PART I (A)

EFFECT OF URETHANE ON CHICK EMBRYOS CULTURED IN VITRO AND SUBSEQUENT TREATMENT WITH VARIOUS METABOLITES
INTRODUCTION
It is universally accepted that the structure of animal tissues can be altered when subjected to different stresses. Whatever the mode of application, because of the softness of the tissues and the rapid changes in the cells which are going on at the time of development, young ones and embryos are more sensitive to these stresses than adults. The study of the effects of chemical agents on embryonic development has gained a lot of importance as a tool in elucidating the process of morphogenesis. Effect of different chemicals with known reactions and their interference with morphogenesis, might be useful to find out their probable target regions. Of late the study of chemical interference in the natural process of morphogenesis has become a fruitful field of investigation.

**Multiple biological effects of urethane**

Urethane has been shown to be a unique agent in its effect upon cell division, growth and differentiation. As shall be seen later, it often acts as a teratogen, mutagen and a multi-potent carcinogen.

Urethane (ethyl carbamate) is a relatively simple substance which was synthesized by Duman as early as in 1834 (c.f. Haddow, 1963). Since then it has proved to exhibit an astonishing diversity and versatility in its biological interactions. It has been used therapeutically as early as in
1885 (Sinclair, 1950). On the European continent it was used as a sedative for the insane and in the control of hysterias (Sinclair, 1950). Sieveking (1886) reported its successful use in controlling intractable pain from many severe conditions, including massive internal cancers. In the early days, it was used as an anaesthetic in experimental animals and continues to be so used even in recent years. It has been used as an anaesthetic in fishes (Ball & Cowen, 1959), in rats (Bieger et al., 1977; Krieg et al., 1977 and Scott & Aaron, 1977), in cats (Dubrovsky & Barbas, 1977), in rabbits (Domer & Kaiser, 1977), and in guinea pigs (Jankowski et al., 1971). Later it was used as a hypnotic in human medicine and especially in the realm of obstetrics (c.f. Haddow, 1963). It has been used as a narcotic in rats (Sinz, 1971) and in mice (Lehr, 1972). As a biochemical agent, its gamut of potentialities traverses many aspects of chemistry, biology and medicine, from purely chemical studies through microbiology, marine biology and plant physiology, to developmental physiology, cytology, pharmacology and toxicology, pathology, endocrinology, immunology and chemotherapy (c.f. Haddow, 1963).

Urethane, a carcinogenic and carcinostatic agent

Carbamates were used in earlier survey for selecting compounds for testing against tumours. It was Lefevre (1939) who exposed the plants to ethyl phenyl carbamate and noticed that
it showed growth retardation. This was later extended by a systematic survey of aryl carbamates and related substances as plant growth inhibitors (Templeton & Sexton, 1945). These studies adequately pointed to the possible use of this class of compounds in the retardation of neoplastic growth. However, urethane (ethyl carbamate) would have been neglected because of its lack of response in the initial screening, if the data from plant studies alone had been relied upon. Fortunately, Haddow and Sexton (1946) included urethane among the carbamates tested against the walker rat carcinoma, 256 and a mouse spontaneous mammary adenocarcinoma and reported some interesting observations.

The earlier studies on urethane, a carbamate encouraged extensive studies of its specificity among the carbamates. It was shown by Warburg (1910, 1911) that among the homologous series of carbamates, the higher members of the series are more effective than urethane, in retarding cell division. This was later confirmed by Cornman (1950). Larsen (1946, 1948) showed that tumour formation is restricted almost entirely to urethane with some carcinogenic activity residing in a few simple congeners. Haddow and Sexton (1946) and Dustin (1947) report that urethane is more effective than phenylurethane or isopropyl phenyl carbamate, in the treatment of animal tumours and animal leukaemia. Division inhibition, narcosis and toxicity progressively increase as one goes up in the series, but
carcinogenic and carcinoclastic activity is scattered sparsely among compounds not much different from urethane (Cornman, 1954). Amongst several carbamates that have been studied urethane seems to have a unique effect against mouse leukaemia (Skipper & Bryan, 1949), and against mouse sarcoma 180 (Stock et al, 1953). The carcinogenic property of urethane appears to be as unique among carbamates as its antileukemic action. Some other activities of urethane are, however, shared with other carbamates, e.g. hypnosis (Goodman & Gilman, 1941), mitotic inhibition (Cornman, 1950) and leucopenic action (Skipper et al, 1948).

It was Mndzhoyan et al (1963) who in their studies on the antitumour activity of some groups of urethane derivatives found that ethyl carbamate showed the highest antitumour activity. Mirvish et al (1969), from their comparative study of lung carcinogenesis, reported urethane to be more active as a lung carcinogen than N-hydroxyurethane, N-methoxyurethane and N-propyl carbamate. Pound (1972) in his studies on tumour formation in mice by urethane and its homologous derivatives found that neither the homologous esters nor the N-substituted derivatives had any influence on the yield of tumours of the skin, lung or liver. Urethane displayed a high degree of blastomogenic activity with none in methyl carbamate (Yagubov & Suvalova, 1973).

It is seen from reports in the past that N-hydroxyurethane is an essential and active metabolite in the biological
action of urethane (Boyland & Nery, 1965 and Boyland et al., 1965). Both urethane and N-hydroxyurethane are reported to be equally carcinogenic (Berenblum et al., 1959). Kaye (1960) suggested that there is no specific metabolite of urethane, which could be involved in the process of carcinogenesis. Later he stated that the carcinogenic action of N-hydroxyurethane is due to its rapid conversion back into urethane (Kaye & Trainin, 1966). He thus challenged the view put forth by Boyland and coworkers (Boyland & Nery, 1965 and Boyland et al., 1965), according to whom the carcinogenic and antileukemic effects attributable to urethane are probably caused by the hydroxyurethane metabolites. Mirvish (1969) proposed that the active carcinogen may be urethane instead of N-hydroxyurethane as proposed by Boyland and Nery (1965). Bhide et al. (1974) have supported this view put forth by Mirvish (1969).

Toxic effects

The toxicology of urethane has been widely investigated in many species, for example the cat, and the guinea pig (Scanu & Gallo, 1954 and Scanu et al., 1954). It was Kerenyi and Rona (1955) who studied the effects of urethane on the renal, glomerular and capillary systems in rabbits, dogs, guinea pigs, mice and rats. The induction of renal glomerular lesions with urethane, in inbred mice susceptible to spontaneous glomerulonephritis, was described by Kirschbaum and Bell (1947).
Topete (1951) reported a lowering of the partial pressure of oxygen in arterial blood as caused by urethane in common with other hypnotics. Exposure to urethane brought about vasodilation and distension of the vascular bed in *Rana pipiens* embryos (McMillan & Battle, 1954). Roe (1957) described haemorrhagic lesions in the livers of mice following urethane application. It was Levy and Duke (1956) who reported a human case of fatal urethane poisoning as a result of topical therapy with the carbamate, in an attempt to control bacterial infection in multiple burns. As a result, urethane was no longer indicated for this purpose. It was Bertrand and Quivy (1952), who showed that urethane brought about a reduction of fertility in adults and follicular atrophy and an arrest of spermatogenesis in the young. Influence of urethane on the vaginal sex cycle was studied by Fuhrmann (1950) and its action as an antagonist of folliculin was reported by Saurer (1945). It was seen that in rats when urethane was given as an anaesthetic before the critical period, it blocks the ovulatory release of LH or delays and reduces the release of this hormone (Lincoln & Kelly, 1972). A number of workers have reported enhancing effect of urethane on the severity of infection with certain murine viruses, as for instance those of viral pneumonia PVM (Mirick et al., 1952), specific strains of coxsackie viruses (Wallis & Sulkin, 1953) and of viral hepatitis (Braunsteiner & Friend, 1954 and Friend & Yuceoglu, 1955). During immunological studies it was seen that administration of
urethane ten days prior to injection of diphtheria toxoid, eliminated antibody production (Borneff & Lennert, 1950), and that this effect was correlated with atrophy of the lymphoid follicles of the spleen, especially of the larger lymphocytes (Lennert & Borneff, 1951). Tal'chuk (1974) reported that urethane treatment caused a decrease in the weight of the spleen.

Because of its possible use as an adjuvant it was combined with other drugs and Peple (1949) reported two deaths from such a combination with penicillin. It is also used as a cosolvent in many water-insoluble drugs and Nomura (1975) has warned of the possible hazards arising due to possible carcinogenic effects of urethane in humans. Treatment of beverages with diethyldicarbonate leads to the formation of urethane, as a result of which a cancer risk to man from urethane cannot be excluded. Therefore, Schmaehl et al. (1977), have suggested the replacement of diethyldicarbonate by a toxicologically unobjectionable compound.

In addition to the biological responses already quoted, urethane gives rise to many other effects, some of them almost bizarre, as the production of oedema by urethane in monkeys (Weston, 1942) and the restoration of gastric motility by urethane after sectioning of the vagus nerve for peptic ulcer (Machella et al., 1947).
Nucleotoxic and Cytotoxic effects

In 1943, Oehlkers reported induction of large chromosomal aberrations by urethane (c.f. Freese, 1967). Extensive literature surveys regarding the effects of urethane in the mitotic processes in plant and animal cells exist (Gayer & Claus, 1947; Hohl, 1947; Dustin, 1947; Hirscher & Laur, 1950; Gattiker, 1952; Driessens, 1952 and Cormann, 1954). In general these studies have revealed that at high concentrations a block and at medium concentration a retardation occurs in cell division. Others have more specifically shown an impairment of the action of spindle fibres (Ennis, 1948), chromatin condensation and the appearance of double nuclei (Osgood & Chu, 1948), an elevated mitotic index due to a decrease in prophases and an increased metaphase index, (Rosin, 1951 and Rosin & Goldhaber, 1956, 1958) or an inhibited entry into prophase (Deysson & Truhaut, 1957 and Truhaut & Deysson, 1957). In his cinematographic studies on chick frontal bone, Hughes (1950) has shown that urethane is a metaphase inhibitor.

Nucleotoxic and cytotoxic effects of urethane on mitosis and meiosis in the grasshopper have been reported by Nambiar (1955). Boyland & Koller (1954) showed that urethane produced chromosomal damage in the rat tissue similar to that produced by irradiation. Inhibition of cleavage in chaetopterus and sea urchin eggs, on treatment with urethane have been shown
Failure of organisation of chick embryonic cells (Adhikari, 1961), and various anamolies of frog embryos (Joshi, 1967) have been attributed to urethane induced cellular misdivision. Deshpande (1970) studied the nucleotoxic and cytotoxic effects of urethane in the primitive erythrocytes of chick. Timson (1970) reported urethane to be cytotoxic in human lymphocytes. Urethane was said to have caused meiotic anamolies in hamsters (Lavappa & George, 1971). Pogosyants et al (1968) reported that urethane induces chromosome abnormalities both in primary cultures of mouse embryo lung cells and chinese hamster cell line. Bhattacharya (1974) studied the cytological and cytochemical effects of urethane on meiotic cells of Rhoeo discolor. Recently, Ishidate and Odashima (1977) reported chromosomal aberrations in chinese hamster cells on treatment with urethane.

**Effect on Plants**

Urethane brings about inhibition of photosynthesis of some algae namely *Chlorella*, *Nostoc* sp., *Rhodospirillum rubrum*, *Oscillatoria* sp., *Chlamydomonas* sp. and one angiosperm namely *Lemna arnhiza* (Bierhuizen, 1957).

