HYALURONIDASE ACTIVITY IN STYRENE TREATED CHICK EMBRYO

DISSERTATION SUBMITTED TO THE JAWAHARLAL NEHRU UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF PHILOSOPHY
1980

ii, 44p.

MARY JULIANA D'SOUZA

SCHOOL OF ENVIRONMENTAL SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067
INDIA

C O N T E N T S

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.

Mary. D'Souza.
(MARY J. D'SOUZA)
Candidate

Kasturi Datta
(Dr.(Mrs.) KASTURI DATTA)
Supervisor

(Prof. J.M. DAVE)
Dean

May, 1980.

School of Environmental Sciences,
Jawaharlal Nehru University,
New Delhi - 110067.

School of Environmental Sciences
Jawaharlal Nehru University
New Mehrauli Road,
New Delhi-110067.

ACKNOWLEDGEMENTS

I am indebted to Dr.(Mrs.) Kasturi Datta for her inspiring guidance throughout this course of work. I am also grateful to her for her valuable suggestion, constructive criticism and affection shown to me in making the manuscript.

I would like to express my gratefulness to Prof. J.N. Dave, Dean, School of Environmental Sciences for providing all the facilities. I am also thankful to Prof. H.K. Das for providing some facilities.

I extend my sincere thanks to Mr. A.R.K. Prasad, Mr. V. Ramesh and Mr. J. Shanker for their enthusiastic cooperation.

I extend my sincere thanks to Mr. G. Ramaswamy, Jose A. Mallekal, Mr. Sampath Kumar, Miss Rekha, Mr. Kailash and other friends. I am also extremely grateful to Mr. Balam Ram for his help throughout this work.

I am thankful to Mr. R.N. Saini afor drawing and Mr. Ram Prasad for typing the manuscript.

Finally, I am grateful to School of Environmental Sciences for providing me the financial assistance in the form of a Junior Research Fellowship.

Mary J. D'Souza

AIM AND SCOPE

Styrene is extensively used in the manufacture of various plastics, synthetic rubbers, latexes and resins and may be considered as an occupational health hazard agent. The general population may also be exposed to styrene since it appears as a contaminant in drinking water, cigarette smoke, coal gas, coal tar and petrol produced by cracking processes.

Zlobina et al. (1) have reported the cases of pathological birth's among women employed in Russian polystyrene production plants. Embryotoxicity of styrene is also reported very recently at the level of 20-100 μmole/egg. Furthermore, from our laboratory it has been shown (2) that styrene even at very low level (5-100 nmole/kg) produces lethality of chick embryo, although no visual malformation has been detected. Stewart et al. (3) has reported transient neurological impairment with styrene exposure. Styrene is also known as mutagenic agent (4, 5). In the present study, chick embryo is used as a model system to study the effect of known quantity of styrene exposure (5-100 nmole/kg egg wt.) at sublethal dose during different stages on embryonic development as it can be directly injected into the incubating eggs to eliminate the variables of maternal influences. The chick embryo is also easily accessible to pollutants as compared to mammals. Since the avian and rodent embryo react

similarly in most teratological studies (6), we propose to use chick embryo as a model system for evaluating the teratogenicity of unknown compounds.

On the other hand, the role of hyaluronic acid, the major glycosaminoglycan on regulating the onset of differentiation has been reported (7). Moreover, the level of hyaluronite and hyaluronidase in immature developing brain has been correlated with brain morphogenesis (65-69). The modulation of the levels and distribution of hyaluronic acid has altered under different physiological conditions (8-14) and on neoplastic transformation (14-21). Though the activity of hyaluronidase, the enzyme responsible of hyaluronic acid degradation, has also found to be diminished in the sera of cancer patient (22), the physiological significance of hyaluronidase on differentiation process is not clearly defined at present.

Further, a close relationship exists between thyroxine action and hyaluronate catabolism, chondrogenesis and brain development. Normal skeletal development is dependent on the action of thyroxine (23). Chondrogenesis in vitro is stimulated by thyroxine (24) which is known to antagonise to hyaluronate (25). It has been further shown

that the period of morphogenesis is associated with increased hyaluronidase activity and loss of hyaluronate (26).

From the other report (27) it is evident that styrene exposure creates the hormonal imbalance at the level of pituitary, thyroxine and adrenaline. Therefore, it is our speculation that styrene may impair brain morphogenesis through the derangement, made at the level of hyaluronic acid and hyaluronidase. It may mediate through thyroxine imbalance.

Since the possibility of styrene interaction at sublethal dose with brain development cannot be ruled out, it is of our interest to study whether styrene at low level can produce any harmful effect on brain development in chick embryo, if it has been injected during different developmental stages. An emphasis will be given to correlate the biochemical basis of styrene neural toxicity with alteration in brain hyaluronidase activity. Our aim is to understand the role of brain hyaluronidase on styrene toxicity during embryonic development and to examine whether it can be correlated with the onset of brain development. As an integral part of the programme, we have studied the *in vitro* action of styrene on brain hyaluronidase, isolated from 14 day old normal chick embryo.

We will be involved very shortly in determining the level of acid mucopolysaccharides like hyaluronic acid and chondroitin sulphate, as well as their synthesizing and degradative enzymesin brain and other tissues of styrene exposed chick embryo. Further studies on this aspect will reveal the role of hyaluronic acid on differentiation process and its mechanism.

INTRODUCTION

Now a days due to heavy industrialization, the environmental contamination with different pollutants are becoming a serious health hazardous problem. Styrene (phenyl ethylene, vinyl benzene) the precursor of many thermoplastic polymers, is extensively produced and used in large quantities. Styrene is used in toys, appliances, houseware, plastic, synthetic rubber etc. Large number of workers are directly exposed to it during its synthesis and polymerization phases. General population are exposed by inhaling styrene and by the use of various plastic items e.g. polystyrene foam cups and food containers which contain 80-3600 ppm of styrene, with leaching of 0.02-35 ppm into water, 50% alcohol (28). Structurally styrene resembles with vinyl chloride which is shown to be mutagenic (43, 5) clastogenic (29) and carcinogenic compounds (30,31). 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 congenital malformations (32).

Styrene is quickly metabolised after subcutaneous administration to rats, 85% of the dose being eliminated within 24 h. The major portion (71%) appears as water soluble metabolites in the urine (33). Little is excreted in feces (3%) whilst 15% is exhaled as CO_2 and as unchanged

styrene in the ratio of 4:1. An important fraction of styrene metabolites is recovered as mandelic acid or phenylglycoxylic acid in occupationally exposed humans (34, 35). There is also evidence for significant hippuric acid excretion upon intensive human exposure (36). It is established now that epoxidation in liver microsomes is the primary step in styrene metabolism and produces styrene oxide which acts as biological precursor of mandelic acid, phenylglycoxylic acid and hippuric acid (37). The in vitro oxidation of styrene into phenyl glycol is evident in microsomes in the presence of a NADPH generating system (38).

Styrene oxide (phenyl ethylene oxide) has been detected as a volatile component of tobacco concentrate (39), as a by product in commercial samples of styrene chlorohydrin (40) and in effluent water from various latex manufacturing plants (41). It is used as a reactive diluent in epoxy resins (42) and as an intermediate in the preparation of agricultural and biological chemicals, cosmetics, coatings and in treatment of textiles and fibres.

Both styrene and styrene oxide have mutagenic activity in bacterial system (43, 5). Styrene oxide, is mutagenic without metabolic activation, to Salmonella typhimurium (43, 44), while styrene has been reported to have some

mutagenic activity, but only after metabolic activation (43, 45). Styrene is characterized as a potent mutagen in production of forward mutation in mammalian somatic cells in culture (45). It is more active than mutagenic agent ethyl methane sulfonate (46). Styrene oxide is an epoxide and is potentially an alkylating agent. The ultimate toxicants alkene and arene oxide have been suggested to bind covalently to macromolecules leading to carcinogenic (47), mutagenesis (48) and cytotoxicity (49).

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 duodenum than that in blood, lungs and spinal cord (50). Recently, it has been reported that Drosophila melanogaster were exposed to styrene and styrene oxide which induced recessive lethals frequently (51). The use of phenobarbitone as a pretreatment further increased the recessive lethals frequency after exposure to styrene and styrene oxide.

The augmentation of styrene toxicity by phenobarbitone is due to accelerated formation of the more toxic intermediate styrene oxide (37) from styrene.

SKF 525 (a potent inhibitor of hepatic microsomal enzyme system) inhibits the epoxidation of styrene in rats, thus blocking its microsomal conversion. Conversely, phenobarbitone brings about aggravation of styrene toxicity (52).

