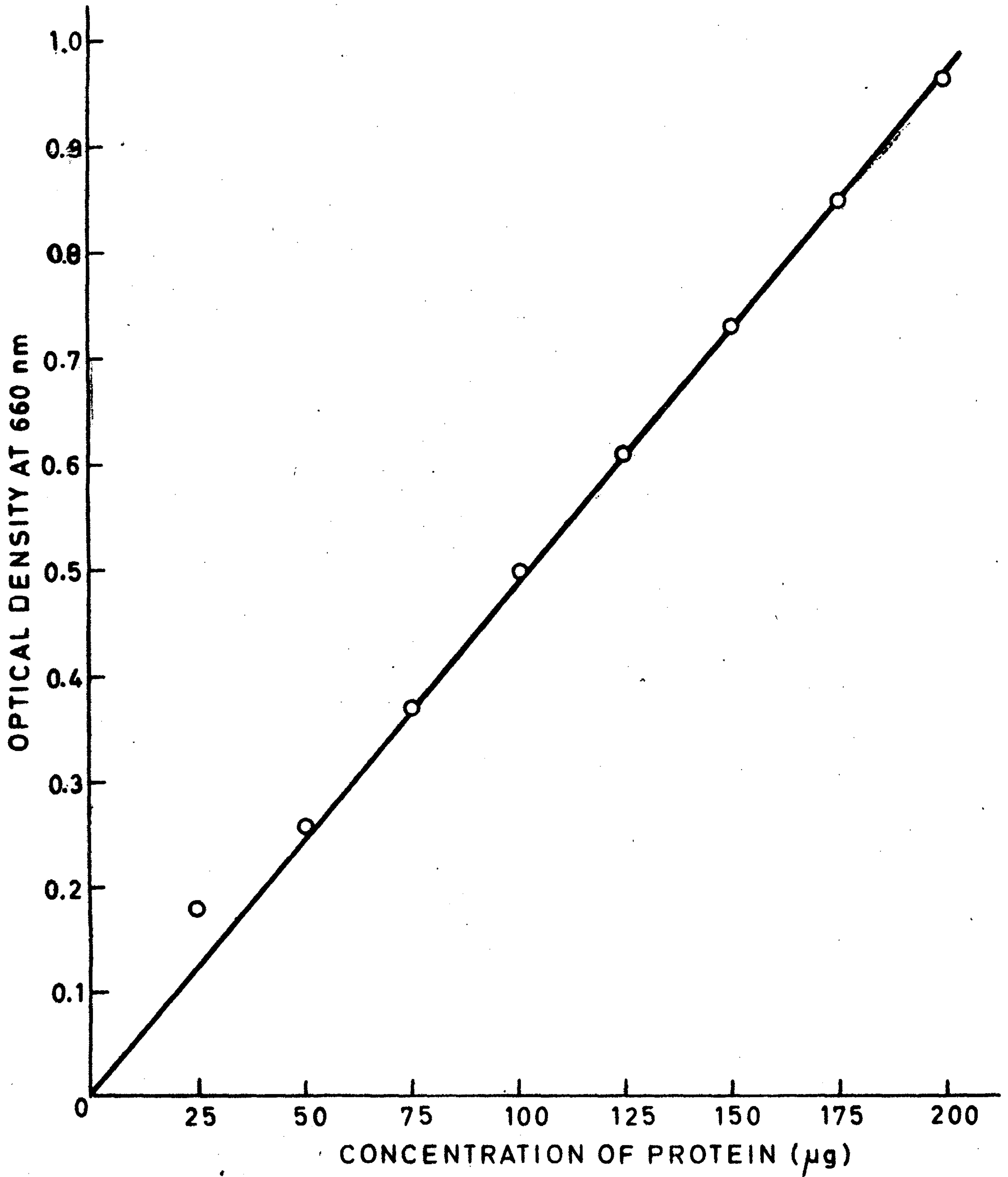

Fig. 1: Standard curve for protein.

measured according to the method of Piedra *et al.* (116). ALA synthetase catalyses an irreversible conjugation of succinyl Co-A and glycine to from ALA. ALA synthetase activity is measured by the rate of formation of ALA.

Reagents used :

0.9% NaCl solution containing 0.5 mM EDTA and 10 mM Tris-HCl, pH 7.4;

50 mM Tris-HCl buffer, pH 7.2.

16 mM EDTA.

Glycine (200 mM) : 1.2016 gm of glycine was dissolved in 10 ml of 0.03 M Tris-HCl buffer, pH. 7.2.

25% TCA : 25 gm of Trichloroacetic acid was dissolved in 100 ml of distilled water.

1 M acetate buffer, pH 4.6.

Procedure:

Chick liver was pooled and homogenised rapidly with 3 volumes of 0.9% NaCl solution containing 0.5 mM EDTA and 10 mM Tris-HCl, pH 7.4 to make 25% homogenate and was used as enzyme source. The incubation mixture contained (in μmole) in a final volume of 2.0 ml; glycine, 200; EDTA, 20; Tris-HCl (pH 7.2), 150 and 0.5 ml of enzyme preparation. For carrying the blank the contents were mixed in a vortex mixture and 0.5 ml of 25% TCA was added in the beginning of incubation.

Incubation was terminated by addition of 0.5 ml of 25% TCA. After centrifuging for 10 minutes at 6,600 x g. 1 ml of the clear supernatant was further assayed for ALA formed.

Estimation of Amino levulinic acid :

ALA was determined according to the method of Granick et al. (115), modified by Piedra et al. (116) by Ehrlich reaction.

Reagents used:

Stock ALA solution: 8.380 mg of pure crystal ALA HCl was dissolved in 100 ml of distilled water to get 5 mM solution.

Preparation of modified Ehrlich reagent: 1.0 gm of p-dimethylaminobenzaldehyde was dissolved in about 30 ml of glacial acetic acid. 8.0 ml of 70% perchloric acid was added and the solution was diluted to 50 ml with acetic acid. The reagent is unstable and should be used on the day on which it was made and remainder was discarded.

1M acetate buffer, pH 4.6.

Ethyacetoacetate: Imported from Sigma Chemical Co., U.S.A.

Ethylacetate : E. Merck.

Procedure:

1.5 ml of sample containing ALA was made upto 4.0 ml with 1M acetate buffer, pH 4.6. Then 0.2 ml of ethylaceto-

acetate was added and allowed to react in a boiling water bath for 10 minutes. After cooling, 3 ml of ethyl acetate was added and tubes were shaken for 10 secs and centrifuged at 4,000 x g to extract the amino-levulinic-acid-pyrrole.

2 ml of the upper layer and equal volume of modified Ehrlich reagent were mixed together and the absorbance at 553 nm was measured after 10 minutes, against a blank containing 2 ml of ethyl acetate and 2 ml of Ehrlich reagent. The standard curve for ALA is shown in Fig. 2. ALA synthetase was expressed as p mole of ALA formed/mg protein/90 min at 37°C.