**Mutagenic effects**

Urethane was found to induce mutations in bacteria (Latarjet et al, 1950) and in Drosophila (Vogt, 1948, 1950).
**Teratogenic effects**

Högland (1952) while studying the effects of urethane on reproduction in mice observed that it caused a marked reduction in the number of living offsprings. Influence of urethane on development and teratogenesis in Amphibia was investigated by Stroink (1951) and Joshi (1967). In the teleost, *Brachydanio rerio* urethane produced retardation of growth and differentiation in early development (Battle & Hisaoka, 1952). Similarly, McMillan and Battle (1954) found that the blastulae, gastrulae and newly hatched larvae of the leopard frog, when exposed to urethane, underwent retardation of growth and development, the earlier stages being more susceptible. Landauer (1956), studied the influence of urethane in hereditary and induced cross beak of fowl. Takaori et al (1966) showed that urethane caused developmental abnormalities in the rat embryos, mainly in the skeletal system. On the same lines, Ferm (1966, 1969) reported developmental malformations such as spina bifida, exencephaly, abnormally coiled cardiac tubes and open neural tube on urethane treatment in the embryos of hamster. Urethane treatment produced subcutaneous haemorrhages and eye defects in the mouse (Diwan et al, 1970 and Diwan & Batra, 1972). Urethane was found to be teratogenic in mice (Nomura & Okamoto, 1972). In 1974, Diwan and Mulherkar reported abnormal development of chick embryos treated with N-ethyl urethane in *vitro* for 21 hours.
Joashi (1975) in his studies on the effect of urethane on developmental stages of the yellow meal worm *Tenebrio molitor* L. showed that there was total lack of normal development. Bressman and Seto (1976) reported histologically obvious degenerative changes in the neural tissue of mouse embryos when treated with urethane. Digital anomalies of mouse limbs on urethane treatment were reported by Yasuda (1977).

Our studies with urethane

The studies till today, however, give relatively very little detailed information about the mechanism of action of urethane during the fundamental processes of growth, differentiation and also embryogenesis. None of these observations either singly or in combination afford any convincing interpretation and explanation as to the mode of action of urethane.

The chick embryo which is a nutritionally self-contained experimental entity has been widely used as a test system for the study of inhibitory action of drugs, other inhibitory substances and teratogenic agents, (Cravens, 1952; O'dell & McKenzie, 1963; Billet et al., 1965 and 1971; Bowman, 1967; Caplan et al., 1968; Caplan, 1971; Gebhardt, 1972; Katdare & Mulherkar, 1972; Lee & Cortes, 1972; Lee et al., 1974 and Telang & Mulherkar, 1974). The method of culturing chick embryos *in vitro* described by New
(1955) has several advantages over the in ovo methods of testing the effects of various substances. The main advantages of such a system lie in the possibility to study embryotoxic effects with well defined concentrations of drug or its metabolites without any external interference and as an available model system which in some respects is more easily understood than the complex developmental processes occurring in ovo. Though the in vitro method is more elaborate and time consuming than simple in ovo injection, it probably ensures uniform diffusion of the chemicals into the tissues. Thus a relatively small number of specimens may be sufficient to assess the effects. Further the method can be adapted to experiments involving removal of the material and growing the embryos in media with or without other chemicals.

It was thus felt desirable to study the effects of urethane on the early embryonic development of chick. By studying its influence on metabolically active embryonic system one may have a better idea of the possible mode of action of this versatile substance.

In this part, firstly, effect of urethane on chick embryos cultured in vitro was seen and then the effect of subsequent treatment with various metabolites on these urethane treated embryos was studied.
MATERIALS AND METHODS
Effect of urethane on the development of chick embryos has been studied earlier (Adhikari, 1961 and Diwan & Mulherkar, 1974). Earlier in this laboratory Diwan and Mulherkar (1974) cultured the chick embryos in vitro and treated them with 0.25% urethane for 20-21 hours. For reversal studies they injected urethane in ovo. After this, these treated embryos were cultured in vitro during which the chemical for subsequent treatment was added. We wanted to carry out both these treatments in vitro, as this method ensures uniform diffusion of the chemicals into the tissues. Further, the method can be adapted to experiments involving removal of the teratogen and growing the embryos in media with or without other chemicals. To achieve all these objectives, the duration of urethane treatment had to be shortened. Therefore a series of experiments were carried out to fix the stage of the embryo, concentration of urethane and duration of the treatment.

Asceptic conditions were maintained throughout the experiments by sterilizing the glassware, metallic instruments etc. and by autoclaving separately the constituent solutions of the Pannett Compton's saline (P.C. saline) at 15 lbs. pressure for 45 to 60 minutes.
Fresh, fertilized eggs of white leghorn hens brought from a local poultry farm were incubated at 37.5° ± 1°C for adequate number of hours to get the mid-primitive streak stage or definitive primitive streak stage (stage 3* or 4 of Hamburger & Hamilton, 1951; Text Figs. 2 and 3, Plate I).

The incubated eggs were then cooled down to room temperature. The embryos were explanted from the incubated eggs and mounted in vitro by the method described by New (1955) (Text Fig. 1, Plate I). It consists of cutting the vitelline membrane along the equator of the egg proper. The vitelline membrane is then carefully peeled out along with the blastoderm. The yolk sticking to the vitelline membrane is removed by gentle jets of saline produced with the help of a dropper. The vitelline membrane along with the blastoderm is then transferred on to a watch glass with the endodermal side facing the observer. The edge of the membrane is folded over the ring on all sides thereby keeping the vitelline membrane with the blastoderm considerably stretched. The watch glass is then transferred to a petridish lined with moist cotton wool. Thin albumen is added around the ring and a few drops (0.1 ml) of Pannett Compton saline inside the ring for nutrition of the developing embryo. The petridish is covered.

The cultures were employed in studying the effects of
Ethylurethane. Ethylurethane, an E. Merck product, was employed in the present work. Its structural formula and other relevant details are as follows:

Structural formula:

\[
\begin{array}{c}
\text{H} \\
\text{N} - \overset{\circ}{\text{O}} - \overset{\circ}{\text{C}}_2\text{H}_5 \\
\text{H}
\end{array}
\]

Urethane (Ethyl Carbamate)

Molecular formula: \( \text{H}_2\text{N COO C}_2\text{H}_5 \)

Molecular weight: 89.10

Urethane is an integrated chemical of an ethyl, an amino and a carboxyl radical. It is also known as Ethyl Carbamate. It is structurally one of the simplest carcinogens soluble both in water and lipids. In fact, it was the first water soluble carcinogen to be discovered. It is a white solid with m.p. 48.19° and b.p. 185.25°. It sublimes, absorbs moisture and is soluble in lower alcohols, ketones, ethers, esters, chlorinated hydrocarbons and water. It is partially soluble in aromatic hydrocarbons and insoluble in aliphatic hydrocarbons. It contains amide and ester groups, and is readily hydrolyzed by hot acid and alkali to give \( \text{NH}_3, \text{CO}_2 \) and ethanol. It, however, exhibits only a few distinct chemical reactions (Mirvish, 1968). The chemical is in a crystalline form. A fresh solution of urethane was prepared every time in Pannett Compton saline.
From amongst the various concentrations of urethane used (Table 1) on different developmental stages (Table 2) and for different time durations (Table 1), the concentration of 0.168 M at the mid-primitive streak stage (stage 3*) or definitive primitive streak stage (stage 4) with 4 hours duration was found suitable (Table 2), and hence was used throughout the experiments.

From the explanted embryos, half of them were treated with 0.1 ml of 0.168 M urethane solution (experimentals) and were kept for 45 minutes at room temperature for proper diffusion of the chemical. The other half was treated with 0.1 ml of Pannett Compton saline (controls), and kept at room temperature for identical period. 60 embryos each of experimental and control set were kept.

All the embryos were then incubated for 4 hours at 37.5°C ± 1°C after which the urethane from the treated embryos was completely removed. This was done by the following procedure. The liquid egg albumen outside the ring was removed, inside of the ring flooded with Pannett Compton saline, and then the watch glass along with the ring was gently immersed in a petridish full of Pannett Compton saline. All the embryos were thus thoroughly washed three times for complete removal of any unbound urethane. Having done this, the watch glass along with the ring was then transferred back on to the cotton bed. Superfluous Pannett
Compton saline was removed and a few drops of fresh Pannett Compton saline were added inside the ring, and thin albumen on the outside of it. The controls were also operated in the same way so as to constitute identical treatment.

All the embryos were further incubated for 18-21 hours. After this the gross morphological peculiarities of all these embryos were noted down by observing them under 10 x magnification, They were then fixed in Bouin's fixative. A few representative embryos from each of the two sets were processed for entire mountings, stained in diluted Delafield's haematoxylin, differentiated in acid water, cleared in xylene, mounted in D.P.X. and photographed.

**SUBSEQUENT TREATMENT WITH VARIOUS METABOLITES**

Having studied the effective sublethal optimum concentration of urethane, 0.168 M for 4 hours, the next step was to study the reversal if any, of the teratogenic effects of urethane by subsequent treatment with various metabolites viz., Aspargine, Carbamyl-aspartic acid, Dihydro-orotic acid, Orotic acid, Uridine, Thymidine, Choline, Folic acid, Vitamin B₁₂, p-aminobenzoic acid, Vitamin C, Methionine, Homocysteine, Glycine, Serine, Histidine and Inosinic acid.

In these experiments, the urethane treated embryos,
after the 4 hour treatment were thoroughly washed three times in Pannett Compton saline as described in details earlier (Refer page 16). Half of them were treated subsequently with 0.1 ml of approximately equimolar solution of the above mentioned chemicals. The other half of the urethane treated embryos were treated with Pannett Compton saline. In this series the urethane treated embryos served as controls and the ones treated subsequently with the various above mentioned metabolites served as experimental sets. All the embryos from both the control and the experimental sets were then incubated at 37.5° ± 1° for 18-21 hours, after which they were observed under 10 x and gross morphological peculiarities noted down. They were fixed in Bouin's fixative. Few representative embryos from each set were processed for entire mountings and photographed.
EXPERIMENTAL RESULTS
As mentioned earlier, effects of various concentrations of urethane at different stages for different durations on the developing chick embryo were studied. From amongst the various concentrations of urethane used (Table 1) at different developmental stages (Table 2), the concentration of 0.168 M urethane for four hours duration at stage 3+ and 4 gave the best results in terms of high incidence of abnormality (Text Fig. 4, Plate II) and low rate of mortality (Text Fig. 5, Plate III). It was observed that the concentration higher than the above mentioned optimum sublethal concentration (0.168 M for 4 hours), such as 0.225 M for 4 hours or 6 hours duration proved lethal to the embryos (Table 1; Text Fig. 5, Plate III; Text Fig. 7, Plate V) and hence showed poor incidence of abnormalities in the survivals (Text Fig. 4, Plate II; Text Fig. 6, Plate IV). The development of these embryos was arrested at stage 4 and most of the embryos showed complete degeneration after 20-21 hours of incubation. On the contrary at a concentration lower than the optimum sublethal concentration (0.168 M for 4 hours), such as 0.112 M for 4 hours and 6 hours there was low incidence of abnormality and mortality (Text Figs. 4, 5, 6 and 7; Plates II, III, IV and V). The embryos failed to exhibit optimum effects of urethane and showed a slight retardation of growth (Table 1).
The concentration of 0.168 M at 6 hours proved lethal to the embryos (Table 1, Text Fig.7, Plate V). Therefore, the duration of the treatment was decreased to 4 hours, which was found to be effective in exerting optimum sublethal potentially reversible effects (Table 1). In this series of experiments 120 chick embryos were explanted in vitro, out of which 60 embryos were treated with 0.168 M urethane for 4 hours (experimental). An equal number of embryos treated with Fannett Compton saline instead of urethane were kept as controls. The embryos thus treated with 0.168 M urethane showed abnormal development of almost all the organ systems and are described in detail subsequently (Table 3, Text Figs.9,11,13,15 and 17; Plate VI, VII and VIII). On the other hand it was seen that the controls showed normal development (Text Figs.8,10,12,14 and 16; Plate VI, VII and VIII).