Acute exposures to styrene causes a reduction of leucocytes in the blood (53). It causes irritation of eye, nasal, and mucus membrane of upper respiratory tract, nose and mouth, followed by nausea, cramps and death due to respiratory center paralysis. Prolonged or repeated exposure may lead to dermatitis due to deflating of skin. Animals exposed to 2500 ppm showed weakness and stupor, followed by incoordination, tremor, coma and death in eight hours (54). Electrophysiological findings of a large number of workers with low level of styrene exposure describe subtle electroencephalographic changes as well as decreased conduction velocity in the peripheral nerves (55, 56). Stewart et al. (3) also reported transient neurological impairment, as well as eye and nasal irritation in human volunteers exposed to 376 ppm of styrene for upto 7 hours.

Styrene stands third in rank by volume in the utilization of plastic industries after polyethylene and polyvinyl chloride, but surprisingly few studies have been done with styrene treatment. These few studies on styrene

toxicity has been done on adult animals at a high level of styrene exposure and very little attention has been given to find the toxicity of styrene at sublethal doses to the embryo. However, there is one report (57) that both styrene and styrene oxide can produce malformation and 60% death of the embryos, if they are injected at 50 umole and 25 umole/egg respectively. To evaluate the embryotoxicity of inhaled styrene, Ragulo (58) exposed pregnant rats for 4 h/day to 0.35, 1.2 or 12 ppm of the compound throughout gestation. An increased occurrence of resorptions was reported at the level of exposure, but the incidence of fetal malformations was unaffected. Zlobina et al. (1) reported a high incidence of 'pathological births' among pregnant women working in polystyrene plants in Russia. The influence of hazardous agent styrene causes general endocrinal dysfunction of the pituitary, thyroid and adrenal glands (27).

All these investigations suggest that embryotoxicity of styrene, although the actual mechanism is still not clear. In the developing embryo, the cell division and differentiation lead to the increase of enzyme activity and protein synthesis. The rate of permeability of toxic agents depend on the physical and chemical properties, as well as lipid solubility and degree of ionization and membrane permeability

to reach the developing embryo. Therefore, the enzymes participating in the developing embryo are more susceptible to the toxic agents which permeate the placental barrier. In the present investigation, it is our interest to study the effect of styrene on brain development and attempt will be made to correlate with biochemical aspect of chondrogenesis.

Morphogenetic Role of Hyaluronate in Brain and Other Tissues:

Morphogenesis of an organ or tissue is a complex and specific series of events leading to a unique, functional organization of cellular and extracellular elements. A distinctive aspect of recent studies of the role of extracellular materials in morphogenesis and differentiation is the attempt to deal with known glycosaminoglycans.

Glycosaminoglycans are highly charged polyanions, composed of repeating disaccharide units which bear either a carboxyl or sulphate group of both and which except for Keratan sulphate, consist of uronic acid and hexosamine moieties. Hyaluronate, chrondroitin, chondroitin-4 and 6-sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin are naturally existing glycosaminoglycans. With the exception of hyaluronate, glycosaminoglycan do not ususally exist <u>in vivo</u> as isolated polysaccharide chain

but as proteoglycans where glycosaminoglycans chains are linked covalently to protein (59-64). In relation to the involvement of hyaluronic acid with morphogenesis, considerable evidence has now been obtained to suggest a role in development and possibly also in physiological repair and remodeling.

Immature developing brain contains greater amounts of glycosaminoglycans (65-70), larger areas of intercellular space (71), and higher water content (67, 68, 70, 72) than mature brain. The decrease in hyaluronate content during brain development is considerably more marked than in other glycosaminoglycan. Hyaluronate content has been related to water content in many other system and thus may detect high degree of hydration of immature brain and in turn the size and properties of the intercellular space.

Correlation of Hyaluronate Synthesis and Hyaluronidase Activity with Morphogenesis.

The observation that hyaluronate is relatively enriched in embryonic or young tissues in comparison to adult tissues including brain has been made by several investigators(65-67, 70, 73-78). Hyaluronate plays a major role in the regulation of onset of differentiation in chick embryo (7). Excess hyaluronate is found in the

early stages of development of the chick embryo cornea (26) and vertebral column (79) and of regeneration of the amputated newt limb blastema (80). Thus by studying hyaluronate metabolism at discrete stages in early development at times and in tissues where striking morphogenetic cell movements or differentiations are taking place, close correlations between hyaluronate synthesis and cell migration and between hyaluronidase activity and cell differentiation are expected.

Ray and Revel (81) have described the course of events during early corneal morphogenesis is chick embryo in detail. It is interesting to note that the leading migratory mesenchymal cells always move adjacent to the corneal epithelium (81), which is the source of hyaluronate. The appearance of hyaluronidase activity in corneal tissue coincides with the cessation of migration and deswelling (26) which follows the further development along with the change in major glycosaminoglycan from hyaluronate to sulfated glycosaminoglycan subsequently (82, 83). In support of this view, it has been shown further (79) that the enzyme hyaluronidase appears precisely at that time when cell begins to produce cartilage matrix after concluding their migration around the notochord. Pratt et al. (84) have shown that a major component of the extracellular matrix

into which cranial neural crest cells migrate, is hyaluronate and these investigations correlate the production of hyaluronate closely with a large increase in volume of extracellular matrix and the initiation of crest cell migration. Cartilage redifferentiation is maximal when hyaluronidase activity is maximal.

Hyaluronidase has been isolated from skin (85), spleen (86), urine (87,88); plasma (89,90), brain (91), heart (92), serum (93), ~~liver (98)~~, testis (94), neural crest (95), liver (98) and synovial fluids and shown to act as an endoglycosidase capable of depolymerizing hyaluronate and chondroitin sulfates to form a mixture of oligosaccharides ranging from tetra to decasaccharides (96-98). These oligosaccharides are reduced to smaller units by exoglycosidase. A significant amount of hyaluronidase has found in the serum and organs of rabbit, bearing Brown Pierce tumor (99). In contrast, other investigation (100) has shown he the decreased activity of hyaluronidase in the serum of tumor bearing rats. Moreover, decreased activity of this enzyme has been detected in the sera of cancer patient (22). Although, several studies have been made, the role and mechanism of hyaluronidase action on tumor growth and development are still not known.

An interesting observation has been made by Toole (25) that thyroxine can antagonize the action of hyaluronate and it can be correlated with the dependence of brain development on thyroxine. Corneal morphogenesis is also associated with hyaluronidase activity and the loss of hyaluronate (26). The skin of hypothyroid rats contains elevated level of hyaluronate, probably due to decreased degradation which can be rectified with thyroxine administration. Normal skeletal development is dependent on the action of thyroxine (23) and chondrogenesis in vitro is stimulated by this hormone (24). It is proposed by Toole (7) that developmental sequences can be mediated firstly by morphogenetic phase during which cells accumulate by proliferation and migration at a suitable location and in the required number and characterized by hyaluronate production. This has been followed by the second phase of overt differentiation which is characterized by hyaluronidase activity.

The quantiation of brain hyaluronidase is of importance not only to determine the phase of overt differentiation in brain but the significant alterations in levels of brain hyaluronidase in styrene exposed chick embryo may indicate toxicity or carcinogenecity or tumorogenicity of test compounds. Therefore, in the present investigations, we propose to study

the effect of styrene exposure, made on both initial (7 day) and later stage (14 day) of embryonic development, on the level of brain hyaluronidase. Attempt will be made to correlate the development of brain with the appearance of hyaluronidase in styrene exposed chick embryo.

MATERIALS AND METHODS

Hyaluronic acid, p-dimethylaminobenzaldehyde, Triton X-100, N-acetylglucosamine were purchased from Sigma Chemical Company, U.S.A. A few of the other chemicals were purchased from BDH (AR grade) sodium formate, formic acid (E. Merck). Sodium acetate, acetic acid, hydrochloric acid were brought from commercial sources. Styrene was purchased from SISCO. Microsyringe has been purchased from Hamilton & Co. Switzerland.

Maintenance of the Eggs:

The fresh, white leghorn strain zero day old eggs were brought from Govt. Poultry Farm, Satbari, New Delhi. The eggs were cleaned, weighed and marked properly for the experiment. The eggs were incubated at 37°C and 60% humidity in BOD incubator. The humidity was maintained by keeping an tray, full of water in presence of a hygrometer.

Eggs were rotated manually once a day and were examined through the candler everyday 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

1. 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 - 100 nmole/kg egg wt. in 50 µl were given on different days of development (7th and 14th day). These doses can also be expressed as 0.52-10.4 ppmm Control eggs were given the same amount of solvent injection on the same day of incubation. For giving an injection, the eggs were thoroughly wiped with distilled alcohol and placed on the candler to determine the position of the embryos so that a scratch was made on the shell directly over the embryo. Injections were given into the yolk sac of embryos with the help of micro-syringe. Soonafter giving the injections the eggs were resealed with surgical tape to prevent the contamination. Sterile syringe, scalpel were used throughout the experiment. After giving the injections the eggs were kept in the incubator with minimum disturbance. They were candled every day for detecting their mortality. Brains were dissected out on 19th day and weight of the brain were noted.