Assay of liver ALA dehydratase :

The enzyme ALA dehydratase mediates irreversibly the dehydratative cyclization of ALA to porphobilinogen. For assaying the dehydratase activity, liver homogenate was made in 0.02 M phosphate buffer, pH 6.7 containing 0.15 M KCl (118). The assay of ALA dehydratase is based on measurement of the amount of porphobilinogen produced from the substrate ALA with the help of modified Ehrlich reagent as described by Granick et al. (115). The reaction was carried out in presence of dithiothretol (DTT), as an activator, because reduced form of glutathione is an essential component for ALA dehydratase activity. The reaction was stopped by the addition of TCA containing $HgCl_2$

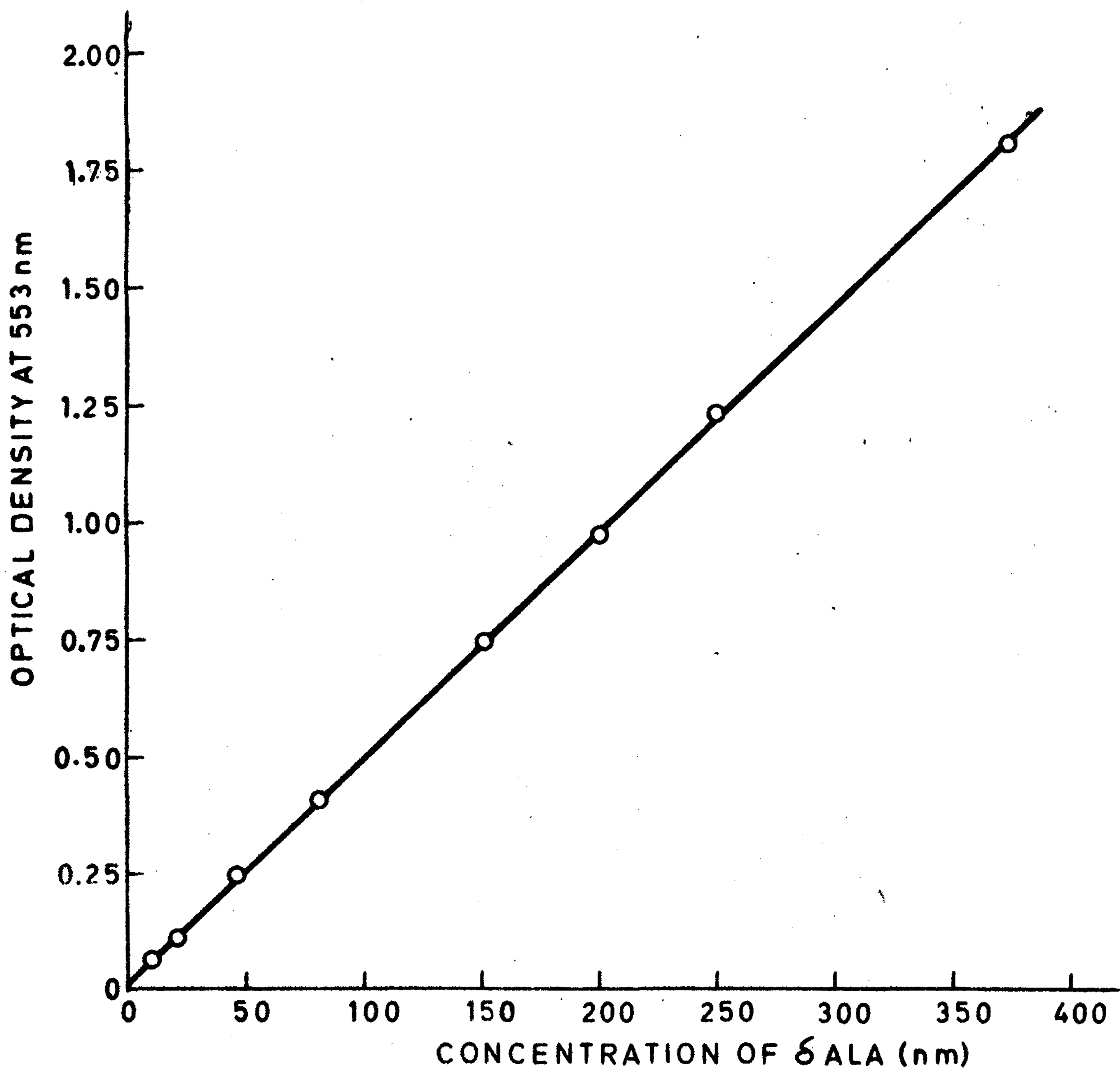

Fig. 2: Standard curve for Amino levulinic acid (ALA).

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and centrifuged. An aliquot of the supernatant was added to Ehrlich's reagent and the resulting Ehrlich colour salt, a condensation product of p-dimethylaminobenzaldehyde with porphobilinogen was measured at 553 nm. The $HgCl_2$ in the TCA mixture was used to remove sulfhydryl compounds from reaction mixture in order to avoid frequent interaction of it with the Ehrlich reagent. The pH of the reaction mixture was chosen 6.5 because it is the optimum pH of ALA dehydratase and also conversion of porphobilinogen to uroporphobilinogen becomes negligible as the enzyme, UPG I synthetase, responsible for this conversion has optimum, pH 7.8. Sodium phosphate was used because the potassium forms an insoluble perchlorate salt with the Ehrlich reagent which interferes with colour reaction.

Reagents used:

0.2 M sodium phosphate buffer, pH 6.7 containing 0.15 M KCl.

0.1 M sodium phosphate buffer, pH 6.5.

2 mM ALA HCl: 1.67 mg of ALA HCl was dissolved in 2.5 ml of 0.1 M sodium phosphate buffer, pH 6.5.

20 mM Dithiothretol: 7.75 mg of DTT was dissolved in 25 ml of 0.1 M sodium phosphate buffer, pH 6.5.

TCA-$HgCl_2$ solution : 4 gm of TCA and 2.7 gms of $HgCl_2$ were dissolved in 100 ml of the distilled water.

Modified Ehrlich reagent: 1 gm of p-dimethylamino-benzaldehyde was dissolved in 30 ml of glacial acetic acid

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and then 8 ml of 70% perchloric acid was added. It was made upto 50 ml with acetic acid. It was prepared just before the addition of the reagent.

Procedure :

Chick liver was pooled and homogenized rapidly in 0.02 M sodium phosphate buffer, pH 6.7 containing 0.15M KCl to make 20% homogenate and was used as enzyme source. The assay mixture contained (in μmoles) in a total volume of 500 μl: ALA, 0.2; dithiothretol, 2.0; sodium phosphate buffer (pH 6.5) 10.0 and enzyme. After incubation for the 1 hr at 37°C, the reaction was stopped by adding 2 ml of TCA-$HgCl_2$ solution. After centrifugation for 10 mins, 1.5 ml of the supernatant was taken for the spectrophotometric estimation of porphobilinogen by Ehrlich reaction. Standard zero minute enzyme control and dithiothretol control were assayed in the same manner.

Estimation of porphobilinogen by Ehrlich reaction :

Reagents used:

4 mM porphobilinogen.