The urethane treated embryos exhibit a profound stage specificity in manifestation of its effects. A differential response to urethane treatment was seen depending on the stage of development of the embryos at the time of treatment. Differences in the response were seen with different durations and different concentrations. Thus urethane treated embryos showed lethal, toxic effects at stage 3, optimum sublethal effects at stage 3* and 4 and none at stage 5 (Table 2). Stage specificity on similar lines was reported by Lakshmi(1962),

The failure of urethane to act at stage 5 could be explained on the basis of the following possibilities as mentioned by Telang (1974):

(a) The concentration and/or duration of urethane treatment is not optimum to the embryos at this particular stage of development.

(b) Some mechanism for the detoxification of urethane is evolved at this particular stage of development.

(c) Folic acid mediated conversions, which are derailed or impaired at stage 3 and 4, as discussed later, are not required at stage 5.

(d) The requirement of the above mentioned conversions is fulfilled by some alternative pathway.

From the data in Table 3, it could be seen that the organ systems predominantly affected by urethane treatment are the fore brain, the mid and the hind brain, the neural tube, the optic vesicles, and the somites which show abnormal differentiation to the extent of 76.6%, 90.0%, 91.6%, 83.3% and
80.0% respectively.

Due to the abnormal differentiation of the brain there is very frequently present a solid mass of tissue in place of a well developed brain, clearly differentiated into prosencephalon, mesencephalon and rhombencephalon (Text Fig. 9, Plate VI). Sometimes, forebrain in the form of a plate like structure is seen (Text Fig. 11, Plate VI).

The malformations of the optic vesicles or their total absence could be correlated with the abnormal differentiation of the forebrain (Text Fig. 9, Plate VI). Thus in embryos where total development of the forebrain is inhibited the optic vesicles are absent (Text Fig. 11, Plate VI). While in embryos where neural folds at the forebrain region level remain separate, the optic vesicles are only partially differentiated (Text Fig. 13, Plate VII).

The abnormal differentiation of the neural tube is frequently seen as the wide separation of neural folds and as a result of this the neural folds never fuse to form the neural tube (Text Figs. 11 and 13; Plates VI and VII).

The brain abnormality especially the microcephalous condition along with the shortening of the embryonic axis, brings about the anterior placement of the heart (Text Fig. 15, Plate VII). In such cases the heart lies anterior to the diminutive
brain. Inspite of the change in position the rhythmic contractions of the heart are very well seen, suggesting normal functional differentiation. As already mentioned (Table 3) the heart is one of the least affected organ systems after urethane treatment. The abnormal differentiation of the heart, which is seen only in 46.6% of the treated embryos (Table 3), is usually seen as the arrest of its development at the time of fusion of the omphalo-mesenteric veins. The heart in such cases is seen as a non-pulsatile straight tube (Text Figs.11,13,14,17 and 18; Plates VI,VII and VIII).

It has been reported by Spratt(1952 and 1956) that the brain and the heart in chick embryos exhibit a differential inhibitory response to treatment with azide and fluoride, suggesting that the formation of these organs is underlain both quantitatively and qualitatively by different metabolic processes, since azide preferentially inhibits brain differentiation, while fluoride inhibits the differentiation of the heart. On somewhat similar lines, Telang(1974) reported higher sensitivity of brain differentiation than that of heart to hydrazine treatment in chick embryos.

In the present study, it is reasonable to conclude that metabolic processes responsible for differentiation of brain are more sensitive to urethane treatment than those responsible for
The abnormal differentiation of the somites which is to the extent of 80.0% is usually manifested as diffused somites (Text Fig. 17, Plate VIII). The somites on the whole show reduction in number and sometimes lack their counterpart (Text Figs. 11, 15 and 17; Plates VI, VII and VIII) on the other side.

As a result of urethane treatment there is a considerable shortening of the embryonic axis (Text Figs. 9, 11 and 17; Plates VI, VII and VIII). It is seen to be significant in 73.0% of the treated embryos. This malformation, however, is invariably present along with other malformations described previously, such as abnormal forebrain, retardation or total absence of the mid and hind brain, persistently separated neural folds, retarded heart and diffused somites.

The embryos from the control series, on the whole, showed normal development of all the organ systems such as brain, neural tube, optic vesicles, heart and somites (Text Figs. 8, 10, 12, 14 and 16; Plates VI, VII and VIII).

**Subsequent treatment with various metabolites**

To understand the mechanism of action of urethane, in the present study, the urethane treated embryos were subsequently treated with various metabolites listed in Tables...
In this series of experiments, the urethane treated embryos were equally divided into two groups, each group consisting of 30 embryos. Of these two groups of urethane treated embryos, one group served as the controls, as mentioned earlier in Materials and Methods (see page 16). The embryos in the other group were subsequently treated with various substances listed in Table 4a, 4b and 4c, and served as experimentals.

From amongst the individual amino acids, used for the subsequent treatment of urethane treated embryos (Table 4a), glycine (Text Figs. 19 and 21, Plate IX), methionine (Text Figs. 27 and 29, Plate XI), were capable of reversing the urethane induced abnormalities to the extent of 63.3% and 60.0% respectively, while serine (Text Figs. 23 and 25, Plate X), homocysteine (Text Figs. 31 and 33, Plate XII) and histidine (Text Figs. 35 and 37, Plate XIII) gave reversal only to the extent of 23.3%, 20.0% and 33.3% respectively. Asparagine gave reversal only to the extent of 33.3% (Text Figs. 39 and 41, Plate XIV). Urethane controls for these above mentioned series showed abnormal development (Text Figs. 18, 20, 26, 28, 22, 24, 30, 32, 34, 36, 38 and 40).

In the experiments where nucleic acid precursors were used for the subsequent treatment of urethane treated embryos
(Table 4b) it was observed that thymidine was capable of reversing the urethane-induced abnormalities to the extent of 66.6% (Text Figs. 43 and 45; Plate XV), while uridine could reverse these effects only to the extent of 16.6% (Text Figs. 47 and 49; Plate XVI). Orotic acid which is important for general pyrimidine biosynthesis, however, could give reversal only to the extent of 26.6% (Text Figs. 51 and 53; Plate XVII). Even other nucleic acid precursors such as dihydro-orotic acid, carbamyl aspartic acid gave reversal only as much as 23.3% and 15.6% respectively (Text Figs. 55 and 57, Plate XVIII; Text Figs. 59 and 61; Plate XIX). Inosinic acid gave reversal to the extent of 56.6% (Text Figs. 63 and 65, Plate XX). Urethane controls for the above respective series showed abnormal development (Text Figs. 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, and 64).

The data in Table 4c shows the effects of certain vitamins and other related substances on urethane treated embryos. It is evident that folic acid as well as its precursor substance, p-aminobenzoic acid, are successful in bringing about the reversal to the extent of 70.0% and 73.5% respectively (Text Figs. 67 and 69, Plate XXI; Text Figs. 71 and 73, Plate XXII). Vitamin B₁₂ (cyanocobalamin), and choline are also effective in bringing about the reversal of urethane effects, both to the extent of 60.0% (Text Figs. 75 and 77, Plate XXIII; Text Figs. 79 and 81, Plate XXIV). Vitamin C brought about the reversal in
56.5% of the embryos (Text Figs. 83 and 85; Plate XXV). The urethane controls for this series showed abnormal development (Text Figs. 66, 68; 70, 72; 74, 76; 78, 80, 82 and 84).
TABLE 1

EFFECT OF DIFFERENT CONCENTRATIONS OF URETHANE FOR DIFFERENT DURATIONS ON CHICK EMBRYOS AT MID PRIMITIVE STREAK STAGE (STAGE 3+) AND DEFINITIVE PRIMITIVE STREAK STAGE (STAGE 4).

Stage of development (H.H.): 3+ and 4

<table>
<thead>
<tr>
<th>Conc. of urethane used</th>
<th>Duration of treatment</th>
<th>Results</th>
<th>Conclusions</th>
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<tbody>
<tr>
<td>0.112 M</td>
<td>4 HRS. &amp; 6 HRS.</td>
<td>Retardation of growth, Low mortality. Abnormalities not pronounced. All the organ systems except heart shows retarded development. Heart singularly unaffected.</td>
<td>Treatment sublethal but not optimum.</td>
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<tr>
<td>0.168 M</td>
<td>4 HRS.</td>
<td>Almost all the organ systems show abnormal differentiation. Mortality is negligible.</td>
<td>Treatment sublethal and optimum.</td>
</tr>
<tr>
<td></td>
<td>6 HRS.</td>
<td>Arrest of embryonic growth at stage 4. Some signs of degeneration evident. Mortality is high.</td>
<td>Treatment lethal to the embryos.</td>
</tr>
<tr>
<td>0.225 M</td>
<td>4 HRS. &amp; 6 HRS.</td>
<td>Primitive streak degenerated. Total absence of growth and differentiation. 90% of the treated embryos died.</td>
<td>Treatment is highly lethal to the embryos.</td>
</tr>
</tbody>
</table>

Stages of development according to Hamburger, V., and Hamilton, H.L. (1951).

A series of normal stages in the development of the chick embryo.

## TABLE 2

**DIFFERENTIAL RESPONSE OF EMBRYONIC STAGES TO URETHANE TREATMENT**

No. of embryos treated: **30 for each concentration**

Concentration of urethane used: **0.168 M**

Duration of treatment: **four hours.**

<table>
<thead>
<tr>
<th>Stage of development (H.H.)</th>
<th>Results</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>All the treated embryos develop normally.</td>
<td>Stage shows resistance to urethane treatment.</td>
</tr>
<tr>
<td>4</td>
<td>Almost all the organ systems show abnormal differentiation. No trace of degeneration. Mortality very low.</td>
<td>Concentration is effective, optimum sublethal.</td>
</tr>
<tr>
<td>3*</td>
<td>Almost all the organ systems show abnormal differentiation. No trace of degeneration. Mortality very low.</td>
<td>Concentration is effective, optimum sublethal.</td>
</tr>
<tr>
<td>3</td>
<td>Embryotoxic effects observed.</td>
<td>Embryos highly susceptible to urethane treatment.</td>
</tr>
<tr>
<td></td>
<td>No differentiation due to arrested growth at stage 4.</td>
<td></td>
</tr>
</tbody>
</table>


A series of normal stages in the development of the chick embryo.