2. Treatment of Chick Embryos for the Studies on Brain Hyaluronidase under Styrene Exposure:

For studying the effect of styrene exposure on brain hyaluronidase activity, the embryos were injected on 7th or

14th day of incubation with different concentration of styrene (5, 25, 50 and 100 nmole/kg egg wt.). Embryos were opened on 18th or 19th of incubation and brain was collected for further studies on hyaluronidase activity.

3. Assay of Hyaluronidase Activity from Brain:

Brain hyaluronidase activity of chick embryos was measured by the method of Polansky & Toole (66). Hyaluronidase activity is determined by the measurement of the terminal N-acetylglucosamine in oligosaccharides released during incubation of hyaluronate with the enzyme, prepared from chick brains.

Reagents used:

a) Hyaluronic acid: 0.8 mg/ml of hyaluronic acid (Sigma type Grade I) in 0.1M sodium formate buffer, pH 3.7 containing 0.15M NaCl.

b) Sodium formate buffer : 0.1M of sodium formate in 0.1M fornic acid, pH 3.8.

c) 1% Triton-X : 0.01 ml of Triton-X was dissolved in 10 ml of 0.1 M sodium formate buffer, pH 3.8.

Preparation of Enzyme Hyaluronidase from Brain of Chick Embryo

The brain of 19th day old chick embryos were dissected out and kept in ice. For the assay of hyaluronidase, the

brains were weighed and homogenized in 10 volumes of 0.05M sodium formate buffer pH 3.7 containing 0.05M NaCl. The homogenate was centrifuged at 25,000 x g for 20 minutes in Sorvall-RC-5, which resulted in the separation of brain hyaluronidase (localized in the precipitate). The pellet, containing the enzyme was resuspended in the 0.1M formate buffer, pH 3.7 containing 0.15M NaCl and 0.1% Triton-X for six hours at 0-4°C. This fraction was used as an enzyme source for hyaluronidase assay.

Determination of Hyaluronidase Activity from the Brain of Chick Embryo:

The incubation mixture containing 0.6 ml of hyaluronic acid (0.6 mg/ml of 0.1M sodium formate buffer, pH 3.7 containing 0.15M NaCl) and 0.4 ml of enzyme preparation was incubated for six hours in a water bath maintaining the temperature at 37°C. The blank consisted of 0.4 ml of enzyme preparation oin 0.1% Triton-X suspension and 0.6 ml of 0.1 M sodium formate buffer, pH 3.7 containing 0.15 M NaCl. Enzymatic reaction was terminated by boiling the tubes at 100°C for three minutes. The enzymatic activity of blank was terminated at zero minute. Release of terminal reducing N-acetyl hexosamine as a result of hyaluronidase action was measured according to Reissig et al. (101)

4. Estimation of N-Acetylglucosamine by a Modified Colorimetric Method by Reissig et al.:

a) Potassium tetraborate: A solution of 0.8M borate was prepared, and the pH was adjusted to 9.2 with NaOH. Some borate salt crystallised out on standing. The supernatant was used for assay.

b) p-Dimethylaminobenzaldehyde : 10 gm of p-dimethylaminobenzaldehyde was dissolved in 100 ml of glacial acetic acid which contained 12.5% (volume per volume) 10 N HCl (analytic reagent). This reagent could be stored at 2° for a month without significant deterioration shortly before use it was diluted with nine volumes of glacial acetic acid.

c) N-Acetylglucosamine : To prepare the standard solution of N-acetylglucosamine 4.442 mg of N-acetylglucosamine was dissolved in 10 ml of distilled water.

Procedure:

N-acetylglucosamine was estimated according to Reissig et al. (101). To 0.5 ml of samples containing different concentration of N-acetylglucosamine 0.1 ml of Na-tetraborate is added. The tubes are heated in a vigorously boiling waterbath for exactly 3 minutes and cooled in tap water. 3 ml of DMAB reagent are then added and immediately after mixing the tubes are placed in a bath at 36-38°C. After precisely 20 minutes the tubes are cooled in tap

water and read without delay at 578 mu. The colour produced in this reaction is proportional to the amine content of the solution assayed. The standard curve for N-acetyl-glucosamine is given in Fig. 1.

5. Estimation of Protein:

Protein was assayed by the method of Lowry et al. (1951)(102).

Reagent 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 µg), 2.5 ml of protein reagent was added and after

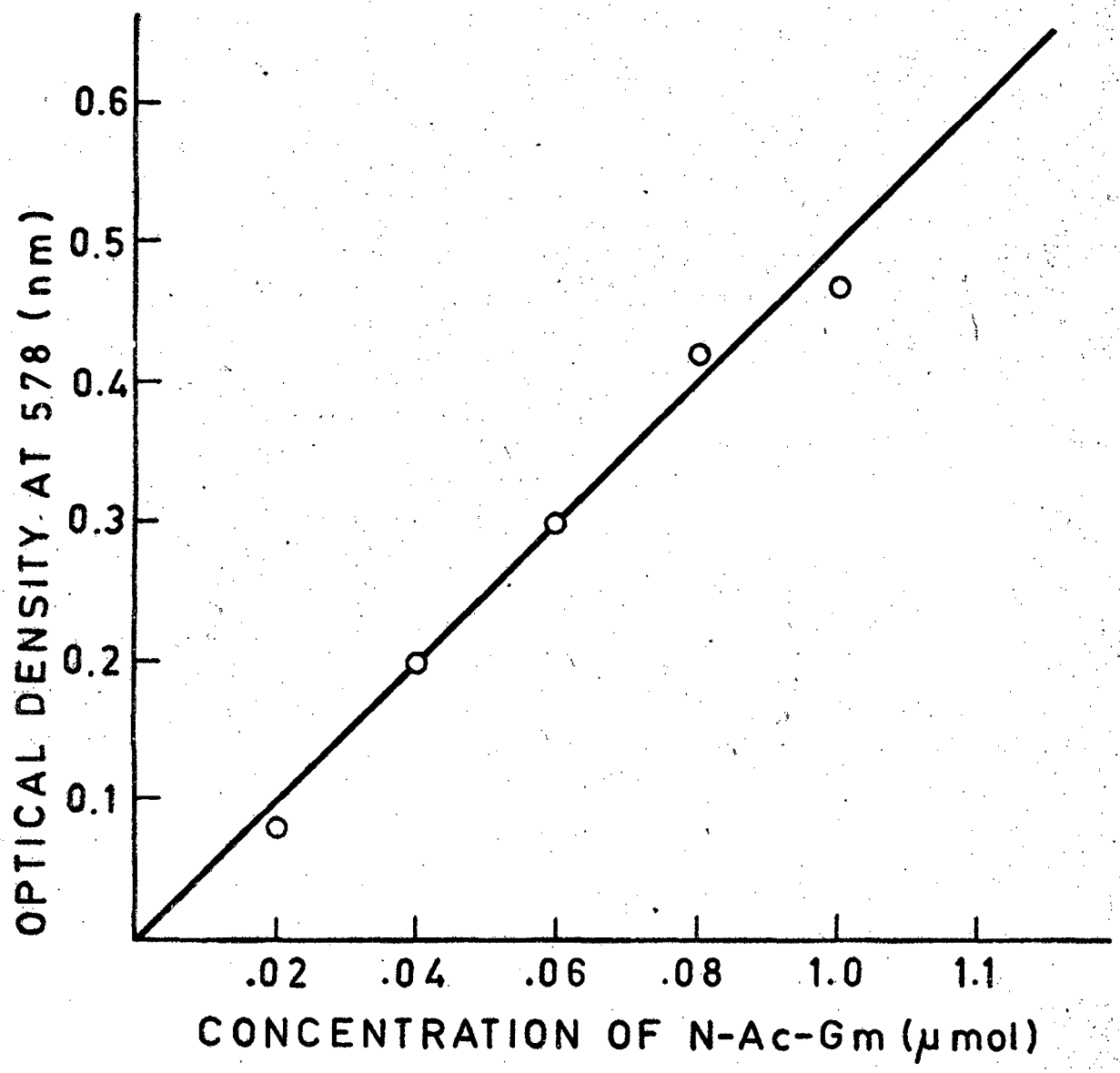

Fig. 1: Standard curve for N-Acetylglucosamine.

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. (2).

6. Studies on the Effect of Styrene in vitro on Brain Hyaluronidase Activity:

To study the effect of in vitro addition of styrene on the activity of hyaluronidase from normal brain, the enzyme activity was determined in presence of varying concentration of styrene (1-5 nmole). All other experimental procedures are the same as described earlier.