0.1 M sodium phosphate buffer, pH 6.5.

Modified Ehrlich reagent: 1 gm of p-dimethylaminobenzaldehyde was dissolved in 30 ml of glacial acetic acid and then 8.0 ml of 70% perchloric acid was added. It was prepared every time freshly.

Procedure :

To 1.5 ml sample containing porphobilinogen, 1.5 ml of freshly prepared modified Ehrlich reagent was added. After 10 minutes, the absorbance at 553 nm was measured in spectrophotometer. The colour produced in this reaction is proportional to porphobilinogen content of the solution assayed. The standard curve is given in Fig. 3.

ALA dehydratase activity is expressed as p mole of porphobilinogen formed/mg of protein/hr at 37°C.

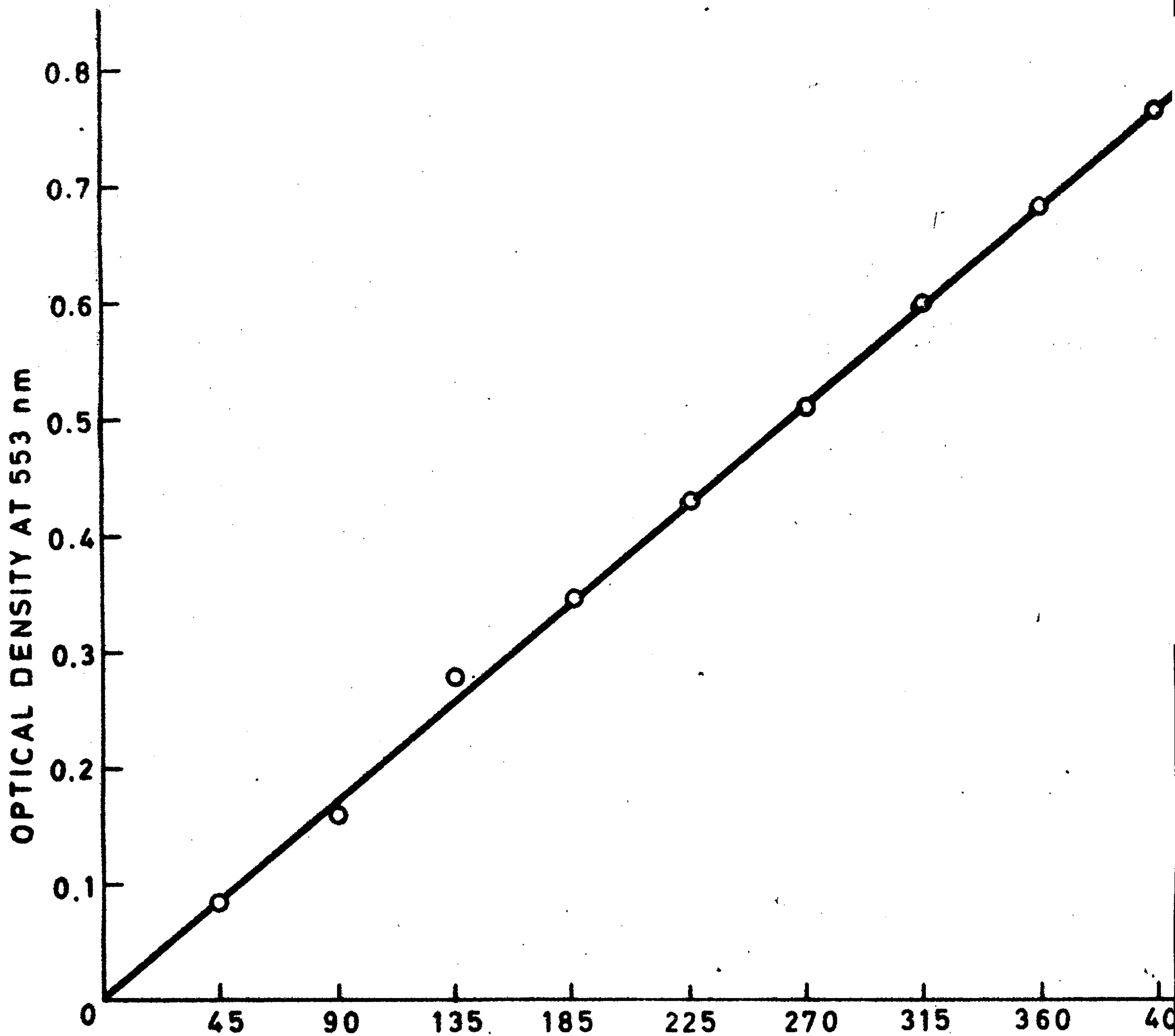


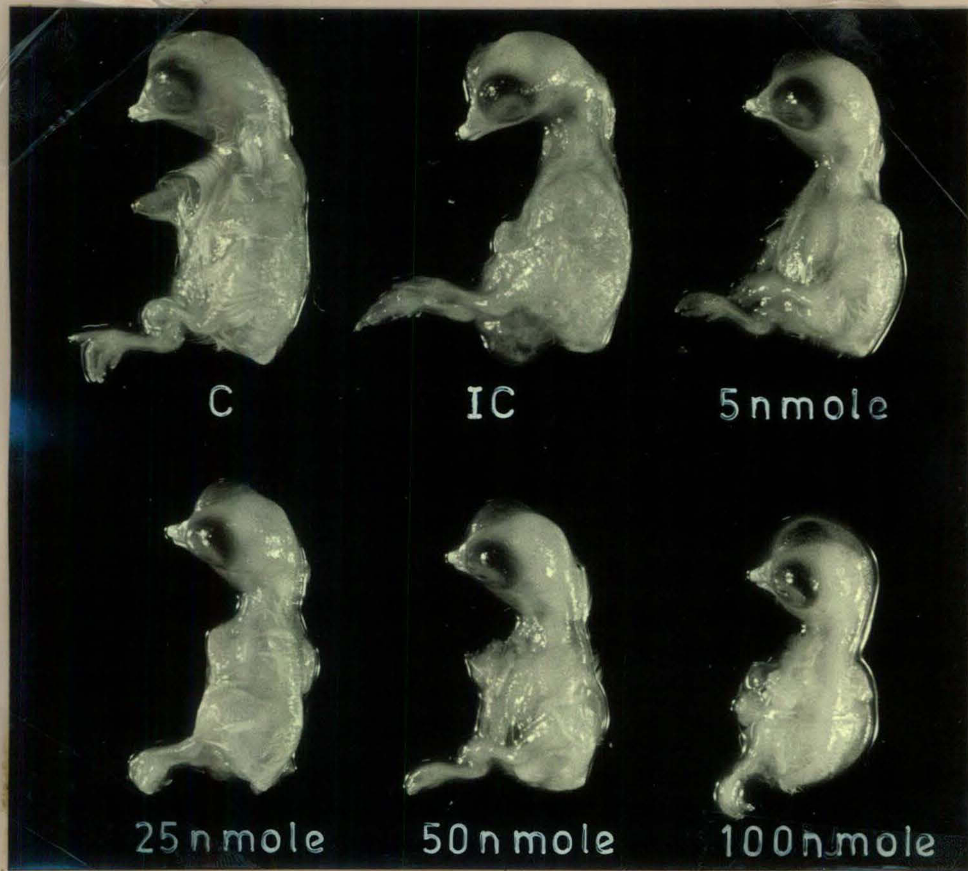
Fig. 3: Standard curve for porphobilinogen (PBG).