*J. Morph.*, **88**: 49-92.
### TABLE 3

**EFFECT OF URETHANE ON EMBRYOS CULTIVATED IN VITRO**

<table>
<thead>
<tr>
<th>Organ systems</th>
<th>No. of embryos showing normal development</th>
<th>No. of embryos showing abnormal abnormalities</th>
<th>% of abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con</td>
<td>Exp</td>
<td>Con</td>
</tr>
<tr>
<td>1. Forebrain</td>
<td>55</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>2. Midbrain and Hindbrain</td>
<td>54</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>3. Neural tube</td>
<td>56</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4. Optic vesicles</td>
<td>53</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>5. Heart</td>
<td>58</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>6. Somites</td>
<td>55</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>7. Embryonic Axis</td>
<td>57</td>
<td>24</td>
<td>3</td>
</tr>
</tbody>
</table>

**Total number of embryos:** Con. : 60 ; Exp. : 60.

**Con:** Control series, untreated embryos grown in Pannett Compton saline.

**Exp:** Experimental series, urethane treated embryos grown in Pannett Compton saline.

**Stage of the embryo:** 3<sup>rd</sup> or 4.

**Concentration of urethane:** 0.168 M

**Duration of treatment:** 4 hours.
### TABLE 4(a)

**EFFECT OF VARIOUS METABOLITES (AMINO ACIDS) ON URETHANE TREATED CHICK EMBRYOS CULTURED IN VITRO**

<table>
<thead>
<tr>
<th>Metabolite used</th>
<th>No. of embryos showing normal development</th>
<th>No. of embryos showing abnormal development</th>
<th>Percentage of reversal obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>19</td>
<td>11</td>
<td>63.3</td>
</tr>
<tr>
<td>Serine</td>
<td>7</td>
<td>23</td>
<td>23.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>18</td>
<td>12</td>
<td>60.0</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>6</td>
<td>24</td>
<td>20.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>10</td>
<td>20</td>
<td>33.3</td>
</tr>
<tr>
<td>Asparagine</td>
<td>10</td>
<td>20</td>
<td>33.3</td>
</tr>
</tbody>
</table>
### TABLE 4(b)

**EFFECT OF VARIOUS METABOLITES (NUCLEIC ACID PRECURSORS) ON URETHANE TREATED CHICK EMBRYOS CULTURED IN VITRO**

<table>
<thead>
<tr>
<th>Metabolite used</th>
<th>No. of embryos showing normal development</th>
<th>No. of embryos showing abnormal development</th>
<th>Percentage of reversal obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine</td>
<td>20</td>
<td>10</td>
<td>66.6</td>
</tr>
<tr>
<td>Uridine</td>
<td>5</td>
<td>25</td>
<td>16.6</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>8</td>
<td>22</td>
<td>26.6</td>
</tr>
<tr>
<td>Dihydro-orotic acid</td>
<td>7</td>
<td>23</td>
<td>23.3</td>
</tr>
<tr>
<td>Carbamyl-aspartic acid</td>
<td>5</td>
<td>25</td>
<td>16.6</td>
</tr>
<tr>
<td>Inosinic acid</td>
<td>17</td>
<td>13</td>
<td>56.6</td>
</tr>
</tbody>
</table>
### TABLE 4(c)

**EFFECT OF VARIOUS METABOLITES (VITAMINS) ON URETHANE TREATED CHICK EMBRYOS CULTURED IN VITRO.**

<table>
<thead>
<tr>
<th>Metabolite used</th>
<th>No. of embryos showing normal development</th>
<th>No. of embryos showing abnormal development</th>
<th>Percentage of reversal obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic acid</td>
<td>21</td>
<td>9</td>
<td>70.0</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>22</td>
<td>8</td>
<td>73.3</td>
</tr>
<tr>
<td>Vitamin B(_{12})</td>
<td>18</td>
<td>12</td>
<td>60.0</td>
</tr>
<tr>
<td>Choline</td>
<td>18</td>
<td>12</td>
<td>60.0</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>17</td>
<td>13</td>
<td>56.6</td>
</tr>
</tbody>
</table>
DISCUSSION
Normal development depends on a harmonious sequence of closely interdependent events. All of these events presumably are consequences of concurrent chemical and physical processes. Disturbances in either spatial or temporal synchronization of the many developmental interrelationships may lead to abnormal individuals and structures.

Embryological studies must eventually provide the basis for an analysis of the problems in human teratology. Since most investigations of the experimental production of abnormal development have been undertaken on invertebrates and lower vertebrates, one of the problems has been to what extent the observations and conclusions derived from these studies may be applicable to human beings.

The etiology of abnormal development is either environmental or hereditary. Mall (1908), one of the most influential teratologists of his time, emphasized that cell anomalies were the result of external influences on normal ova. Now these influences may be chemical, physical or mechanical. Virtually every environmental factor has been instrumental in producing anomalies in some organism or other, provided that the agency affects it in the proper way and at the proper stage. Temperature variations, mechanical disturbances (vibration, pressure etc.), a host of chemicals, irradiations with rays
(x-rays) and ultraviolet rays, and other radiations, and modification of the gaseous environment, have all been shown to produce effects when properly applied (Zwilling, 1956).

Whatever the cause of the abnormal development may be, all these malformations are the visible expressions of disturbances of physiological processes at the biochemical or molecular level.

In the present study, various effects produced by urethane on the morphological organisation of the embryo have been found to be similar to the teratological forms reported by other investigators, who employed a variety of chemical agents during the early developmental stages of other vertebrates.

The effects of urethane on zebra fish were studied by Battle and Hisaoka (1952). The brain region showed lack of differentiation on continuous exposure while on shorter exposures parts of the brain appeared to be enlarged and malformed. Regional duplication of the central nervous system occurred in varying degrees. Retardation was particularly observed in the cephalic region of the embryos of Leopard frog, *Rana pipiens* by McMillan and Battle (1954). Klima and Wengraff (1943), observed that human patients treated with urethane exhibited psychic and neurological disorders caused by urethane, induced damage to gangliar cell apparatus of the brain and
spinal cord. Adhikari (1961) has shown that the organizational set up of urethane treated chick embryos shows a marked retardation of development.

In the present work, as seen from Table 3, it is obvious that the fore brain shows abnormalities in 76.6% cases, the mid and the hind portion of the brain was abnormal in 90.0% cases. The percentage of fore brain abnormality (76.6%) is less than that in case of posterior brain portion. These observations are in accord with the views held by Waddington (1932), Spratt (1942, 1947) and Rudnick (1938) that the forebrain differentiates autonomously. The same was shown to be true by Joshi (1964) and Telang (1974) in urethane and hydrazine treated chick embryos respectively. In most cases the brain lacks differentiation into prosencephalon, mesencephalon and rhombencephalon (Text Figs. 9 and 11, Plate VI). In some cases, fore brain shows an open nature (Text Fig. 11, Plate VI) while, in some the mid brain region is left open (Text Fig. 13, Plate VII). It seems obvious that the chemical has affected the movements leading to the closure of the brain.

In the present studies, in few cases where both the optic vesicles are formed, they are bent and pointed upwards showing the tendency towards cyclopia (Text Fig. 9, Plate VI). In some cases, only one of the two optic vesicles is formed (Text Fig. 13, Plate VII), while in some they are totally absent
(Text Fig. 11, Plate VI). Adhikari (1961) has recorded the absence of eye-cup formation in 48 hour chick embryo which were treated in ovo with urethane. Battle and Hisaoka (1952) treated the zebra fish eggs with urethane at the gastrula stage. The eyes sometimes failed to develop, were abnormally small or exhibited partial to complete median fusion. The types of abnormalities produced in urethane treated zebra fish eggs are similar to those recorded in teleosts following subjection to colchicine (Waterman, 1940) and phleomycin (Lee et al., 1972). The type of abnormalities recorded in this work have been shown by Joshi (1964) in chick embryos in ovo on urethane treatment for 21 hours.

Neural tube defects in the present investigation are very interesting. They are to the extent of 91.6%. In many cases they show a zigzag and open nature. Neural folds are wavy and wide apart in certain regions (Text Fig. 11, Plate VI). Adhikari (1961) could also obtain the bifurcation of the neural tube at the anterior region. Urethane treated zebra fish embryos showed shortening and deformation of spinal cord (Battle & Hisaoka, 1952). Waddington and Carter (1953) obtained abnormal closure, 'bends' and 'kinks' in the neural tube by treatment with trypan blue of mouse embryos.

In the present work, the somites showed various
abnormalities. In most cases, somites are diffused in nature while in some their counterparts are missing (Text Figs.9, 11 and 17; Plates VI and VIII). On the whole there is reduction in the number of somites. The percentage of abnormality of somites is 80.0%.

Battle and Hisaoka (1952), showed reduction in the number of myotomes in the zebra fish embryos which resulted in shortening of the body axis. Curious flexures of the tail were sometimes attributable to an unusual arrangement of the musculature. Adhikari (1961) has observed the failure on the part of mesodermal derivatives to establish, in case of 48 hour chick embryos treated with urethane.

**Possible mechanism of action of urethane**

An answer to the mechanism of action of urethane is most likely to be found in the multifarious expressions of urethane's biology and though there are certain tangible clues the problem is ever elusive and extremely fascinating.

Various explanations have been propounded for the mechanism of action of urethane. Narcotic action of urethane was attributed to simple adsorption (Warburg, 1921), which was later contradicted. Later both narcosis and inhibition of cell division were associated with a general inhibition of dehydrogenases (Haddow, 1963). It was then suggested that urethane brings
about its effect by formation of a not easily dissociable complex composed of dehydrogenase substrate and cytochrome B₁ and thus makes it inaccessible to the portion of the system reacting with oxygen (Keilin & Hartree, 1939; Keilin, 1929 and Keilin, 1930). Boyland and Rhoden (1949) examined the effect of urethane treatment on enzymes and concluded that urethane may inhibit such phosphokinases which are concerned in metabolism.

None of these observations either singly or in combination provided any conclusive answer. Therefore, an explanation on altogether different lines had to be sought, such as urethane as a competitor in specific biosynthetic processes. It was felt that urethane might become involved in the urea cycle or pyrimidine biosynthesis through competition with carbamyl phosphate, or carbamyl aspartate. It was later proposed by Todd (c.f. Haddow, 1963) that urethane might conceivably act by competing with some natural metabolic intermediate involved in the biosynthesis of nucleotides, possibly pyrimidine nucleotides. In the intervening years this idea became more attractive on the grounds of evidence (Timmis, 1961).

In the present investigation, in order to understand the mechanism of urethane induced teratogenic effects in chick embryos, various amino acids, nucleic acid precursors, vitamins were used for the subsequent treatment of urethane
treated embryos. Analysis of the effects on controlled administration of active agents, such as urethane and its reversal by certain substances as seen from the present studies, reveal certain potentialities that have not been previously demonstrated.

Urethane treated embryos when subsequently treated with glycine showed reversal to the extent of 63.3% (Table 4a, Text Figs.19 and 21, Plate IX), while those embryos which were subsequently treated with serine showed only 23.3% reversal (Table 4a, Text Figs.23 and 25, Plate X), thus indicating that urethane has paralyzed the capacity of converting serine into glycine in the treated embryos. Folic acid serves as a coenzyme in the conversion of serine to glycine by serine hydroxymethylase, in which the β-carbon of serine is converted into 'active formaldehyde' in 5,10-methylene FH₄ (Kisliuk & Sakami, 1954 and Blakley, 1954 A,B). Ellwyn and Sprinson (1950) have demonstrated the impairment of serine to glycine conversion in rats, deficient in folic acid. Braunstein and Vilenkina (1952) showed that liver preparations of folic acid deficient chicks and rats do not synthesize glycine from serine.

In the present study the subsequent treatment with methionine gave 60% reversal (Table 4a, Text Figs.27 and 29, Plate XI) while homocysteine was able to counteract the urethane effects only to the extent of 20% (Table 4a, Text Figs.31 and 33,
Plate XII), thus indicating that urethane has crippled the conversion of homocysteine to methionine. The participation of folic acid in this conversion has been suggested by Bennett (1949), Hatch et al. (1961), Sakami and Uksins (1961), Herbert and Zalusky (1962), Buchanan et al. (1964) and Guest et al. (1964).

Choline has been implicated as a cofactor in one carbon metabolism because of the significant role of its 'labile' methyl group in methylation reactions, hence the effects of the subsequent treatment using choline, methionine and homocysteine are being discussed together. In the present investigation, choline brings about reversal in 60% of the cases (Table 4c, Text Figs. 79 and 81, Plate XXIV). The comparison of the results obtained by the subsequent treatment using methionine (60% reversal), choline (60% reversal) and homocysteine (20% reversal) suggests that a 'labile' methyl group containing substance is capable of counteracting the urethane-induced abnormalities. Thus methionine and choline are effective, whereas, homocysteine without any 'labile' methyl group is not very effective.

Histidine when used subsequently on urethane treated embryos, was able to bring about the reversal only to the extent of 33.3% (Table 4a, Text Figs. 35 and 37, Plate XIII). Histidine has been shown to be catabolised to a one-carbon fragment like
formimino glutamic acid (Rabinowitz, 1960; Stockstad & Oace, 1965 and Sullivan, 1967). It has also been observed that in folate deficiency further conversion of formimino-glutamic acid (formed from histidine breakdown), into glutamic acid is impaired and that there is increased excretion of formimino glutamic acid after feeding histidine to the folic acid deficient animals (Silverman & Pitney, 1959 and Broquist & Luhby, 1959). In view of these observations, it is interesting to note that histidine could counteract urethane induced abnormalities, partially i.e. to the extent of 33.3%.