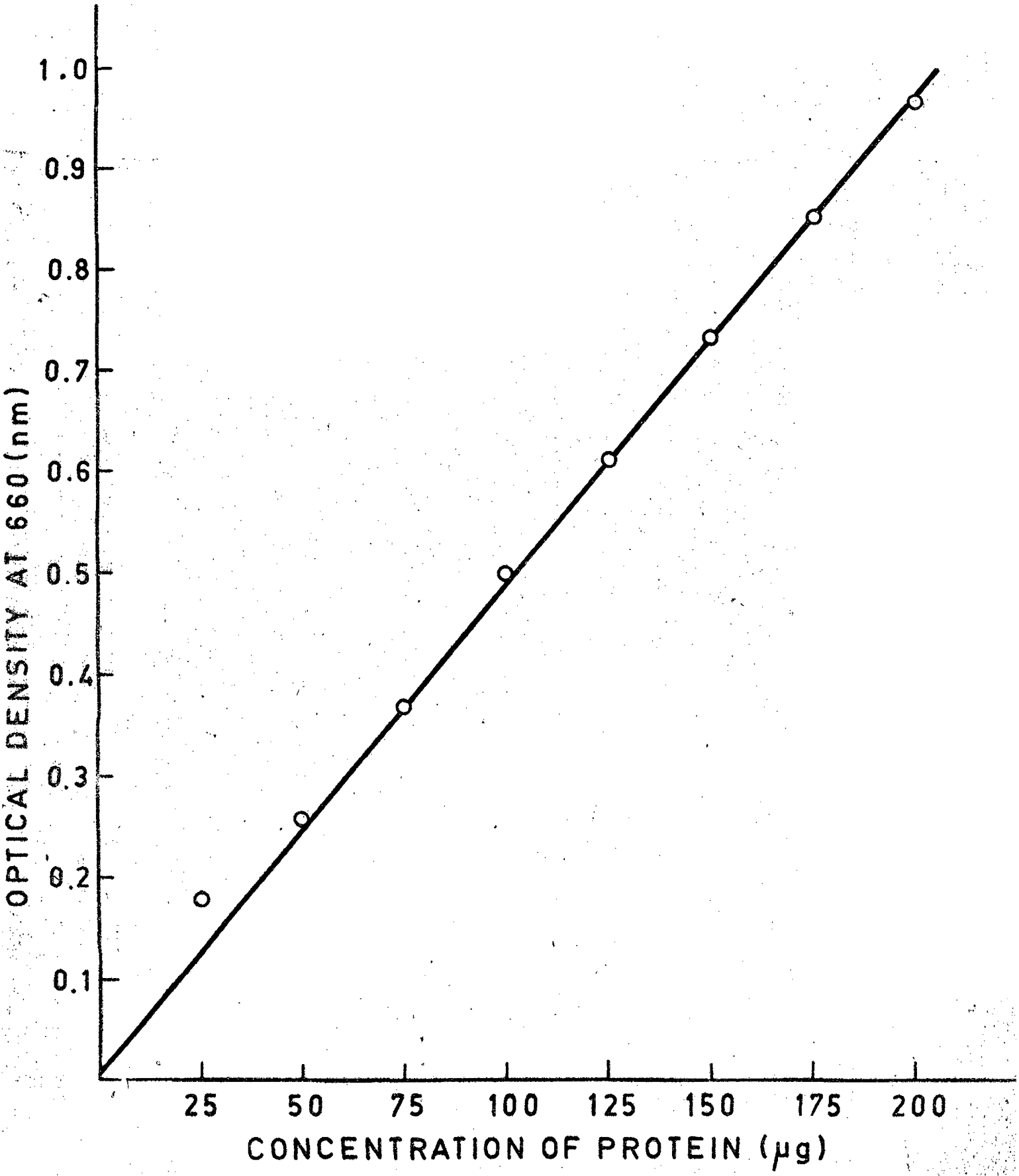

Fig. 2: Standard curve for protein.

RESULTS

BRAIN DEVELOPMENT OF CHICK EMBRYO UNDER STYRENE TREATMENT

The effect of styrene (5-100 nmole/kg. egg wt.) treatment during different development stages, like 7th and 14th day of incubation on the brain weight of chick em bryo is represented in Table I. The chick embryos were injected with styrene at varying concentrations either on 7th or on 14th day of incubation and they were sacrificed on 19th day of incubation and the weight of the brain was noted. It has been shown that styrene treatment on both the days (7th or 14th day) of incubations resulted on the reduction in brain weight. Styrene injections on 7th day decreased the brain weight with the increasing concentrations of styrene. The brain weight was also observed to reduce with varying concentration of styrene treatment on 14th day. In contrary to 7 day old styrene treated chick embryo, it has been observed that irrespective of what dose of styrene used, the same degree of reduction in brain weight was obtained. However, there was no detection of tumor in the brain. The decrease of weight of the brain of 7th day and 14th day styrene treated chick embryo may be an indication of embryotoxicity of styrene, even at sublethal dose, e.g. 5-100 nmoles/kg. egg wt (equivalent to 0.52-10.4 ppm). This result is (supported by the previous findings from our laboratory (2) which indicates the growth retardation and

TABLE I

BRAIN WEIGHT OF 19th DAY CHICK EMBRYO TREATED WITH STYRENE ON 7TH OR 14TH DAY

Treatment with styrene (nmole/kg.egg wt.)	Weight of brain (gm) of chick embryo treated with styrene on	
	7th day[a]	14th day[a]
Control	0.8316	0.8316
Injected + Control	0.8036	0.8036
5	0.7548	0.6190
25	0.7130	0.6548
50	0.6389	0.6532
100	0.6156	0.6726

a = A minimum number of 30 embryos were used in each group.

improper development of styrene (5-100 mmole/kg) treated chick embryo.

Fractionation of Brain Hyaluronidase :

Brains from both normal and styrene treated chick embryo were collected on 18th-19th day of incubation. All operations were carried out between 0° and 5°C. Brains were homogenized in 0.05M sodium formate buffer pH 3.7, containing 0.05M sodium chloride and were centrifuged at 25,000 g for 20 minutes. The pellet was extracted with the detergent Triton-X-100 (0.1%) in ice for 6 hours. The enzyme activity obtained in this fraction is significantly higher than the homogenate. Throughout the experiment, this fraction has been used as an enzyme source.

KINETIC PROPERTIES OF THE PARTIALLY PURIFIED BRAIN HYALURONIDASE

a) Effect of incubation time on reaction velocity of the enzyme: The initial rate of an enzyme catalyzed reaction should not change with time of incubation at infinite substrate concentration. But due to instability of enzyme and other factors, the rate may change appreciably within a short period of incubation. The time course of brain hyaluronidase activity is shown in Fig. 3. It can be seen that the rate of reaction, catalysed by this enzyme is constant for about 6 hours.