RESULTS

Development and mortality of chick embryo under Styrene Treatment :

Growth retardation and improper development in styrene treated chick embryos was observed as there was reduction in size and weight as compared to the controls (Figs. 4-6). However, there was no visual appearance of malformations (Fig. 4). The effect of styrene injection (5-100 nmole/kg. egg wt.) on 7th or 14th day of development on the length of embryo was shown in Fig. 5. The embryos were opened on 18th day of incubation and length was measured and expressed as percent decrease from normal value. The length of 18 day old control embryo was 9 cm, but when 5, 25, 50 and 100 nmole/kg. egg wt. of styrene was injected on 7th day of the development, the length of the embryo was reduced by 10%, 16%, 18% and 22% respectively. Similarly, when the same doses of styrene was injected on 14th day of development, the length on 18th day was observed with the reduction of 6%, 9%, 13% and 15%. Styrene treated embryos have also lost the body weight (Fig. 6). The weight of 18 day old embryos was 36.5 gm, but when (5, 25, 50 and 100 nmole/kg. egg wt.) of styrene was injected on 7th day of the development the weight of the embryos was decreased by 16%, 23%, 26% and 30% respectively.

Fig. 4: Typical development of 14 day chick embryo injected with styrene on 7th day (C: control; IC : injected control; 2, 25, 50 and 100 nmole of styrene/kg. egg wt.).



C
IC
5nmole
25nmole
50nmole
100nmole

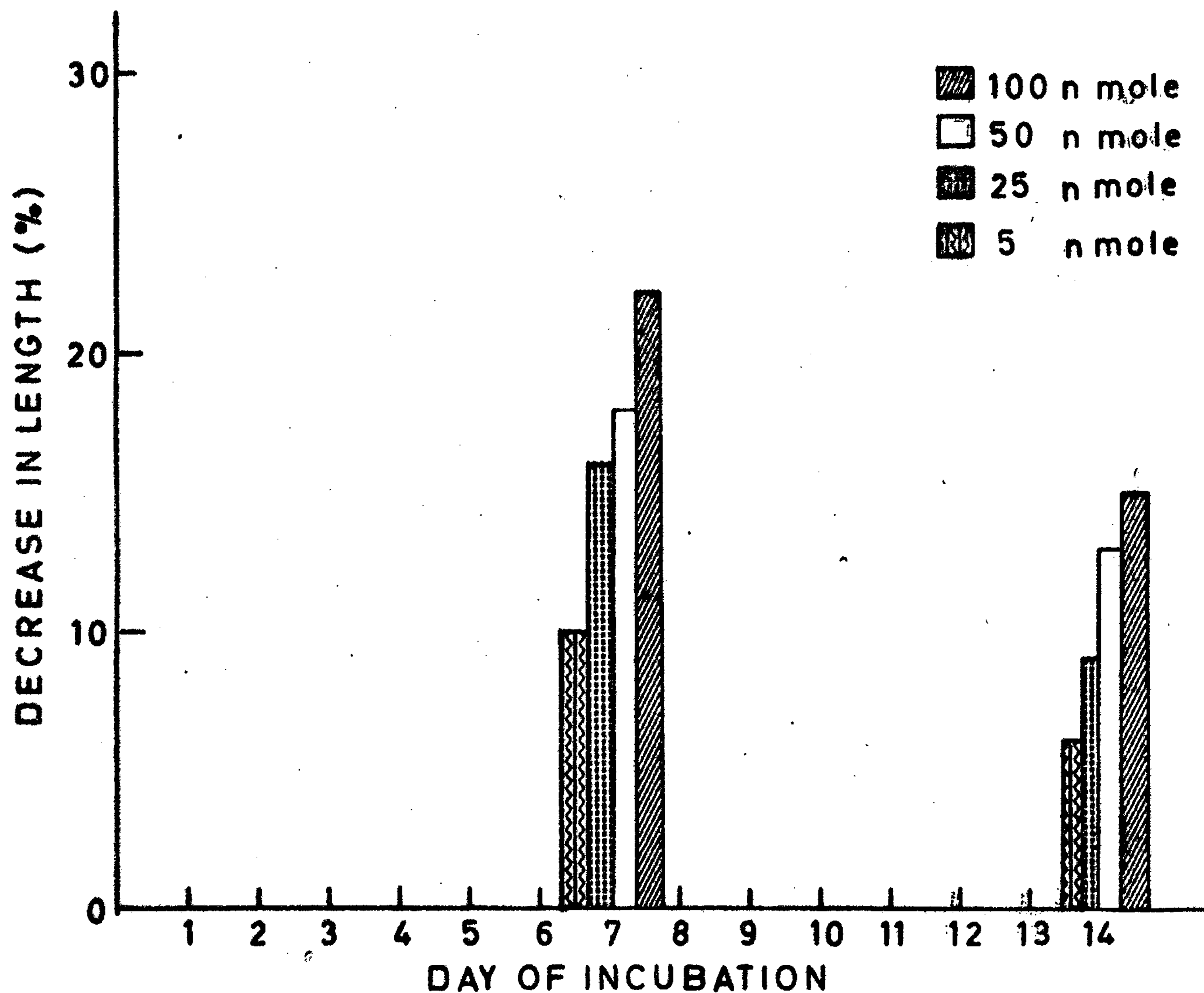


Fig. 5: Effect of styrene (5-100 nmole/kg. egg wt.) injection on the length of 7th day or 14th day old embryo in comparison with control.