In the present work, urethane treated embryos when subsequently treated with thymidine, gave reversal in 66.6% of the treated embryos (Table 4b, Text Figs.43 and 45, Plate XV), while uridine could reverse the urethane effects only to the extent of 16.6% (Table 4b, Text Figs.47 and 49, Plate XVI). This indicates that urethane treated chick embryo is unable to convert uridine into thymidine. This conversion is also dependent upon folic acid and hence cannot possibly take place in the deficiency of folic acid, which in turn creates the need for an exogenous supply of thymidine. It was Dustin (1947) who showed that urethane chiefly interferes with the DNA metabolism by inhibiting thymine synthesis. Friedkin and Roberts (1956) have demonstrated the conversion of deoxyuridine into thymidine of DNA in chick embryos as well as in rabbit and chick bone marrow
preparations. The participation of folic acid in the above mentioned conversion is indicated by the fact that amethopterin, a folic acid antagonist, has an inhibitory effect on this conversion. Similarly, Humphreys and Greenberg (1958) have shown that soluble extract from rat thymus can bring about the conversion of deoxyuridylic acid to thymidylic acid which requires folic acid.

Interference of urethane with thymine metabolism has been reported by several workers. McKinney (1950) showed urethane to be a specific inhibitor for transmethylation reactions when present in low concentrations.

Boyland and Koller (1954) in their reversal studies found that the incidence of chromosome fragmentation and anaphase bridges induced by urethane in the Walker rat carcinoma was reduced by thymine but not by uracil. Deshpande (1970) in his chromosome studies reported that nucleotoxic and cytotoxic effects of urethane could be reversed by thymidine but not by uridine. Tolkacheva et al. (1973) in their studies suggested that the mutagenic action of urethane may be connected with a disturbance in the synthesis of thymine.

The reversal data with thymidine in the present investigation suggests that synthesis of thymidine from uridine is the sensitive metabolic reaction. Similar observations have
been made in frog embryos treated with folic acid analogue, amethopterin (Grant, 1960). The pattern of thymidine reversal suggests, that thymidine is probably an end product of a reduction sequence requiring a folic acid coenzyme. The relatively little reversal observed with uridine also proves that the site of interference is a pathway concerned with thymidine formation. In his speculation on the importance of synthesis of thymidyllic acid Freidkin (1959) suggests that thymidine biosynthesis and the role of tetrahydrofolinic acid therein, may serve to regulate cellular proliferation.

The major role of folic acid in various metabolic pathways such as biosynthesis of purine nucleotides, as well as thymine and in the biosynthesis of certain amino acids is very well known (Huennekens et al., 1958; Hartman & Buchanan, 1959; Buchanan, 1960; Rabinowitz, 1960 and Sullivan, 1967).

Pyrimidine biosynthesis is generally known to route through orotic acid. From the results obtained in the present investigation it can however be seen that orotic acid as well as dihydro-orotic acid, carbamyl aspartic acid could bring about the reversal only to the extent of 26.6%, 23.3% and 16.6% respectively (Table 4b; Text Figs. 51 and 53, Plate XVII; Text Figs. 5.5 and 57, Plate XVIII and Text Figs. 59 and 61, Plate XIX). These results are not in agreement with the in vivo
observations of Rogers (1957) in case of mice. Giri and Bhide (1968) in their studies on the effect of urethane in Swiss mice showed that urethane brings about a decrease in the activity of the enzyme, aspartic transcarbamylase which is alleged to play a regulatory role in the biosynthesis of nucleic acids (Bresnick, 1960). It appears from our observations that any direct interference of urethane with pyrimidine synthesis may be of a lesser significance in the system under study. The reversal obtained by thymidine but not by uridine, can be explained on the basis of interference of urethane with the methyl group transfer reaction which is folate dependent.

Inosinic acid plays a significant role as a precursor in the biosynthesis of nucleic acids. The fact that the subsequent treatment with inosinic acid gives 56.6% reversal (Table 4b, Text Figs. 63 and 65, Plate XX) speaks in support of its significant position. 4-5-amino imidazole carboxamide ribotides not being available, could not be tested. However, one may not expect any significant reversal.

In the present embryonic system, if urethane is exerting its effects by bringing about a deficiency of folic acid as suspected from our earlier observations, then folic acid itself as well as its precursor substance should be able to alleviate the urethane induced malformations in a significant percentage
of the embryos. This has been very clearly indicated by the data in Table 4c, where folic acid and its precursor p-aminobenzoic acid, have counteracted the urethane effects to the extent of 70% and 73.3% respectively, (Table 4c, Text Figs. 67 and 69, Plate XXI and Text Figs. 71 and 73, Plate XXII).

The ability of p-aminobenzoic acid to function as a precursor substance for folic acid has been suggested by Woods (1950) and Hendlin et al. (1953).

From amongst the antianemic substances Vitamin B_{12} (cyanocobalamin) and Vitamin C were tried. These substances were capable of bringing about the reversal to the extent of 60% and 56.6% respectively (Table 4c, Text Figs. 75 and 77; Plate XXIII and Text Figs. 83 and 85, Plate XXV). Vitamin B_{12} is also reported to play a role in certain methylation reactions mediated by folic acid in mammalian systems and this vitamin is known to be required for normal folic acid metabolism (Herbert & Zalusky, 1962 and Buchanan et al., 1964). Both these vitamins are required for normal growth of animals (Bennett, 1950). Vitamin C (ascorbic acid) could also reverse the urethane effects to the extent of 56.6% (Table 4c) which is in agreement with its role in potentiating the antianemic property of folic acid. The disturbances in functioning of neural system as a result of folic acid and particularly Vitamin B_{12} deficiency are
well established. These observations are also of interest in view of the narcotic action of urethane.

The results given in table 4a, 4b and 4c suggest a striking resemblance between biochemical lesions due to folic acid deficiency noted in microbial systems (Shive, 1950) and the chick embryonic system used in the present investigations. The chick embryo seems to have a derailed metabolism of one-carbon units and, therefore, an exogenous supply of products arising from methylation is needed. The results suggest that urethane is possibly interfering in normal methylation process during embryogenesis by inducing a deficiency of folic acid, possibly by interference with its biogenesis in a significant percentage of the experimental population.

Folic acid has been attributed a central role in the metabolism of one carbon units at three levels of oxidation, methyl, formyl and formate respectively (Friedkin, 1963). Thus folic acid has been implicated in the reactions involving one carbon moieties such as formyl, hydroxymethyl foraminino and methyl groups (Handschumacher & Welch, 1960). These folic acid mediated reactions are manifested as serine to glycine conversion, homocysteine to methionine conversion, breakdown of histidine, conversion of 4,5-aminolimidazole carboxamide ribotide to inosinic acid during the biosynthesis of purines,
and the methylation of uridine to thymidine during the biosynthesis of thymidine (Huennekens et al., 1958; Huennekens & Osborn, 1959; Buchanan, 1960; Rabinowitz, 1960; Jaenicke, 1964; Stockstad & Oace, 1965; Stockstad & Koch, 1967 and Sullivan, 1967). Methylation of cytosine to 5-methyl cytosine and subsequent conversion to thymidine providing an alternate pathway for thymidine formation, conversion of cytosine to 5 hydroxymethyl cytosine in certain viruses are also other reactions in which folic acid participates. Possible role of folic acid in the formation of leucine and threonine has been postulated.

The role of folic acid in the biosynthesis and maturation or functional activation of protein, RNA, DNA is discussed later (see page 99).

Urethane, as seen from the present study, most probably causes a folic acid deficiency and interferes in the reactions which involve one carbon unit metabolites which are critically required for cellular maturation and hence possibly for normal morphogenesis. Supplementation of urethane treated embryos with one carbon metabolites, therefore, brings about the reversal of urethane-induced malformations.
### LIST OF ILLUSTRATIONS

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<th>Plate No.</th>
<th>Text Figs.</th>
<th>Particulars</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Schematic representation of New's In Vitro Culturing Technique.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Chick embryo at mid primitive streak stage.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Chick embryo at definitive primitive streak stage.</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>Histogram showing percentage of abnormality in urethane treated chick embryos after 4 hour treatment at three different stages (3*, 4, and 5) and with three different concentrations (0.112 M, 0.168 M and 0.225 M).</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>Histogram showing percentage of mortality in urethane treated chick embryos after 4 hour treatment at three different stages (3*, 4 and 5) and with three different concentrations (0.112 M, 0.168 M and 0.225 M).</td>
</tr>
<tr>
<td>Plate No.</td>
<td>Text Figs.</td>
<td>Particulars</td>
</tr>
<tr>
<td>-----------</td>
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</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>Histogram showing percentage of abnormality in urethane treated chick embryos after 6 hour treatment at three different stages ($3^+, 4$ and $5$) and with three different concentrations ($0.112$ M, $0.168$ M and $0.225$ M).</td>
</tr>
<tr>
<td>V</td>
<td>7</td>
<td>Histogram showing percentage of mortality in urethane treated chick embryos after 6 hour treatment at three different stages ($3^+, 4$ and $5$) and with three different concentrations ($0.112$ M, $0.168$ M and $0.225$ M). Treatement with urethane</td>
</tr>
<tr>
<td>VI</td>
<td>8 and 10</td>
<td>Whole mount photographs of the embryos from master control series.</td>
</tr>
<tr>
<td></td>
<td>9 and 11</td>
<td>Whole mount photographs of the embryos from urethane control series.</td>
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<tr>
<td>VII</td>
<td>12 and 14</td>
<td>Whole mount photographs of the embryos from master control series.</td>
</tr>
<tr>
<td></td>
<td>13 and 15</td>
<td>Whole mount photographs of the embryos from urethane control series.</td>
</tr>
<tr>
<td>Plate No.</td>
<td>Text Figs.</td>
<td>Particulars</td>
</tr>
<tr>
<td>-----------</td>
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</tr>
<tr>
<td>VIII</td>
<td>16</td>
<td>Whole mount photographs of an embryo from the master control series.</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>Whole mount photograph of an embryo from the urethane control series.</td>
</tr>
<tr>
<td></td>
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<td><strong>SUBSEQUENT TREATMENT USING AMINO ACIDS</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Subsequent treatment with glycine</strong></td>
</tr>
<tr>
<td>IX</td>
<td>18 and 20</td>
<td>Urethane controls</td>
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<td>19 and 21</td>
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**Subsequent treatment with dihydroorotic acid**

| **XIX**   | 58 and 60  | Urethane controls |
|           | 59 and 61  | Experimentals |

**Subsequent treatment with carbamyl aspartic acid**

| **XX**    | 62 and 64  | Urethane controls |
|           | 63 and 65  | Experimentals |

**SUBSEQUENT TREATMENT USING VITAMINS AND COFACTORS**

| **XXI**   | 66 and 68  | Urethane controls |
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**Subsequent treatment with folic acid**

|         | 70 and 72  | Urethane controls |
|         | 71 and 73  | Experimentals |

**Subsequent treatment with p-aminobenzoic acid**
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PLATE I

Text Fig. 1  A schematic representation of \textit{in vitro} culturing of the chick embryos by the method of New.

Text Fig. 2  Chick embryo at mid-primitive streak stage (stage 3\textsuperscript{+}) which is used for the experiments.