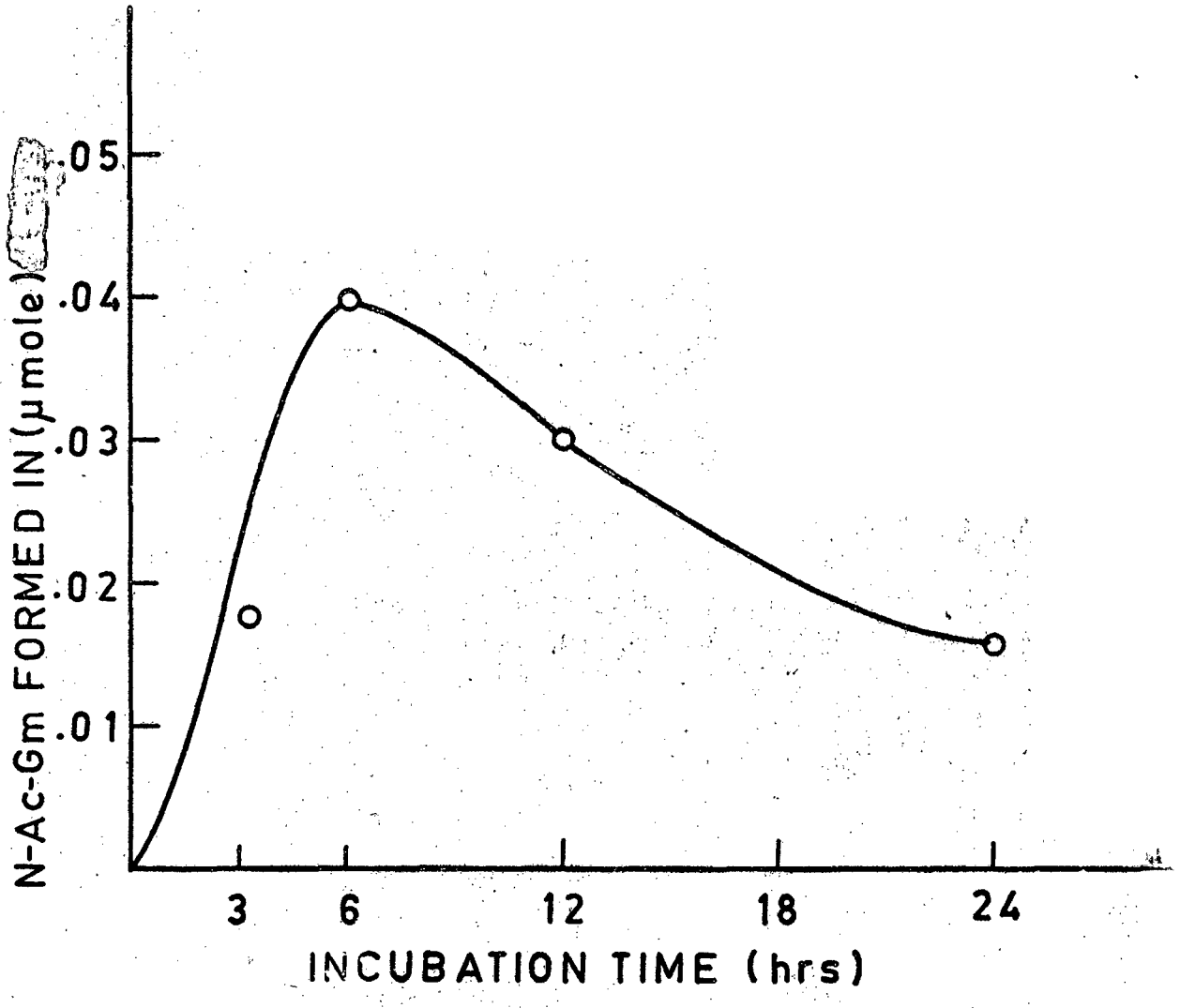

Fig. 3: Effect of incubation time on reaction rate of brain hyaluronidase of normal chick embryo.

b) Optimum pH : H^+ ion concentration of the incubation system has profound effect on the activity of the enzyme and for every enzyme there is a pH or a pH range within which the enzyme is most active. The effect of pH on the rate of hyaluronidase activity varies with the source of enzyme, the salt concentration and the test method.

Brain hyaluronidase activity was determined at various pH values as shown in Fig. 4. The sets of buffer used were namely sodium acetate (pH 3.5 and 5.0) sodium formate (pH 3.0, 3.7; 4.0; 4.5) and potassium phosphate buffer (pH 7.4). The enzyme exhibits a pH optima at 3.7. The study indicates clearly that brain hyaluronidase has a pH optima at 3.7, unlike that of testicular hyaluronidase, which has pH optima at 4.5 and 5.7.

BRAIN HYALURONIDASE ACTIVITY WITH STYRENE TREATMENT

a) Effect of styrene treatment to 7 day old chick embryo on brain hyaluronidase activity : Brain hyaluronidase activity of 19 day old chick embryo, treated with styrene at varying concentration from 5-100 nmole/kg on 7th day of development was measured (Table II). The results of Table II showed that the brain hyaluronidase was inhibited significantly to an extent of 74%, 77%, 77% and 88% of normal value with the treatment of styrene at the level of 5, 25, 50 and 100 nmole/kg. respectively, injected on 7th day of development. Moreover, it was also found that with 100 nmole. Styrene

TABLE II

EFFECT OF STYRENE EXPOSURE TO 7 DAY OLD CHICK EMBRYO ON BRAIN HYALURONIDASE ON DAY 19.

Treatment with styrene (nmole/kg. egg wt.)	Hyaluronidase Activity[a] (nmole of N-AcGm formed/ mg of protein/6 hrs at 37°C)	Inhibition (%)	p value
Control	2.2469 ± .05[b]	-	-
Control (Injected)	2.0738 ± .02	-	-
5	0.6531 ± .09	74	.001
25	0.5241 ± .04	77	.001
50	0.5241 ± .01	77	.001
100	0.2821 ± .04	88	.001

a - Represents mean value of hyaluronidase activity ± Std. error.

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

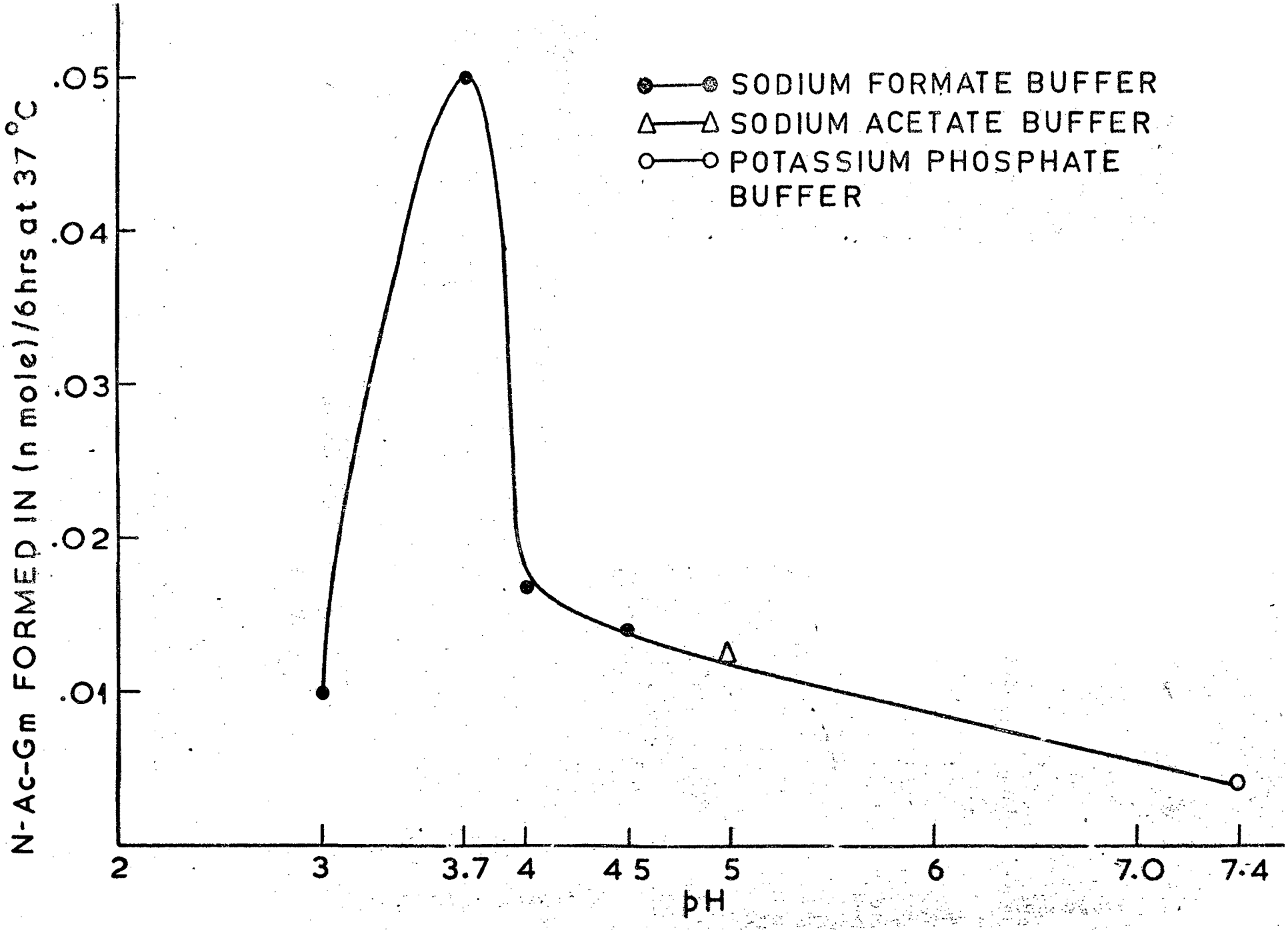

Fig. 4: pH activity curve for brain hyaluronidase.

treatment, the maximum inhibition of 68% in the brain hyaluronidase activity was obtained.

b) Effect of styrene treatment to 14 day old chick embryo on brain hyaluronidase: The effect of styrene exposure on day 14th to embryo on the brain hyaluronidase activity, of 19 day old chick embryo was studied (Table III). As shown in Table III, styrene exposure to 14 day old chick embryo increased the brain hyaluronidase activity significantly as compared to normal level, on day 19 of incubation. The maximum stimulation (67%) of brain hyaluronidase was obtained with 50 nmole of styrene injection, made on 14th day of incubation. Surprisingly, no stimulatory effect on brain hyaluronidase has been observed with 100 nmole of styrene injection indicating that styrene acts as a stimulator at the level of 25-50 nmole/kg.

Stimulatory Action of Styrene in vitro on Brain Hyaluronidase

Since we have seen the stimulatory role of styrene on brain hyaluronidase with 25 and 50 nmole/kg styrene injection, our interest was to examine the effect of styrene in in vitro on brain hyaluronidase activity. The effect of styrene (1-5 nmole) addition in vitro on brain hyaluronidase activity isolated from 14 day old normal chick embryo has been represented in Fig. 5. It is evident from Fig. 5. that styrene itself has some stimulatory role on brain

TABLE XII

EFFECT OF STYRENE EXPOSURE TO 14 DAY OLD CHICK EMBRYO ON BRAIN HYALURONIDASE ON DAY 19.