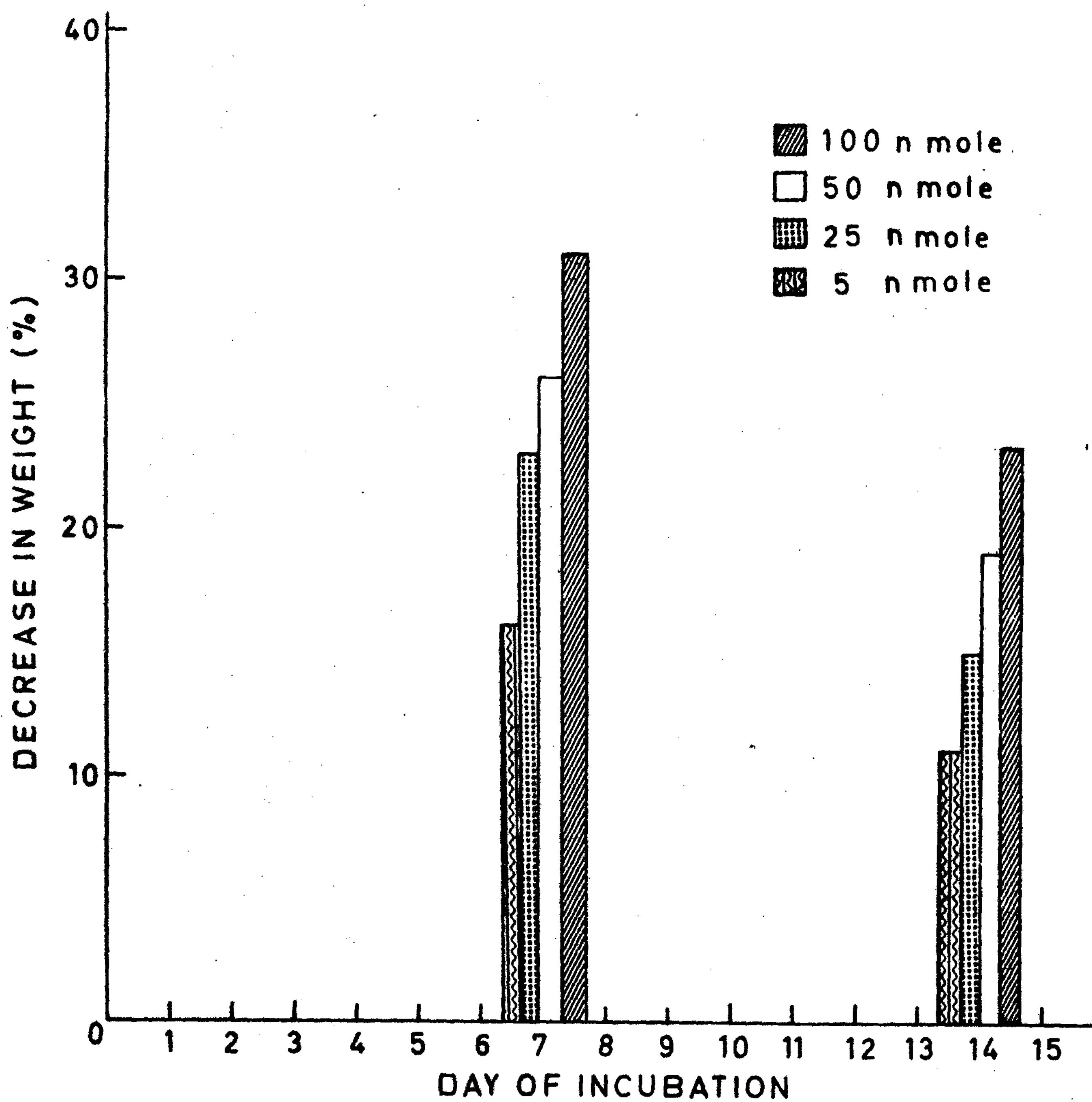


Fig. 6: Effect of styrene (5-100 nmole/kg. egg wt.) injection on the weight of 7 day or 14 day old embryo.

Similarly, when styrene (5-100 nmole/kg. egg wt.) was injected on 14th day, the weight on 18th day/was obtained with reduction of 6%, 9%, 13% and 15%. This data suggests that embryos were gaining resistance towards external agents with the ages.

The effect of varying concentrations of styrene injection to chick embryo during different days of development (3rd, 7th and 14th day) on mortality rate of chick embryo is presented in (Tables I-III). In each experiment, one group of eggs was kept as uninjected control and to another group of eggs, the same amount of solvent (used for styrene solution) was injected on the same day. These eggs were considered as injected controls.

The effect of varying concentrations of styrene (5-100 nmole/kg. egg wt.) injection to 3 day old embryo in the mortality rate was shown in Table I. A gradual increase in the death rate of chick embryos was observed with increasing concentration of styrene. In the uninjected control group, all the embryos were alive throughout the experiment, whereas in injected controls 83% of the embryos were survived. Besides, the rise in death rate with increasing concentration of styrene injection, the time required for the death was also varied accordingly with different doses. The embryos that received less amount of

TABLE I

EFFECT OF STYRENE INJECTION ON MORTALITY OF 3 DAY OLD CHICK EMBRYO

Treatment with styrene (nmole/kg.egg wt.)	Rate of mortality of chick embryo* Dead embryo %	Death occurred on the day
Control	0	-
Control (injected)	17	17-18
5	60	10-11
25	66	7-8
50	66	6
100	77	6

*Mortality was checked evry day thæough candler and was confirmed visually by opening the embryo. A minimum of 30 eggs were used in each group.

styrene(5 and 25 nmole/kg. egg wt.) died on 7-11th day of development. Whereas embryos receiving a higher concentration of styrene (50 and 100 nmole/kg. egg wt.) died on 6th day of development. This clearly indicates that styrene, injected to the yolk sac of the fertilized egg, had an entry into the embryo. Also varying concentrations of styrene (5-100 nmole/kg. egg wt.) injection were tested on 7th day of development (Table II). In this experiment, too, the death of styrene treated chick embryo was linearly proportional to the increasing concentration of styrene. In this case, 3% and 13% death rate was observed in uninjected control and injected control embryos respectively. Besides, decreased viability, there was alot of difference in time, that was required for the death. The embryo, where less doses of styrene (5 and 25 nmole/kg. egg wt.) was given, death occurred and17th and 19th day of incubation, but with higher doses of styrene (50 and 100 nmole/kg . egg wt.), there was an advancement of death i.e. on 9-15th day of development.

In the Table III, the effect of same concentrations of styrene to chick embryo was studied on the 14th day of development. In this experiment, 16%, 16% and 23% mortality was observed in 5, 25 and 50 nmole of styrene injected chick embryos respectively. The maximum mortality of 34% was

TABLE II

EFFECT OF STYRENE INJECTION ON MORTALITY OF 7 DAY OLD CHICK EMBRYO

Treatment with styrene (nmole/kg. egg wt.)	Rate mortality of chick embryo[a] Dead embryo %	Death occurred on the day
Control	3	21
Control (injected)	13	21
5	32	18-19
25	43	17-18
50	64	13-15
100	70	9-10

[a] Mortality was checked every day through candler and was confirmed visually by opening the embryo. A minimum of 30 eggs were used in each group.

TABLE III

EFFECT OF STYRENE INJECTION ON MORTALITY OF 14 DAY OLD CHICK EMBRYO

Treatment with styrene (nmole/kg. egg wt.)	Rate of mortality of chick embryo* Dead embryo %	Death occurred on the day
Control	0	-
Control (injected)	0	-
5	16	21
25	16	20
50	23	19
100	34	17-18

*Mortality was checked every day through candler and was confirmed visually by opening the embryo. A minimum of 30 eggs were used in each group.

obtained with 100 nmole styrene injection. No death was observed in both the groups of uninjected control and injected control. Apart from the death rate, there was considerable variation in the time requirement for the occurance of death. These results indicate that 14 day old embryos were less susceptible to styrene intoxication as compared to 3 day and 7 day old embryos.