Text Fig. 3  Chick embryo at definitive primitive streak stage (stage 4) which is also used for the experiments.
In vitro CULTIVATION OF CHICK EMBRYO
By NEW’S TECHNIQUE
A histogram representing the percentage of abnormality in urethane treated chick embryos after four hour treatment, in three different stages 3+, 4 and 5, and with three different concentrations 0.112 M, 0.168 M and 0.225 M. Note chick embryos at stages 3+ and 4 with 0.168 M of urethane, show maximum incidence of abnormality and stage 5 is refractory.
PERCENTAGE OF ABNORMALITY
Duration of Treatment: 4 Hours
Concentrations of URETHANE
A = 0.112 M  B = 0.168 M  C = 0.225 M

Stages:
3* → □□□□
4 → □□□□
5 → □□□□

URETHANE CONCENTRATION →
PERCENT →
100
90
80
70
60
50
40
30
20
10
A  B  C
PLATE III

Text Fig. 5

A histogram representing the percentage of mortality in urethane treated chick embryos after four hour treatment, in three different stages, 3', 4 and 5, and with three different concentrations 0.112 M, 0.168 M and 0.225 M.

Note chick embryos at stages 3' and 4 with 0.225 M of urethane show maximum incidence of mortality and stage 5 is refractory.
PERCENTAGE OF MORTALITY
Duration of Treatment: 4 Hours
Concentrations of URETHANE
A = 0.112 M  B = 0.168 M  C = 0.225 M
Stages:
3
4
5

PERCENT
100
90
80
70
60
50
40
30
20
10
0

URETHANE CONCENTRATION
A histogram showing the percentage of abnormality in urethane treated chick embryos after six hour treatment, in three different stages 3\textsuperscript{+}, 4 and 5, and with three different concentrations 0.112 M, 0.168 M and 0.225 M. Note percentage of abnormalities is insignificant at all 3 stages and with all three concentrations. Chick embryos at stage 3\textsuperscript{+} and 4 with 0.168 M concentration show highest percentage of abnormality.
PERCENTAGE OF ABNORMALITY
Duration of Treatment = 6 Hours
Concentrations of URETHANE
A = 0.112 M  B = 0.168 M  C = 0.225 M

Stages: 3 - 4 - 5

PERCENT

URETHANE CONCENTRATION
A histogram showing the percentage of mortality in urethane treated chick embryos after six hour treatment, in three different stages 3*, 4 and 5 and with three different concentrations 0.112 M, 0.168 M and 0.225 M. Note chick embryos at stages 3* and 4 with 0.168 M and 0.225 M urethane show maximum incidence of mortality. Stage 5 is refractory.
PERCENTAGE OF MORTALITY
Duration of Treatment: 6 Hours
Concentrations of URETHANE
A = 0.112 M
B = 0.168 M
C = 0.225 M
Stages: 3* 4  5

URETHANE CONCENTRATION

PERCENT
Plate VI

Text Fig. 3  Embryo grown in Pannett Compton saline for 18-21 hours without urethane treatment, showing normal development of brain (B), optic vesicles (OV), neural tube (NT), heart (H) and somites (S). No shortening of the embryonic axis (Master Control) x 24.

Text Fig. 9  Urethane treated embryo grown in Pannett Compton saline for 18-21 hours. Note the malformed brain (B), unfused neural folds (NF). The heart (H) has shifted anteriorwards. The embryonic axis is shortened. (Urethane Control) x 24.

Text Fig. 10  Embryo grown in Pannett Compton saline for 18 - 21 hours without urethane treatment. All the organ systems such as brain (B), optic vesicles (OV), neural tube (NT), somites (S) and heart (H), show normal development (Master Control) x 24.

Text Fig. 11  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours, showing retarded development of brain (B), unfused neural folds (NF) and heart (H) is retarded and somites (S) are diffused in nature and fewer in number. Embryonic axis is shortened. (Urethane Control) x 24.
Text Fig. 12 Embryo grown in Pannett Compton saline for 18 - 21 hours without urethane treatment. Note the normal development of all the organ systems such as brain (B), optic vesicles (W), neural tube (NT), somites (S) and heart (H). (Master Control) x 24.

Text Fig. 13 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours, showing retarded development of brain (B), retarded heart (H), diffused somites (S) some of which only unilaterally developed (Urethane Control) x 24.

Text Fig. 14 Embryo grown in Pannett Compton saline for 18 - 21 hours without urethane treatment. Note the normal development of brain (B), optic vesicles (W), neural tube (NT), somites (S) and heart (H). (Master Control) x 24.

Text Fig. 15 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the extremely malformed brain (B) and widely separated neural folds (NF) at the level of somites. Somites (S) show reduction in number and heart (H) is present anterior to the diminutive brain (Urethane Control) x 24.
Text Fig. 16  Embryo grown in Pannett Compton saline for 18 - 21 hours without urethane treatment. Note the normal development of brain (B), optic vesicles (W), neural tube (NT), heart (H) and somites (S). Embryonic axis is not shortened (Master Control) x 24.

Text Fig. 17  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. The somites (S) are diffused and heart (H) is showing retarded development and is present as a straight tube without its characteristics curvature. (Urethane Control) x 24.
Text Fig. 18
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours, showing retarded brain (B), widely separated neural folds (NF) in the region of somites. Heart is totally absent (Urethane Control) x 24.

Text Fig. 19
Urethane treated embryo subsequently treated with glycine for 18 - 21 hours. The normal development of brain (B), neural tube (NT), heart (H) and somites (S) indicates that urethane-induced abnormalities are reversed. (Experimental) x 24.

Text Fig. 20
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Brain (B), lack its normal differentiation, neural folds (NF) remain widely separated and heart (H) shows retardation.

Text Fig. 21
Urethane treated embryo subsequently treated with glycine for 18 - 21 hours. The normal development of all the organ systems, such as brain (B), neural tube (NT), heart (H) and somites (S), clearly indicates that there is a reversal of urethane induced abnormalities (Experimental) x 24.
Text Fig. 22 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the malformed brain (B), neural folds (NF) are wide apart in the region of somites and heart (H) is anteriorly placed. (Urethane Control) x 24.

Text Fig. 23 Urethane treated embryo subsequently treated with serine for 18 - 21 hours. Note the retardation of brain (B) and optic vesicles (V). Neural folds (NF) are unfused and the heart (H) is retarded. (Experimental) x 24.

Text Fig. 24 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours, showing retarded brain (B), retarded heart (H), unfused neural folds (NF) and diffused somites (S). (Urethane Control) x 24.

Text Fig. 25 Urethane treated embryo subsequently treated with serine for 18 - 21 hours. Note the malformed brain (B), only single optic vesicle (V) and retarded heart (H). (Experimental) x 24.
Text Fig. 26  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours, showing abnormal differentiation of the brain (B). The neural folds (NF) are unfused at the level of somites. Somites (S) show diffused nature and heart (H) lies on top of the malformed brain. Embryonic axis is slightly bent. (Urethane Control) x 24

Text Fig. 27  Urethane treated embryo subsequently treated with methionine for 18 - 21 hours. The normal development of brain (B), optic vesicles (OV), neural tube (NT), heart (H) and somites (S) indicates the reversal of urethane induced abnormalities. (Experimental) x 24.

Text Fig. 28  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the malformed brain (B), unfused neural folds (NF) in the somite region and anterior shifting of the heart (H). Embryonic axis shows a slight bend. (Urethane Control) x 24.

Text Fig. 29  Urethane treated embryo subsequently treated with methionine for 18 - 21 hours. Normal development of brain (B), optic vesicles (OV), neural tube (NT), heart (H) and somites indicates that urethane induced abnormalities are reversed. (Experimental) x 24.
Text Fig. 30
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the malformed brain (B), widely separated neural folds (NF) and malformed somites (S). Heart (H) is present anterior to the brain. (Urethane Control) x 24.

Text Fig. 31
Urethane treated embryo subsequently treated with homocysteine for 18 - 21 hours. Note the retardation of development of brain (B). Somites (S) are diffused and heart (H) shows retardation. All this indicates that there is no reversal of urethane induced malformations. (Experimental) x 24.

Text Fig. 32
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours, showing retarded brain (B) and absence of heart (H). (Urethane Control) x 24.

Text Fig. 33
Urethane treated embryo subsequently treated with homocysteine for 18 - 21 hours. Note the malformed brain (B), unfused neural folds (NF), retarded heart (H) and diffused somites (S). This indicates that there is no reversal of urethane-induced abnormalities. (Experimental) x 24.
PLATE XIII

Text Fig. 34  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded development of the brain (B), unfused neural folds (NF), diffused somites (S). (Urethane Control) x 24.

Text Fig. 35  Urethane treated embryo subsequently treated with histidine for 18 - 21 hours. Note the malformed brain (B), retarded heart (H) and diffused somites (S). These results indicate that there is no reversal of urethane induced malformations. (Experimental) x 24.

Text Fig. 36  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded development of brain (B), optic vesicles (OV) and heart (H). (Urethane Control) x 24.

Text Fig. 37  Urethane treated embryo subsequently treated with histidine for 18 - 21 hours, showing malformed brain (B), displacement of heart (H) from its normal position and abnormal differentiation of somites (S). The embryonic axis is slightly curved. These results indicate no reversal of urethane-induced abnormalities. (Experimental) x 24.
PLATE XIV

Text Fig. 38
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded development of brain (B) and absence of heart (H). Neural folds (NF) in the brain region as also at the level of somites. Somites (S) are few in number and diffused. Embryonic axis shortened. (Urethane Control) x 24.

Text Fig. 39
Urethane treated embryo subsequently treated with asparagine for 18 - 21 hours showing retardation of development of the brain (B), unfused neural folds (NF) retarded heart (H) and abnormal differentiation of somites (S). These results indicate that there is no reversal of urethane-induced malformations. (Experimental) x 24.

Text Fig. 40
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing malformed brain (B), unfused neural folds (NF), unilateral differentiation of somites (S) and displacement of heart (H). The malformed brain is bent on itself. (Urethane Control) x 24.

Text Fig. 41
Urethane treated embryo subsequently treated with asparagine for 18-21 hours. It shows retarded brain (B), unfused neural folds (NF), abnormally differentiated somites (S), extreme displacement of heart (H), which lies on top of the brain. This indicates that there is no reversal of urethane induced abnormalities. (Experimental) x 24.
Text Fig. 42
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded development of the brain (B) and optic vesicles (OV), unfused neural folds (NF) in the brain region and somite region, retarded heart (H). (Urethane Control) x 24.

Text Fig. 43
Urethane treated embryo subsequently treated with thymidine for 18 - 21 hours. Normal development of all the organ systems such as brain (B), optic vesicles (OV), somites (S), neural tube (NT) and heart (H) indicates that there is reversal of the abnormal effects induced by urethane. (Experimental) x 24.

Text Fig. 44
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing malformed brain (B), diffused somites (S), retarded heart (H). (Urethane Control) x 24.

Text Fig. 45
Urethane treated embryo subsequently treated with thymidine, for 18 - 21 hours showing normal development of all the organ systems such as brain (B), optic vesicles (OV), neural tube (NT), heart (H) and somites (S). This indicates that urethane induced malformations are reversed. (Experimental) x 24.
Text Fig. 46  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded development of the brain (B), and abnormally differentiated somites (S). (Urethane Control) x 24.

Text Fig. 47  Urethane treated embryo subsequently treated with uridine for 18 - 21 hours. The malformed brain (B), displaced heart (H), reduced number of somites (S) as well as the curved embryonic axis indicate that there is no reversal. (Experimental) x 24.

Text Fig. 48  Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded brain (B), unfused neural folds (NF) in the region of brain as well as somites (S), total absence of heart (H) and shortened embryonic axis. (Urethane Control) x 24.

Text Fig. 49  Urethane treated embryo subsequently treated with uridine for 18 - 21 hours. Note the retarded brain (B), malformed optical vesicles (OV), reduced number of somites (S). All this indicates that there is no reversal of urethane induced malformations. (Experimental) x 24.
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing retarded development of brain (B), diffused somites (S). (Urethane Control) x 24.

Urethane treated embryo subsequently treated with orotic acid for 18 - 21 hours. Note the mal-formed brain (B), anteriorly placed heart (H), unfused neural folds (NF). All these results indicate that there is no reversal of urethane induced malformations. (Experimental) x 24.

Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the malformed brain (B), unfused neural folds in the region of brain and somites (S) and anterior shifting of the heart (H). (Urethane Control) x 24.

Urethane treated embryo subsequently treated with orotic acid for 18 - 21 hours. The retarded development of brain (B), optic vesicles (OV), heart (H), malformed somites (S), indicate that there is no reversal of urethane induced abnormalities. (Experimental) x 24.
PLATE XVIII

Text Fig. 54
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the malformed brain (B), widely separated neural folds (NF) in the region of brain and somites, few diffused somites and anteriorly placed heart (H). (Urethane Control) x 24.

Text Fig. 55
Urethane treated embryo subsequently treated with dihydro orotic acid for 18 - 21 hours. Note the retarded development of brain (B) and optic vesicles (W), widely separated neural folds (NF), few diffused somites and absence of heart. These results indicate that there is no reversal of malformations induced by urethane. (Experimental) x 24.

Text Fig. 56
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the malformed brain (B), diffused somites (S) and anterior placement of the heart (H). (Urethane Control) x 24.

Text Fig. 57
Urethane treated embryo subsequently treated with dihydro-orotic acid for 18 - 21 hours. The retarded development of brain (B), unilateral development of optic vesicle (W), diffused somites (S) and retarded heart (H), clearly indicate indicate that urethane induced abnormalities have not been reversed. (Experimental) x 24.
Text Fig. 58 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded development of brain (B), widely separated neural folds (NF), diffused somites (S), and absence of heart (H). (Urethane Control) x 24.

Text Fig. 59 Urethane treated embryo subsequently treated with carbomyl aspartic acid for 18 - 21 hours showing extremely malformed brain (B), retarded heart (H). This indicates that there is no reversal of urethane induced malformations (Experimental) x 24.

Text Fig. 60 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours, showing retarded development of the brain (B), unfused neural folds (NF) in the region of brain as well as somites, diffused somites (S), anterior shifting of the heart and shortened embryonic axis. (Urethane Control) x 24.

Text Fig. 61 Urethane treated embryo subsequently treated with carbamyl-aspartic acid for 18 - 21 hours showing retarded brain (B) and retarded heart (H) and diffused somites (S). These results indicate that the urethane induced malformations are not reversed (Experimental) x 24.
Text Fig. 62
Urethane treated embryo grown in Panett Compton saline for 18 - 21 hours showing malformed brain (B) unfused neural folds (NF), anteriorly placed heart (H) and shortened embryonic axis (Urethane Control) x 24.

Text Fig. 63
Urethane treated embryo subsequently treated with inosinic acid for 18 - 21 hours. The normal development of all the organ systems, such as brain (B), neural tube (NT), heart (H) and somites (S) clearly indicates that there is a reversal of urethane induced abnormalities (Experimental) x 24.

Text Fig. 64
Urethane treated embryo grown in Panett Compton saline for 18 - 21 hours. Note the retarded brain (B), diffused somites (S), retarded heart (H) and shortened embryonic axis (Urethane Control) x 24.

Text Fig. 65
Urethane treated embryo subsequently treated with inosinic acid shows normal development of all the organ systems such as brain (B), optic vesicles (Ov), neural tube (NT), heart (H) and somites (S). This indicates that there is reversal of urethane-induced abnormalities (Experimental) x 24.
Text Fig. 66 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded development of brain (B), unfused neural folds (NF) in the brain and somites region, extremely abnormal somites (S). (Urethane Control) x 24.

Text Fig. 67 Urethane treated embryo subsequently treated with folic acid for 18 - 21 hours. The normal development of all the organ systems such as, brain (B), optic vesicles (O), neural tube (NT), somites (S) and heart (H) indicates that urethane-induced malformations are reversed (Experimental) x 24.

Text Fig. 68 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing abnormally differentiated brain (B), unfused neural folds (NF) and anteriorly placed heart (H). (Urethane Control) x 24.

Text Fig. 69 Urethane treated embryo subsequently treated with folic acid for 18 - 21 hours, showing normal development of all the organ systems such as brain (B), optic vesicles (O), neural tube (NT), somites (S) and heart (H). These results indicate that there is reversal of urethane-induced malformations (Experimental) x 24.
**PLATE XXII**

**Text Fig. 70**
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing extremely retarded development of brain (B), unfused neural folds (NF) and absence of heart (Urethane Control) x 24.

**Text Fig. 71**
Urethane treated embryo subsequently treated with p-aminobenzoic acid for 18 - 21 hours. It shows normal development of all the organ systems such as brain (B), neural tube (NT), optic vesicles (OV), heart (H) and somites (S). This indicates that urethane induced abnormalities are reversed. (Experimental) x 24.

**Text Fig. 72**
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the malformed brain (B), unfused neural folds (NF), malformed somites (S), absence of heart and shortened embryonic axis. (Urethane Control) x 24.

**Text Fig. 73**
Urethane treated embryo subsequently treated with p-aminobenzoic acid for 18 - 21 hours showing normal development of all the organ systems such as brain (B), neural tube (NT), optic vesicles (OV), somites (S) and heart (H). This indicates the reversal of abnormalities induced by urethane (Experimental) x 24.
Text Fig. 74 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing retarded brain (B), unfused neural folds (NF), total lack of somites (S) and absence of heart (Urethane Control) x 24.

Text Fig. 75 Urethane treated embryo subsequently treated with vitamin B₁₂ for 18 - 21 hours. Normal development of brain (B), optic vesicles (W), neural tube (NT), somites (S) and heart (H) indicates reversal of urethane induced abnormalities (Experimental) x 24.

Text Fig. 76 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing abnormal brain (B), retarded heart (H), reduction in the somites (S) number (Urethane Control) x 24.

Text Fig. 77 Urethane treated embryo subsequently treated with vitamin B₁₂ for 18 - 21 hours. Note the normal development of brain (B), neural tube (NT), optic vesicles (W), somites (S) and heart (H). It indicates reversal of urethane induced abnormalities (Experimental) x 24.
Text Fig. 78 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing malformed brain (B), unfused neural folds (NF) and anterior placement of the heart (H). (Urethane Control) x 24.

Text Fig. 79 Urethane treated embryo subsequently treated with choline for 18 - 21 hours showing reversal of urethane induced abnormalities. Note the normal brain (B), optic vesicles (OV), neural tube (NT), somites (S) and heart (H). (Experimental) x 24.

Text Fig. 80 Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours. Note the retarded brain (B), unfused neural folds (NF), malformed somites (S) and absence of heart (H). (Urethane Control) x 24.

Text Fig. 81 Urethane treated embryo subsequently treated with choline for 18 - 21 hours showing normal brain (B), optic vesicles (OV), neural tube (NT), somites (S) and heart (H). These results indicate that urethane induced abnormalities are reversed (Experimental) x 24.
Text Fig. 82
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing retarded and malformed brain (B) and malformed somites (S). (Urethane Control) x 24.

Text Fig. 83
Urethane treated embryo subsequently treated with vitamin C for 18 - 21 hours showing normal development of all the organ systems such as brain (B), optic vesicles (V), neural tube (NT), heart (H) and somites (S). This indicates that urethane induced abnormalities have been reversed (Experimental) x 24.

Text Fig. 84
Urethane treated embryo grown in Pannett Compton saline for 18 - 21 hours showing retarded brain (B), retarded heart (H), (Urethane Control) x 24.

Text Fig. 85
Urethane treated embryo subsequently treated with vitamin C for 18 - 21 hours showing normal development of brain (B), optic vesicles (V), neural tube (NT), heart (H) and somites (S) indicating reversal of urethane induced abnormalities (Experimental) x 24.
1. The effect of urethane has been studied on the chick embryos cultivated in vitro by the method devised by New.

2. Of all the concentrations tried, a concentration of 0.168 M urethane when used on mid-primitive streak stage (stage 3*) and definitive primitive streak stage for 4 hours duration gave best results in terms of high incidence of abnormality and low rate of mortality. These studies showed that effect of urethane depends on the stage of development of the embryo at the time of treatment. The embryos at early primitive streak stage (stage 3), when treated with the above mentioned concentration, showed a high degree of mortality, while those treated at the head process stage (stage 5) showed resistance to a remarkable degree.

3. Urethane treated embryos when grown in Pannett Compton saline for 18-20 hours at 37.5 ± 1°C showed abnormalities which were predominantly restricted to brain, neural tube, optic vesicles and somites. The differentiation of heart was also affected but to a lesser extent.

4. Urethane treated embryos were subsequently treated with various amino acids, nucleic acid precursors, vitamins and an attempt has been made to understand its possible mechanism of action.
5. Subsequent treatment with amino acids showed a certain degree of specificity. The amino acid conversions which depend on transmethylation reactions were selectively affected. Thus the urethane-induced abnormalities were effectively reversed by glycine, but not by serine, by methionine but not by homocysteine and by histidine. These results thus suggest that the amino acid conversions which are mediated by folic acid are impaired in the urethane-treated embryos.

6. From amongst the various nucleic acid precursors which were used for the subsequent treatment of urethane treated embryos, thymidine could successfully reverse the inhibitory effects of urethane while uridine could not. Other precursors such as orotic acid, dihydro-orotic acid, carbamyl aspartic acid were not effective in bringing about the reversal. Thus the results obtained by thymidine and uridine can be explained on the basis of a block in normal methylation reactions brought about by urethane.

7. Subsequent treatment with inosinic acid was seen to be effective in bringing about the reversal.

8. Folic acid itself as well as other related substances such as p-aminobenzoic acid, choline, vitamin B₁₂ and vitamin C were also effective in reversing the effects of urethane.
9. From all these observations, the mode of action of urethane is discussed at length in the light of the possible inhibition of normal one carbon and methyl group metabolism due to deficiency of folic acid, induced by urethane.
PART I (B)

EFFECT OF URETHANE ON DNA, RNA AND PROTEIN CONTENT OF CHICK EMBRYOS CULTURED IN VITRO
INTRODUCTION
The role of urethane as a dynamic chemical, which produces diverse biological effects, has already been discussed. It is a versatile carcinogen. Interest in the carcinogenic potential of urethane was aroused by the early work of Nettleship and Henshaw (1943), on the induction of pulmonary adenomas in mice on urethane treatment.

Urethane is a broad spectrum carcinogen having potentialities to induce lung adenomas in mice (Rogers, 1957, Colnaghi et al., 1969; Adenis et al., 1970; Tuhas, 1973; Kolesnichenko, 1974; Nomura, 1974; Menard et al., 1976; Adamson & Klass, 1976; Wykle et al., 1977 and Brightwell & Heppleston, 1977). Pickroth and Kuhne (1962) showed induction of lung tumours in mice exposed to urethane aerosols. For many years the carcinogenic action of urethane was believed to be restricted to the lung.

In 1953 it was shown that urethane is not unique as a lung carcinogen. It initiated skin tumours in mice (Graff et al., 1953 and Salaman & Roe, 1953). The multipotential character of urethane as a carcinogen was exquisitely revealed by Tannenbaum and Maltoni, 1962 and Tannenbaum et al., 1962. Toth et al. (1961) reported multipotential urethane induced carcinogenesis in the Syrian gold hamster. Same was noted by Mohr et al. (1974) in the European hamster. Toth
reported tumours of lungs and ovaries by subcutaneous injection of urethane in the guinea pigs. In the rats urethane administration was associated with vascular tumours and malignant lymphomas (Newberne et al., 1967). Garcia and Guerrero (1969) showed high incidence of tumours in mouse on urethane treatment. Tumour induction by urethane was reported in rats by Dushkin and Podoprigora (1972). Urethane induced tumours in Nicotiana seedlings during early development (Andersen, 1973).