Treatment with styrene (nmole/kg. egg wt.)	Hyaluronidase Activity[a] (nmole of N-AcGm formed/ mg of protein/6 hrs at 37°C)	Stimulation (%)	p valu
Control	2.2469 ± .05[b]	-	-
Control (Injected)	2.1180 ± .08	-	-
5	2.5322 ± .05	-	.01
25	2.7902 ± .08	20.4	.001
50	3.9999 ± .08	67	.001
100	2.2609 ± .1	-	.01

a - Represents mean values of hyaluronidase activity ± Std. error.

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

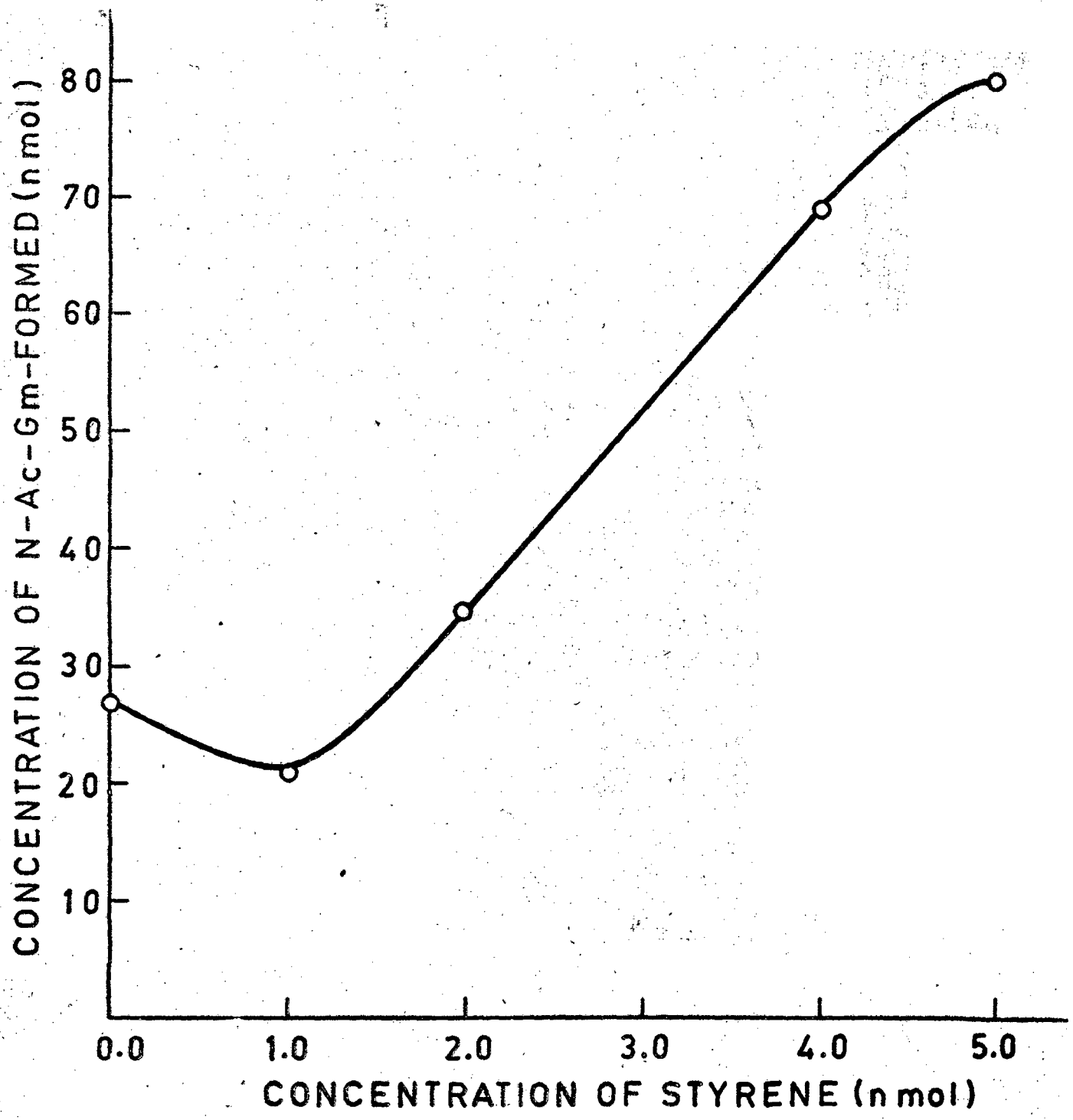

Fig. 5. Activity of brain hyaluronidase with *in vitro* addition of styrene.

hyaluronidase activity, although the nature of modulator is not clarified from this experiment. In future, we propose to study in details about the mechanism of stimulation of brain hyaluronidase by styrene.

DISCUSSION

The present study has revealed some of cellular reactions in response to styrenetoxicity 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 impairs thebrain development of the embryo. Other investigators Vainio et al. (57) have also reported the growth retardation of chick embryo with the injection of styrene to air space at 25-100 µmole/egg. From our laboratory, previously it has been shown that styrene at the level 5-100 nmole/kg increases the mortality of chick embryo (2). In the present study, we have found that during the initial stages of embryonic development like day 7, Styrene can impair thebrain development and such impairment is dose dependent. But, due to styrene exposure on day 14, the same degree of reduction in brain weight is obtained in chick embryo with styrene exposure at the level of 5-100 nmole/kg. The 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 not be toxic to the adults at this level but it can be hazardous to the embryo.

Studies on brain hyaluronidase activity from chick embryo treated with styrene on day 7 or 14 indicate the styrene induced alteration in brain hyaluronidase. Styrene

exposure on day 7 inhibits brain hyaluronidase activity when it is measured on day 19 of incubation. In contrary, due to styrene treatment on later stage of development (day 14) brain hyaluronidase activity is increased on day 19 of incubation. From our present studies, it is further revealed that even in in vitro, condition styrene (1-5 nmole) has a stimulatory role on the activity of hyaluronidase isolated from normal 14 day old chick brain.

In the present investigation, the levels of hyaluronidase in the brain of normal chick embryo during different developmental stages have not been determined. However, the appearance of hyaluronidase activity in the corneal tissue in the chick embryo coincides with maturation process (26). The possible explanation for the inhibition of brain hyaluronidase due to styrene exposure on day 7 is that styrene may inhibit further increase of brain hyaluronidase which may be the regular course of events of normal brain development. But on the later stage the critical level of hyaluronidase may already have been attained and styrene has no further inhibitory action of brain hyaluronidase.

Further studies are necessary to understand how styrene can inhibit brain hyaluronidase during initial stages which is absent during the later stage of development.

Our results related to brain development are in agreement with the previous finding of Vainio et al. (57), who have shown that 7 day old embryos are very much susceptible to styrene toxicity and its toxicity declines rapidly on day 9 of incubation.

It is known fact that normal brain development is dependent on thyroxine. Studies of cerebellar development in hypothyroid rats have indicated that lack of thyroxine results in delayed and aberrant differentiation of purkinje and external granule cells as well as delayed migration of the external granule cells (103, 104). It has been shown that thyroxine also causes a two fold enhancement of hyaluronidase activity in tadpole brain (105) thus highlighting the potential interrelationship between thyroxine and hyaluronate metabolism in morphogenesis. On the other hand, there is a report of general endocrinal dysfunction of the pituitary thyroid, and adrenal glands resulting from the influence of hazardous agents like styrene (27). This indicates that styrene can interact with thyroxine leading to the disturbance created in endocrinal balance. Thyroxine has been proposed as a 'timer' for turning off proliferation necessary for subsequent migration of the differentiated cells (104).

Thus according to the above findings it can be suggested that brain hyaluronate may be affected by styrene intoxication as a result of decrease the level of hyaluronidase in the brain of 7th day styrene treated chick embryo. This depression may be caused by styrene due to endocrinal dysfunction of the pituitary thyroid (27). On the other hand, thyroxine induces hyaluronidase activity thus degrading hyaluronate. Therefore, the disturbance of pituitary thyroid by styrene exposure will ultimately disturb the level of hyaluronidase and subsequently, the brain development may be impaired.

In the both groups, where styrene exposure was made either on 7th day or on 14th day, stunted brain development has been observed, though the response in relation to hyaluronidase activity was not identical in both the cases. This indicates that a critical level of hyaluronidase as well as hyaluronate content is essential for normal brain development.