LD_{50} values of styrene tested on 3 day, 7 day and 14 day old embryo were determined as(25, 50 and 100 nmole/kg. egg wt.) respectively. Dose response data was dependent critically on the age of embryo. Experiments were carried in which different doses of styrene was injected into the eggs on either 3rd or 7th or 14th day of development and percentage of live embryo in experimentalggroups were plotted against the day on which injection was given (Fig. 7). As shown in Fig. 7, the same dose of styrene was more toxic at the initial stages of development than the later stages, since 5 nmole styrene injection appeared to produce 40%, 68% and 84% live embryos on 3rd, 7th and 14th day respectively. Similar pattern of mortality in response to styrene injection on different incubation days i.e. 7th and 14th at the level of 25, 50 and 100 nmole/kg. egg wt. was obtained. However, maximum embryos upto 84% remained alive in the case of 5 and 25 nmole styrene, whereas 77% and 66% of

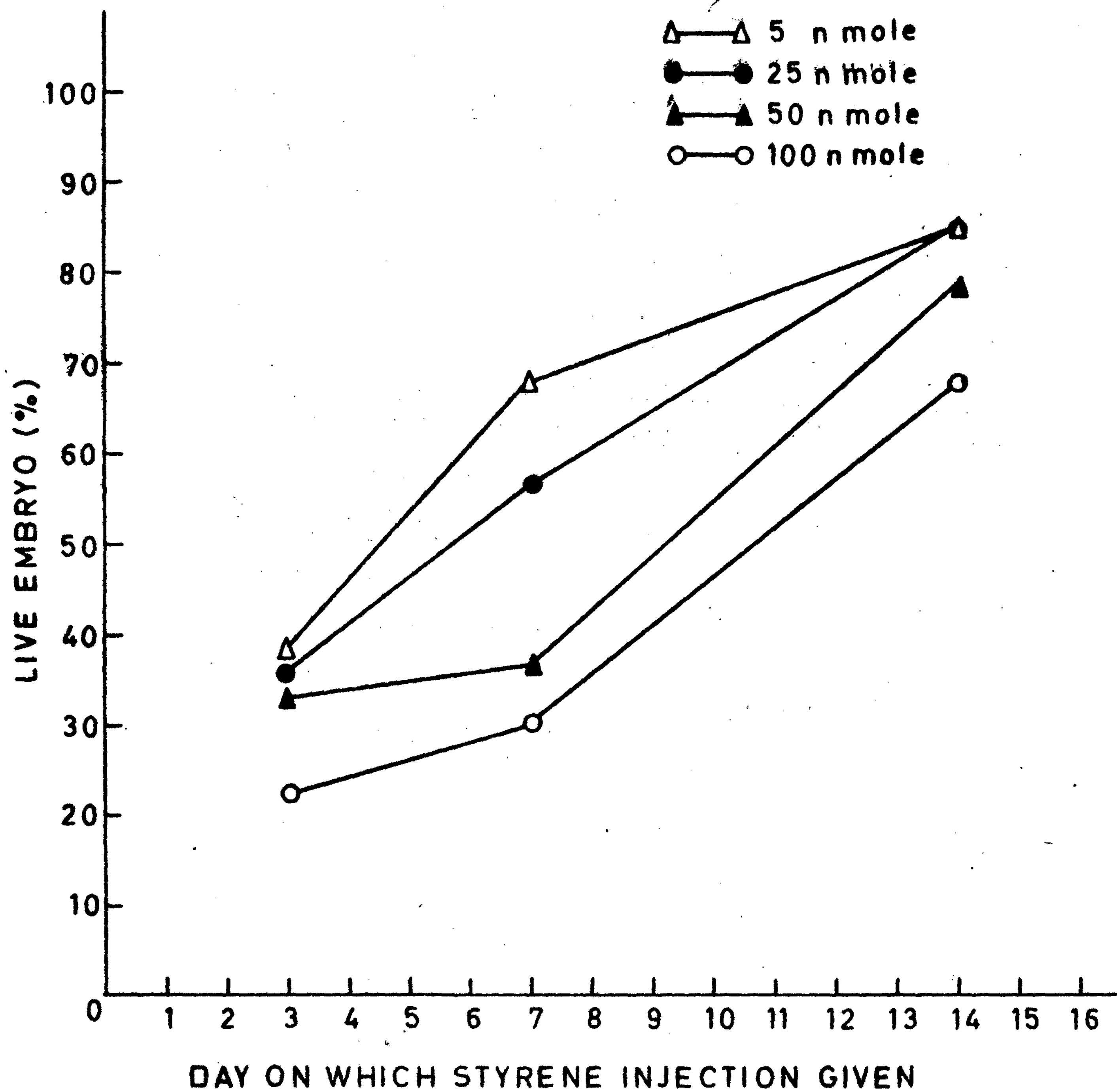


Fig. 7: The effect of varying concentrations of styrene injection on the survivality of embryos at different developmental stages.

embryos survived with 50 and 100 nmole of styrene when injection was made on 14th day of development. These experimental evidences also indicate that the mortality of the embryo at the same dose of styrene was very much dependent on the age of embryo.

Amino levulinic acid metabolism under styrene exposure:

The hepatic ALA level of chick embryo treated with styrene on 7th and 14th day of development was measured (Tables IV and V). These results of Table IV show that ALA level has decreased to an extent of 14%, 25%, 37% and 56% of normal value significantly in the liver of embryos treated with styrene at the level of 5, 25, 50 and 100 nmole/kg. egg wt. respectively on 7th day of development. Similarly, decreased ALA level was observed in chick embryo liver exposed with same doses of styrene on 14th day of development (Table V). From Tables IV and V, it was evident that fall in ALA level due to styrene exposure was marked in 7 day old embryo as compared to that of 14 day old. A 14%, 25%, 37% and 56% decrease in ALA level was found in 5, 25, 50 and 100 nmole/kg. egg wt. of styrene treated 7 day dold chick embryo, whereas 11%, 13%, 27% and 34% fall of ALA level was observed with the same dose of styrene treatment on 14th day of development.

TABLE IV

HEPATIC ALA LEVEL IN STYRENE TREATED 7 DAY OLD CHICK EMBRYO

Treatment with styrene (nmole/kg. egg wt.)	ALA level[a] p mole of ALA/mg protein	Decrease %	P value <
Control	758.0 ± 0.50[b]	-	-
Control (injected)	754.8 ± 0.30	-	-
5	653.5 ± 0.20	14	0.001
25	571.4 ± 0.30	25	0.001
50	480.0 ± 0.40	37	0.001
100	335.4 ± 0.50	56	0.001

a - represents mean values of ALA level ± Std. error.

b - total number of embryos in each group were not less than 12.

TABLE V

HEPATIC ALA LEVEL IN STYRENE TREATED 14 DAY OLD CHICK EMBRYO

Treatment with styrene (nmole/kg egg wt.)	ALA level[a] p mole of ALA/mg protein	Decrease %	P value <
Control	754.6 ± 0.40[b]	-	-
Control (injected)	753.0 ± 0.30	0	-
5	673.0 ± 0.40	11	0.001
25	677.1 ± 0.50	12	0.001
50	552.6 ± 0.40	27	0.001
100	503.2 ± 0.60	34	0.001

a - Represents mean value of ALA level ± Std. error.

b - Total number of embryos in each group were not less than 12.