Though this is the first report where styrene toxicity has been studied in relation to brain hyaluronidase, the depletion of certain amino acids like serine, threonine by chronic exposure of styrene indicating it's involvement with brain development (106) has already been reported. The

experiments on healthy chick embryos exposed to styrene on 14th day show a significant increase brain hyaluronidase activity as contrary to the 7th day injected chick embryo. In the earlier report, stimulation of hyaluronidase activity in the serum and organs of rabbit bearing Brown-Pierce tumor has been observed. In addition to it hyaluronidase has been found as an anticarcinogenic agent (99). However, the mechanism of this effect of hyaluronidase is not known. Styrene inhibits hyaluronidase only when injected on 7th day. The onset of major metamorphic events take place between the 7th and 10th days of treatment (105). Thus in chick both hyaluronate content and hyaluronidase activity are greater during the period of differentiation (7). Therefore, it seems possible that at the stage (14th day of incubation) the pituitary thyroid may overcome the inhibitory action of styrene and as a result the appearance of brain hyaluronidase is not hampered. In this regard, it is interesting to note that thyroxine, cyclic AMP and theophylline have been shown to stimulate chondroitin sulphate synthesis in various systems (24, 107-109).

At this stages, it can be speculated that brain hyaluronidase in 14½ day styrene treated chick embryo may act in concert with β-glucuronidase and β-N-acetylglucosaminidase to reduce the hyaluronate to monosaccharides which do

not have the inhibitory effect on hyaluronidase (7). Furthermore, it is also possible that on 7th day, the level of β-glucuronidase is not enough to react along with hyaluronidase to convert hyaluronate to monosaccharides.

The hyaluronidase of brain exhibites a sharp pH optimum at 3.7 and no activity can be detected at above pH 4.5. Our results are similar to the reports made by other workers (97, 98, 110, 111). However, it stands in contrast to the report of testicular hyaluronidase (112) whose activity shows little variation, between pH 4.5 and 5.7.

Though this study suggests that styrene exposure can alter the pattern of brain development and hyaluronidase activity, the actual mechanism by which it can mediate it's effect is still to be resolved.

SUMMARY

The interactionn of styrene exposure during different developmental steges of chick embryo was studied in relation to appearance of toxicity with brain hyaluronidase. Styrene, at very low level, like 5-100 nmole/kg. egg wt. (equivalent to0.52 to 10.4 ppm) were injected into the yolk sac of embryo on 7th and 14th day of development of the chick embryo and brain weight and hyaluronidase activity were noted on day 19. In the course of our study we had found that the weight of brain of 7 day styrene treated chick embryo reduced and it was dose dependent. In contrast, though the reduction in brain weight was observed in 14 day styrene injected chick embryo, it was not dose dependent. These results indicates the embryotoxicity of styrene at very low level.

The brain hyaluronidase isolated from chick embryo has a sharp pH optimum at 3.7 unlike that the testicular hyaluronidase.

It was further observed that the brain hyaluronidase level in 7 day styrene injected decreased as compared to that of controls and this inhibitory action was proportional to the concentration of styrene injected. Contrary to this, the 14th day styrene treated chick embryo showed an increase in the brain hyaluronidase level as compared to that of normal. This stimulation was observed only at the level of 25 and 50 nmole/kg of styrene injection.

The *in vitro* effect of styrene on brain hyaluronidase of 14 day old normal brain was studied. The stimulatory action of styrene (1-5 nmole) on normal hyaluronidase has been revealed. These results suggested that styrene interacts with normal brain development and this impairment may be correlated with alteration in brain hyaluronidase activity.

REFERENCES

1. Zlobina, N.S., Popova, T.E., Izyumova, A.S., Pereva, G.P. and Ponomareva, N.I., Gig. Trudy Sostoyanie spectsifi-cheskikh Funkts Rat. Neftekhim. Prom. Sti. (1974) 163.
2. Shanker, J.: M.Phil. dissertation, School of Environmental Sciences, Jawaharlal Nehru University, 1980.
3. Stewart, R.D., Dodd, H.C., Baretta, E.D. and Schaffer, A.W. (1968) Arch. Environ. Health. 16 : 656.
4. Mainio, H., Paakkonen, R., Ronnholm, K., Ravnio, V. and Pelkonen, O. (1976) Scand. J. Work Environ. Health. 2 : 147.
5. Lopriene, N., Barak, R., Baroncelli, S., Bartsch, H., Bronzetti, G., Cammellini, A., Corsi, C., Frezza, D., Nierai, R., Leposin, C., Rosellini, D. and Rossi, A.M. (1977) Cancer Res. 37 : 253.
6. Gebhardt, D.O.E., The chick embryo in applied teratology, in D.H.M. Woollam (ed.), Advances in Teratology, Vol. 5, Logos Press, London, 1972, p. 97.
7. Toole, B.P., Morphogenetic roleof glycosaminoglycans (acid mucopolysaccharides, in brain and other tissues) In: "Neuronal Recognition", Barondes, S.H. (ed.) p. 275, New York, Plenum 1976.
8. Morris, C.C. (1960) Ann. N.Y. Acad. Sci. 86 : 878-894.
9. Tomida, M., Koyama, H. and Ono, T. (1975) J. Cell. Physiol. 86 : 121-130.
10. Mosesatelli, D. and Rubin, H. (1977) J. Cell. Physiol. 91. 79-88.

11. Lembeck, K.J. (1976) J. Cell Physiol. 89, 277-288.

12. Castor, C.W., Ritchie, J.C., Scott, M.E. and Whitney, S.L. (1977) Arthritis Rheum. 20 : 859-868.

13. Atherly, A.G., Barnhart, B.J. and Kraemer, P.M. (1977) J. Cell Physiol. 90 : 375-386.

14. Cohn, R.H., Cassiman, J.J. and Barnfield, M.R. (1976) J. Cell Biol. 71 : 280-294.

15. Hamerman, D., Todaro, G.J. and Green, H. (1965) Biochim. Biophys. Acta 101 : 343-351.

16. Ishimoto, K., Temin, H.M. and Strominger, J.L. (1966). J. Biol. Chem. 241 : 2052-2057.

17. Bader, J.P. (1972) J. Virol. 10 : 267-276.

18. Burger, M.M. and Martin, G.S. (1972) Nature New Biol. 232 : 9-12.

19. Satoh, C., Duff, R., Rapp, F. and Davidson, E.A. (1973) Proc. Natl. Acad. Sci. 70 : 54-56.

20. Hopwood, J.J. and Dorfman, A. (1977) J. Biol. Chem. 252 : 4777-4785.

21. Glimelius, B., Norling, B., Westermark, B. and Wasteson, A. (1978) Biochem. J. 172 : 443-456.

22. Nothup, S.N., Stasiw, R.O. and Brown, H.D. (1973) Clin. Biochem. 6 : 220-228.

23. Siesions, H.A. (1971) The growth of bone, In: The Biochemistry and Physiology of Bone, Vol. 3. (G.H. Bourne, ed.) pp. 145-180, Academic Press, New York.

24. Pawelek, J.M. (1969) Dev. Biol. 19 : 52.

25. Toole, B.P. (1973) Science 180 : 302.

26. Toole, B.P. and Trelstad, R.L. (1971) Dev. Biol. 26: 28.

27. Bashirov, A.A. (1969) Gig. Tr. Prof. Zabol., 13 : 40.

*28. Withey, J.R. (1976) Environ. Health Perspect. 12 : 125.

29. Ducatmann, A., Hirschhorn, K. and Selikoff, I.J. (1975) Mut. Res. 31 : 163.

30. Creech, Jr. and Johnson, M.N. (1969) J. Occup. Med. 16 : 150.

31. Taborsshaw, L.R. and Gaffey, W.R. (1974) J. Occup. Med. 16 : 509.

32. Infante, P.F. (1976) Ann. N.Y. Acad. Sci. 271 : 49.

33. Danishevsky, I. and Willhite, M. (1945) J. Biol. Chem. 211 : 549.

34. Bardodej, Z., Bardodejeva, E. (1966) Proc. XVth Int. Congr. Occup. Hlth. Vol. II-I, Vienna, Sept. 457.

35. Getell, P., Axelson, O. and Lindelof, B. (1972) Work-environ. Health. 9 : 76.

*36. Ikeda, M. and Imamura, T. (1974) Int. Arch. Arbeitsmed. 32 : 93.

37. Ohtsuji, H. and Ikeda, M. (1972) Toxicol. Appl. Pharmacol. 18 : 321.

38. Leibman, K.C. and Ortiz, E. (1969) Biochem. Pharmacol. 18 : 552.

*39. Demole, E. and Berthet, D. (1972) Helv. Chim. Acta 55: 1866.

*40. Dolgopolov, V.P. and Lishchets, L.I. (1971) Khimfarm. Zh. 5 : 55.

41. Shackleford, W. M. and Keith, L.H. (1976) Frequency of organic compounds identified in water, p. 214, EPA-600/4-76-062. Athens, Georgia, U.S. Environmental Protection Agency.