Since there is an alteration in ALA level with styrene treatment, the enzyme ALA synthetase, responsible for ALA formation was studied. The effect of styrene (5-100 nmole/kg. egg wt.) injection to 7 day and 14 day old embryo on hepatic ALA synthetase was shown in Tables VI and VII. There was significant inhibition of ALA synthetase activity in styrene treated chick embryo as compared to that of control. Moreover, the inhibition of hepatic ALA synthetase activity was increased proportionaly with the concentration of styrene injection as there was further inhibition of ALA synthetase activity with 100 nmole than with 5, 25 and 50 nmole of styrene injection. Again, from the comparison between the Tables VI and VII, it was found that the degree of inhibition of ALA synthetase activity due to styrene exposure was less in 14 day old embryo as compared to 7 day old embryo.

Table VIII shows the effect of styrene injection, given to 7 day old embryo on the activity of hepatic ALA dehydratase, the next step of the heme biosynthesis. The results from this experiment suggests the stimulatory role of styrene on hepatic ALA dehydratase. It has been also shown that the stimulation of the enzyme activity has increased with increasing concentration of styrene injection. ALA dehydratase activity in 14 day old styrene treated

TABLE VI

HEPATIC ALA SYNTHETASE ACTIVITY IN 7 DAY OLD STYRENE TREATED CHICK EMBRYO

Treatment with styrene (nmole/kg egg wt.) 6	ALA synthetase activity[a] p mole of ALA formed/mg protein/ 90 minutes at 37°C	Inhibition %	P value <
Control	825.0 ± 0.60[b]	-	-
Control (injected)	762.0 ± 0.30	-	-
5	557.0 ± 0.10	33	0.001
25	370.0 ± 0.50	56	0.001
50	352.0 ± 0.30	59	0.001
100	148.0 ± 0.10	82	0.001

a - Represents mean values of ALA level ± Std. error.

b - Total number of embryos in each group were not less than 12.

TABLE VII

HEPATIC ALA SYNTHETASE ACTIVITY IN 14 DAY OLD STYRENE TREATED CHICK EMBRYO

Treatment with styrene (nmole/kg egg wt.)	ALA synthetase activity[a] p mole ALA formed/mg protein/90 min at 37°C	Inhibition	P value <
Control	939.7 ± 0.20[b]	-	-
Control (injected)	881.0 ± 0.30	-	-
5	638.0 ± 0.20	32	0.001
25	593.0 ± 0.20	37	0.001
50	361.0 ± 0.20	61	0.001
100	293.0 ± 0.50	69	0.001

a - Represents mean values of ALA level ± Std. error.

b - Total number of embryos in each group were not less than 12.

TABLE VIII

ALA DEHYDRATASE ACTIVITY IN 7 DAY OLD STYRENE TREATED CHICK EMBRYO

Treatment with styrene (nmole/kg egg wt.)	ALA dehydratase activity[a] p mole formed/mg protein/ 1 hr at 37°C	Stimulation %	P value <
Control	672.28 ± 4.00[b]	-	-
Control (injected)	693.85 ± 1.00	-	-
5	820.00 ± 5.00	22	0.001
25	929.65 ± 3.00	38	0.001
50	946.42 ± 7.00	41	0.001
100	1110.93 ± 3.00	65	0.001

a - Represents mean value of ALA level ± Std. error.

b - Total number of embryos in each group were not less than 12.

chick embryos has been presented in Table IX. The similar trend in stimulation of ALA dehydratase by styrene treatment has been confirmed in 14 day old chick embryo, although there was less stimulation occurred.

The present results suggest that styrene toxicity in chick embryo is highly linked up with ALA metabolism. It decreases the activity of ALA synthetase which is responsible for the formation of ALA and it stimulates ALA dehydratase activity, which is responsible for ALA degradation. So, ultimately the reduction in ALA level has been procceded under this experimental condition.

TABLE IX

ALA DEHYDRATASE ACTIVITY IN 14 DAY OLD STYRENE TREATED CHICK EMBRYO LIVER

Treatment with styrene (nmole/kg egg wt.)	ALA dehydratase activity[a] p mole formed/mg protein/ 1 hr at 37°C	Stimulation %	P value $<$
Control	691.12 ± 0.20[b]	-	-
Control (injected)	692.70 ± 0.10	-	-
5	786.06 ± 0.10	3	0.001
25	904.33 ± 0.10	30	0.001
50	928.14 ± 0.10	34	0.001
100	1095.17 ± 0.10	58	0.001

a - Represents mean value of ALA level ± Std. error.

b - Total number of embryo in each group were not less than 12.

DISCUSSION

The present investigation has revealed some of the cellular reactions in response to styrene toxicity during embryonic development in chick. These results suggest that styrene even at very low level (5-100 nmole/kg. egg wt.), equivalent to 0.52-10.4 ppm, is ~~proved to~~ be lethal to embryo. Treatment of embryos with varying concentrations of styrene at different stages of development indicates that as concentration of the styrene increases, the growth of the live embryo is stunted. The growth retardation of styrene treated embryos is also reported earlier by Vainio *et al*. (2) but with the injection of styrene to air space at 25-100 umole/egg. Although TLV of styrene is considered as 50-100 ppm, since it does not produce any toxicity to adults, our results clearly indicate that it may act as a teratogen at this level.

Furthermore, these experiments reveal that the toxicity of styrene is very much dose dependent, as well as the age of embryo is a critical factor. The most susceptible period for the teratogenic action of styrene is observed to be the early stages of embryonic development. Thereafter, embryotoxicity declines very rapidly to its lowest level on the 14th day. The lower dose of

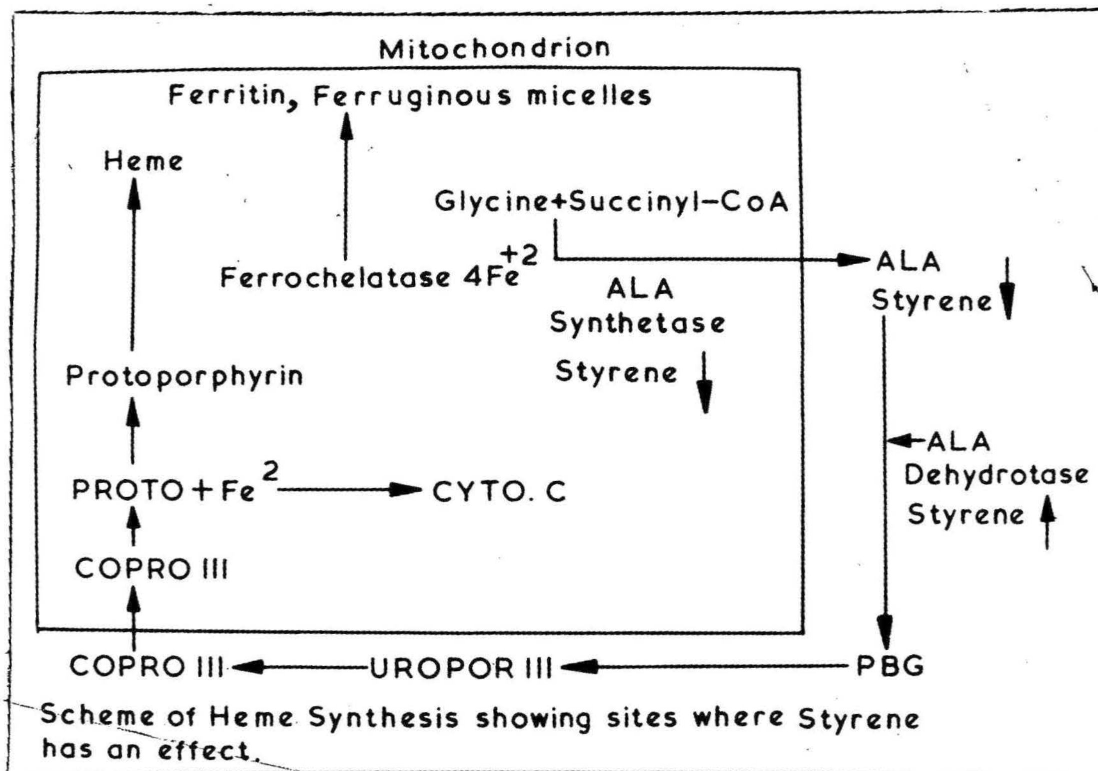
styrene exposure may be lethal during initial stages of development (3-7 day), whereas the same amount of styrene treatment has no effect on embryos during later stages of development (14 day). Recently, Vainio _et al_. (2) report that the embryos are most susceptible to styrene and styrene oxide during early development upto day 7, but then survivality declines again on 9th day. The present study also confirms that 14 day old embryo has a chance to reduce the toxicity in comparison to 3 and 7 day old embryo, if it is exposed to styrene. The one possible explanation can be put forward is that the proper homeostatic mechanism is not developed during early stages of embryonic development. Another possibility is that drug metabolizing enzymes, responsible for pollutant degradation, are absent or present at very low level in normal chick embryo liver, but can be induced by xenobiotics at the early stages of fetal development. Furthermore, due to deposition of styrene or its metabolite in the liver after the exposure, it might also inhibit some of the prime enzymes, which are necessary for growth and development of embryo.

Toxicity of styrene to chick embryo is showed clearly by growth retardation, weight loss and increase in the rate of mortality, but there was no appearance of malformation as in the case of Vainio _et al_. (2). This might be possible due to the fact that malformations are observed only with

higher concentration of styrene, injected to the air space. In the present investigation, styrene is injected at very low concentration to the yolk sac of embryo.

The depletion of microsomal cytochrome P-450, a heme protein, with styrene and styrene oxide exposure has been reported earlier (50), although how it regulates the hemeprotein level is not clarified. The present study clearly indicates that styrene can interfere with heme biosynthesis at the level of ALA metabolism by reducing its hepatic concentration. A schematic presentation of the possible effect of styrene on heme biosynthesis steps is shown in Fig. 8. At least two steps of heme biosynthesis may be affected by styrene intoxidation and thus results in decreasing the concentration of ALA. This depression is caused by two factors: firstly by inhibiting ALA synthetase, the rate limiting enzyme of heme biosynthesis and secondly by the stimulating ALA dehydratase activity, responsible for ALA degradation. Moreover, such alterations in ALA metabolism can be correlated with styrene toxicity developed, as there is a direct relationship between the reduction in ALA level and the mortality rate.

Inhibition of ALA synthetase activity by styrene has not been shown earlier. However, a direct inhibition of



Scheme of Heme Synthesis showing sites where Styrene has an effect.

Fig. 8

the ALA synthetase by heme has already reported with partially purified preparation of the liver enzyme (61, 70, 71, 89). Marked reduction of ALA synthetase activity was also found by ferrous ion (119) and lead (120). Recently, the depletion of certain amino acids like, serine, threonine by chronic exposure of styrene has been reported (121). Furthermore, the increased urinary excretion of hippuric acid, from the styrene exposed humans (122) and rabbits (123) suggest that hippuric acid is the terminal metabolite or detoxified product of styrene (vinylbenzene). Glycine, whether derived from the outside or from endogenous source, is known to be major amino acid , used for hippuric acid (aroyl glycine) formation. All these above observations suggest that level of glycine, the substrate of ALA synthetase, may be a limiting factor with styrene exposure, which ultimately decreases the synthetase activity. In future, we propose to study the inhibitory role of styrene and it's metabolite on ALA synthetase.

Contrary to the inhibition of ALA synthetase by styrene exposure, ALA dehydratase, the next enzyme in the pathways of heme biosynthesis was activated by styrene treatment. In the literature, stimulation of ALA dehydratase activity has been reported with porphyrogenic drugs (59, 107-109), but exact mechanism is not known. Styrene is

known to reduce liver glutathione level (124). Therefore, it is not possible for us to speculate that styrene may stimulate dehydratase activity due to increased availability of reduced glutathione, an essential component of this enzyme. In future, we propose to study the role of protein synthesis on the stimulation of dehydratase activity in presence of styrene.

Furthermore, it has been reported recently by Malik *et al.* (125) that ALA supplementation can stimulate hemeprotein synthesis in Friend Leukemic cell. As shown in the present investigation, styrene can reduce the hepatic ALA level. Thus, styrene can interfere with hemeprotein formation, resulting in decreased level of cytochrome P-450 which is an essential component for the initial step of styrene metabolism. Our interests will be extended in future to study the role of styrene on cytochrome P-450 formation.

SUMMARY

The interaction of styrene exposure during different developmental stages of chick embryo was studied in relation to appearance of toxicity and mortality. Styrene at very low level, like 5-100 nmole/kg egg wt. (equivalent to 0.52 to 10.4 ppm) were injected into the yolk sac of embryo on 3rd, 7th and 14th day of development. Styrene treated embryos were reduced in size and body weight from control, although, there was no visual appearance of malformations. Mortality of styrene treated chick embryo was increased with increasing concentration of styrene exposure and it was dependent on the age of embryo. LD_{50} values of styrene also increased with the age of embryos. LD_{50} values of styrene was determined as 25, 50 and >100 n mole/kg egg wt. on 3rd, 7th and 14th day. These data clearly indicate that styrene may produce embryotoxicity, when they are exposed to sublethal doses.

Attempt has been made to correlate the toxicity developed with the alteration in ALA metabolism which is known to be the regulatory step in heme synthesis. It has been observed that hepatic ALA level in styrene treated chick embryo was decreased as compared to that of controls. In support of this observation, ALA synthetase was found to be inhibited in the liver of chick embryo exposed with styrene, either on 7th day or on 14th day of

incubation. Furthermore, the degree of inhibition of ALA synthetase activity was also dependent on the dose of styrene injection. Contrary to ALA synthetase, the stimulation of ALA dehydratase, the next enzyme of heme biosynthesis pathway was observed with styrene exposure. Such stimulation was also dependent with concentration of styrene injection. These results suggest the possible impairment of heme biosynthesis at the level of ALA metabolism with styrene exposure.

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*Originals not seen.