42. Lee, H. and Neveille, K. Handbook of Epoxy resins, p. 13. McGraw-Hill, New York (1967).

43. Rannug, U., Johnsson, A., Ramel, C. and Wachtmeister, C.A. (1974) Ambio 23, 194.

44. Milvy, P. and Garro, A.J. (1976) Mut. Res. 40 : 15.

45. Loprieno, N., Abbondandolo, A., Barak, R., Baroncelli, S. Bonatti, S., Bronzelti, G., Camellini, Corsi, C., Gorti, G. Fenzza, D., Leparini, C., Mazzaccaro, A., Nieri, R., Rocellini, D. and Rossi, A.M. (1976) Mut. Res. 40 : 317.

46. Abbondandolo, A. (1976) Mut. Res. 32 : 293.

47. Sims, D. and Grover, P.L. (1974) Adv. Cancer Res. 20 : 165.

48. Jerina, D.M. and Daly, J.W. (1974) Science 185, 573.

49. Jallow, D.J., Mitchell, J.R., Zampaglione, N. and Gillettee, J.R. (1974) Pharmacology 11 : 151.

50. Savolainen, H. and Vainio, H. (1977) Toxicol. 8 : 135.

51. Donner, M., Sorsa, M. and Vainio, H. (1979) Mut. Res. 62 : 373.

52. Ikeda, M. (1969) Proc. XVIth Int. Congr. Occup. Hlth. Tokyo, Spet. No. R2-06.

*53. Kokakova, V.V. and Lis, M.B. (1971) Gig. Tr. prof. Zabol. 15 : 54.

54. Marshal, S. (1979) Hazardous and toxic effects of industrial chemicals, Noyes, Data Corporation, New Jersey, U.S.A., pp. 387-388.

55. Seppalainen, A.M. (1975) Scand. J. Work Environ-Health. 1 : 1.

56. Lilis, R., Lorimor, W.V., Diamond, S. and Selikoff, I.J. (1976) Proc. 4th Int. Conf. Medichem. Haifa, No. 44.

57. Vainio, H., Hemminki, K. and Elovaara, E. (1977) Toxicol. 8 : 319.

*58. Rogulo, N.Y. (1974) Gig. Sanit. 11 : 85.

59. Schubert, M. and Hammerman, D. (1968) A primer on connective tissue Biochemistry, Lea and Febiger, Philadelphia.

60. Muir, H. (1969) Am. J. Med. 47 : 673.

61. Sajuera, S.W., Hascall, V.C., Gregory, J.D. and Dziewiatkowski, D.D. (1970) The proteoglycans of bovine nasal cartilage : structure of the aggregate In: Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 2., (E.A. Balazs, ed.) pp. 851-858, Academic Press, New York.

62. Roden, L. (1970) The structure and metabolism of the proteoglycans of chondroitin sulfates and keratan sulfate. In: Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 2, (E.A. Balzs, ed.) pp. 797-821.

63. Roden, L. (1970) Biosynthesis of acidic glycosaminoglycans (mucopolysaccharides). In: Metabolic Conjugation and Metabolic Hydrolysis (Vol. 2, (W. Fishman, ed.) pp. 345-442, Academic Press, New York.

64. Hascall, V.C. and Heinegard, D. (1975) The structure of cartilage proteoglycans. In: Extracellular Matrix Influences on Gene expression, (H.C. Salavkin and R. Crouilch, eds.) pp. 423-434, Academic Press, New York.

65. Singh, N. and Bachawat, B.K. (1965) J. Neurochem. 12 : 519.

66. Singh, N. and Bachawat, B.K. (1968) J. Neurochem. 15 : 249.

67. Young, I.J. and Custod, J.T. (1972) J. Neurochem. 19 : 923.

68. Polarsky, J., Toole, B.P. and Gross, J. (1974) Science 183 : 862.

69. Krusius, T., Finne, J., Karkkainen, J. and Jarnefelt, J. (1974) Biochim. Biophys. Acta 365 : 773.

70. Margolis, R.U., Margolis, R.K., Chang, L.B. and Preti, C. (1975) Biochemistry 14 : 85.

71. Bondareff, W. and Pysh, J.J. (1968) Anat. Rec. 162 : 773.

72. DeSouza, S.W. and Dobbing, J. (1971) Exp. Neurol. 32: 431.

73. Loewi, G. and Meyer, K. (1958) Biochim. Biophys. Acta 27 : 453.

73. Zugibe, F.T. (1962) J. Histochem. Cytochem. 10 : 448.

75. Lipson, M.J., Cerekus, R.A. and Silbert, S.E. (1971) Dev. Biol. 25 : 198.

76. Nanto, V. (1969) Ann. Acad. Sci. Penn. Ser. A5 (Medica) 144 : 1.

77. Breen, M., Weinstein, H.G., Johnson, R.L., Vics, A. and Marshall, R.T. (1970) Biochem. Biophys. Acta 201: 54.

78. Breen, M., Johnson, R.L. Sittig, R.A., Weinstein, H.G. and Vics, A. (1972) Connect. Tissue Res. 1 : 291.

79. Toole, B.P. (1972) Dev. Biol. 29 : 321-329.

80. Toole, B.P. and Gross, J. (1971) Dev. Biol. 25 : 57.

81. Hay, E.D. and Revel, J.P. (1969) Fine structure of the developing avian cornea (A. Wolsky and P.S. Chen, ed.) Vol. I of Monographs in Developmental Biology, Karger, Basel.

82. Praus, R. and Brettschneider, I. (1970) FEBS Lett. 6 : 221.

83. Praus, R. and Brettschneider, I. (1971) Opthalmol. Res. 2 : 367.

84. Pratt, R.M., Larsen, E.A. and Johnston, M.C. (1975) Dev. Biol. 44 : 298-305.

85. Meyer, R.L., Mocholaty, W. and Stauton, D. (1948) Proc. Soc. Expt. Biol. Med. 62 : 529-531.

86. Meyer, K. and Rapport, M.M. (1952) Advances in Enzymology, Vol. 13, Nord, F.F. ed. pp. 199-236, Interscience Publishers, New York.

87. Cinetainsky, A.G., Nature 182 : 1218-1219 (1950).

88. Berlye, G.M. (1960) Nature 185 : 389-390.

89. Kundsen, P.J. and Koefed, J. (1961) Nature 191 : 1306-1307.

90. Cobbin, L.B. and Dicker, S.F. (1962) J. Physiol. 163 : 168-174.

91. Margolis & Margolis (1972) J. Neurochem. 19 : 2325-2532.

92. Markwald, R.K., Fitzharris, T.P.(Bank, H. and Bernanke, D.H. (1976) Dev. Biol. 62 : 292-316.

93. Donner, Jr.W.E. and Cantey, E.Y. (1966) Clinica Chimica Acta 13 : 746-752.

94. Marton, D.D. (1973) Trans. Biochem. Soc. 1 : 385.

95. Derby, M.A. (1978) Dev. Biol. 66 : 321-336.

96. Vaes, G. (1967) Biochem. J. 103 : 802.

97. Aronson, N.N. and Davidson, E.A. (1967) J. Biol. Chem. 242 : 437.

98. Aronson, N.N. and Davidson, E.A. (1967) J. Biol. Chem. 242 : 441.

99. Biryukova, L.S. (1961) Bull Exp. Biol. Med. 50 : 1182-1187.

100. Fleszer-Szafarz, B. and Gullino, P.M. (1970) Proc. Soc. Exp. Biol. (N.Y.) 133 : 597-600.

101. Reissig, J.L., Strominger, J.L. and Leloir, L.F. (1955) J. Biol. Chem. 217 : 959-966.

102. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193 : 265-275.

103. LeGrand, J. (1971) Comparative effects of thyroid deficiency and undernutrition on maturation of the nervous system and particularly on myelination in the young rat. In: Hormones in Development (M. Hamburgh and E.J. Barrington, eds.) pp. 381-390., Appleton-Century-Crofts, New York.

104. Hamburgh, M., Mendoza, L.A., Burkart, J.F. and Weil, P. (1971) In: Hormones in Development (M. Hamburgh and E.J. Barrington, eds.) pp. 403-415, Appleton-Century-Crofts, New York.

105. Polansky, J.R. and Toole, B.P. (1976) Dev. Biol. 53: 30-35.

106. Aliev, T.V., Gadzhiev, F.N. Izv. Akad. Nak. Azerb SSR (1973) Ser. Biol. Nauk. 1 : 80.

107. Meier, S. and Solursh, M. (1973). Dev. Biol. 30 : 290.
108. Martin, T.J., Harris, G.S., Melick, R.A. and Fraser, J.R. (1969) Experientia 25 : 375.
109. Goggin, J.F., Johnson, G.S. and Fanton, I. (1972) J. Biol. Chem. 247 : 5759.
110. Cashman, D.G., Laryea, J.U. and Weissmann, B. (1969) Arch. Biochem. Biophys. 135 : 387.
111. Vaes, G., and Jacques, P. (1965). Biochem. J. 97 : 380.
112. Boyer, K. (1971) The enzymes (Edited by Boyer, P.D.) 3rd ed., Vol. 5, p. 307. Academic Press, New York.

*Originals not seen.