Urethane was found to be carcinogenic in fish (Ball & Cowen, 1959) and embryonic tissues of rat (Napalkov & Alexandrov, 1968 and Kominnei & Choudari, 1970). Carcinomas of the stomach, pancreas were induced by urethane in the guinea pigs (Druckery et al., 1968). Dimethylcarbamyl chloride, an industrial chemical related to urethane, was found to be carcinogenic in mice (Van Duren et al., 1972). Leukaemia was induced in Swiss mice on urethane treatment (Donati et al., 1961). Urethane was shown to have a leukemia promoting action in cattle as well as in laboratory animals (Urbanec & Wittmann, 1970). It was found to be leukemogenic in mice (Kelly et al., 1974).

Studies on the antitumour activity of urethane have been pursued on finding inhibition of several experimental
forms of cancer (Yu, 1947; Murphy & Sturm, 1947; Guthert, 1949 and Landschutz & Müller-Dethard, 1949). Most of these studies were concerned with leukemias, which started with the first clinical report (Patterson et al., 1946). Being a carcinoclast, it has been used as a palliative in human malignant disease (Faber et al., 1956 and Hanh et al., 1966).

Because of the general biological importance of nucleic acid to the maintenance of heritable characteristics and the nuclear poisoning effects of urethane, much attention has been directed towards its possible influence upon nucleic acid synthesis. The available evidence very clearly indicates that urethane is intricately connected with nucleic acid metabolism. This feature presumably has a bearing in the production of cancerous tissue (Boyland & Rhoden, 1949; Skipper et al., 1951, Boyland, 1952, Rogers, 1957, Brachet, 1957 and Bhide & Ranadive, 1966). Boyland and Koller (1954) reported that the damaging action of urethane on the chromosomes in the Walker rat carcinoma could be alleviated to a considerable extent with simultaneous administration of thymine.

Extensive studies have been carried out on the effects of nucleotides and related compounds on urethane carcinogenesis. Thus Cowen(1949) found inhibition of lung tumorigenesis on repeated injections of ribonucleotides at the time of urethane
administration. It was Roe (1955) who showed that skin tumor initiation by urethane was inhibited by the administration of various purine precursors. Spontaneous lung tumorigenesis was, however, significantly repressed by feeding thymine through drinking water (Fink & Fink, 1955 and Kaye & Trainin, 1966). Interference of urethane with pyrimidine metabolism was suspected by Bresnick (1960). Joshi (1967) has observed that in frog embryos the cytological anomalies, resulting from the exposure to urethane, could be reversed by thymidine.

According to Shimkin et al (1969), urethane produces a decrease in thymidine, which suggests an inhibition of DNA synthesis. While studying skin tumorigenesis in mouse, Hennings and Boutwell (1969) showed clear inhibition of DNA synthesis by urethane. Deshpande (1970) showed that the urethane induced nucleotoxic and cytotoxic effects in the primitive erythrocytes of chick could be prevented by thymidine and folic acid. Tolkacheva et al (1973) studied the mechanism of mutagenic action of urethane in the cells of a primary culture of embryonic mouse and lung and transplantable lines of Chinese hamster fibroblasts. From these studies, they suggested that urethane brings about a disturbance in the synthesis of DNA, pyrimidines, more specifically with the synthesis of thymine.
In the regenerating rat liver urethane brought about inhibition of DNA synthesis (Rocchi et al., 1974). Lombardi (1974) has stated that urethane is a chemical which inhibits DNA and RNA synthesis. Diwan and Mulherkar (1974) reported that urethane has an antifolic acid action in the chick embryos and suggested that urethane caused malformations are due to its interference with thymine synthesis in particular and DNA synthesis in general. Prodi et al. (1975) have shown that urethane exerts a strong inhibitory effect on DNA synthesis in the lymphoid organs and bone marrow of the rat.

Nucleic acids and proteins play an important role in animal growth and differentiation (Brachet, 1957) and therefore, an interference involving the nucleic acid and protein synthesis, would give rise to characteristic disturbances in the developmental process. Effects of urethane on chick embryos have been studied (Adhikari, 1961 and Diwan & Mulherkar, 1974). But nothing is known about the direct interference of urethane with DNA, RNA and protein metabolism, alleged to be the key processes for the normal development of an organism. Antifolate action of urethane as suggested by Diwan and Mulherkar (1974) and our observations in the earlier part tempt us to probe further in this regard. In this part, effect of urethane on DNA, RNA and protein formation has been studied so as to reveal the possible mode of action of this multipotential carbamate.
MATERIALS AND METHODS
In this part, the total Deoxyribo nucleic acid (DNA), Ribonucleic acid (RNA) and protein content of treated (experimental) and untreated embryos (control) cultivated in vitro was studied. The objective of the study reported here was to determine the effects of urethane in relation to nucleic acid and protein synthesis.

Chick embryos at mid-primitive streak stage (stage 3*) or definitive primitive streak stage (stage 4, Hamburger & Hamilton, 1951) were processed for in vitro culturing according to the method devised by New (1955), as described earlier (see page 14). The embryos were divided into two sets, the control and the experimental. The embryos in the control set did not receive any treatment and were grown throughout in Pannett Compton saline. The experimental set of embryos was treated with 0.168 M solution of urethane for 4 hours by placing 0.1 ml of the above solution on the blastoderm. The treatment was terminated by plunging the embryos in a series of petridishes containing Pannett Compton saline and by gently pipetting saline around the vitelline membrane as well as over the blastoderm. These embryos were then grown in Pannett Compton saline. Both controls as well as the experimentals were kept at 37.5 ± 1°C for about 18-21 hours after which they
were separately transferred to petridishes containing 0.9% physiological saline. The blastoderms were thoroughly washed so as to remove any yolk still adhering to them. If found necessary, the embryos were given as many as three washes to ensure complete removal of the yolk sticking to the blastoderm. All these washed embryos from the control and the experimental sets were then transferred to two different weighing tubes. The weighing tubes along with the embryos were weighed and the total weight of the embryos from the control and the experimental series as well as number of embryos in each set was carefully noted. In each set, five embryos were used. In all, eight sets each from control and experimental series were used.

These embryos from the control and the experimental sets were then separately transferred to a glass homogenizer. To this a minimum quantity of measured saline was added and embryo was extracted. The embryo extract from control and experimental set was then transferred to two different tubes. The tubes were labelled, plugged with cotton and aluminium foil and stored in the refrigerator till further use.

Nucleic acids, namely, Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA), were separated by Schmidt-Thannhauser procedure (1945) modified by Schneider (1946). Protein was extracted from the residue remaining after nucleic acid removal by overnight incubation at 30°C with 0.5 N NaOH (Chaube &
Extraction of Nucleic Acids

The Schmidt-Thannhauser-Schneider procedure as outlined below, was used to extract DNA, RNA and protein.

(a) Removal of acid soluble compounds :-

1 ml of 20\% tissue homogenate (embryo extract) was mixed with 2.5 ml of cold 10\% Trichloro acetic acid (TCA), and centrifuged. The precipitate was resuspended in 2.5 ml of cold 10\% Trichloroacetic acid (TCA) and centrifuged. These combined extracts constitute the acid-soluble phosphorous fraction.

(b) Removal of phospholipids :-

The tissue residue was suspended in 1.0 ml of water, mixed with 4.0 ml of ethanol and centrifuged. The residue was resuspended in 5.0 ml of ethanol and centrifuged. It was then extracted three times with three portions of 3:1 alcohol : ether at room temperature with brief stirring. The combined extracts constitute the phospholipid phosphorous fraction.

(c) Removal of RNA :-

The residue was treated for 16-20 hours at 37°C with 1\% KCl, approximately 10 ml per gram of fresh tissue, which
results in solution of the tissue. The solution was neutralized with 6 N Hydrochloric acid and the DNA and protein were precipitated by one volume of 5% Trichloroacetic acid. The centrifuged precipitate was washed with 5 ml of 5% Trichloroacetic acid and the extracts were combined to give the RNA fraction plus the inorganic phosphate derived from phosphoprotein.

(d) Removal of DNA:

The residue was suspended in 5.0 ml of 5% Trichloroacetic acid, heated for 15 minutes at 90°C, cooled and centrifuged. The residue was resuspended in 2.5 ml of 5% Trichloroacetic acid and centrifuged and the extracts were combined to form an extract for DNA estimation. The residue is the protein fraction.

(e) Extraction of protein:

The residue remaining after the removal of DNA was incubated at 30°C with 0.5 N NaOH overnight (Chaube & Swinyard, 1975).

Estimation

All fractions were made up to measured volumes and aliquots were removed for analysis. DNA was estimated by diphenylamine method (Burton, 1956); RNA by the orcinol
reaction (Schneider, 1957) and protein by the method of Lowry et al. (1951). The values obtained were referred to those obtained with standard solutions of DNA, RNA and protein, respectively. Calf thymus DNA, yeast RNA and bovine albumin were used as standards for DNA, RNA and protein, respectively.
EXPERIMENTAL RESULTS
The present study was undertaken to understand urethane-induced biochemical lesions in the chick embryo associated with the teratogenic response. Its effect on DNA synthesis was measured since DNA synthesis is said to be sensitive to the action of urethane (Shlimkin et al., 1969; Hennings & Boutilier, 1969, and Rocchi et al., 1974). Since protein synthesis is an important aspect of the process of differentiation and morphogenesis (Davidson, 1968), protein synthesis was measured. The biosynthesis of RNA, in addition, was studied to determine if urethane affected this phase of utilization of genetic information during development.

The data in tables 5, 6 & 7 establish certain quantitative events that occur in chick embryos under the effect of urethane. It is obvious that there is an appreciable decrease in the wet weight of the embryos after the urethane treatment, in all the replicates studied (Text Fig. 86; Plate XXVI). This decrease in wet weight of the embryos could be partly ascribed to the decrease in the cellular constituents, namely deoxyribonucleic acid, ribonucleic acid and protein. It is an established fact that the weight changes of the whole embryo represent the sum of chemical changes taking place in the developing organism (Romanoff, 1967).

It can be seen that there is a considerable decrease in
the protein, deoxyribonucleic acid, ribonucleic acid and content of urethane treated chick embryos as compared to that of those from the control series without any urethane treatment (Text Figs. 87, 88 and 89; Plates XXVI and XXVII).

It is obvious that there is a considerable decrease in the DNA, RNA and protein content of chick embryos treated with urethane. The decrease was found to be statistically significant. It was seen to be in the range of 54%, 37% and 26% for DNA, RNA and protein, respectively. It may be mentioned here that the effect of urethane on protein content has not been reported previously.
### TABLE 5

**TOTAL PROTEIN CONTENT OF UNTREATED (CONTROL) AND TREATED (EXPERIMENTAL) CHICK EMBRYOS.**

<table>
<thead>
<tr>
<th>Set No.</th>
<th>Wet weight of embryos (mgm)</th>
<th>μg Protein</th>
<th>μg Protein/100 mgm wet weight</th>
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</thead>
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<tr>
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</tr>
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<td>4</td>
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<td>1946</td>
</tr>
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<td>1537</td>
</tr>
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<tr>
<td>8</td>
<td>90</td>
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<td>1270</td>
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</table>

**Control**: Untreated embryos grown in Pannett Compton saline.

**Experimental**: Urethane treated embryos grown in Pannett Compton saline.

**Stage of the embryo**: 3+ or 4.

**Concentration of urethane**: 0.168 M

**Duration of treatment**: 4 hours

**Number of embryos**: 5 in each set

**Number of sets**: 8.
### TABLE 6

**TOTAL DNA CONTENT OF UNTREATED (CONTROL) AND UNTREATED (EXPERIMENTAL) CHICK EMBRYOS.**

<table>
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<tr>
<th>Set No.</th>
<th>Wet weight of embryos (mgm)</th>
<th>µgDNA</th>
<th>µgDNA/100 mgm wet weight</th>
<th>µgDNA/gm of protein</th>
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<td><strong>CONTROL</strong></td>
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</table>

**Control**: Untreated embryos grown in Pannett Compton saline.

**Experimental**: Urethane treated embryos grown in Pannett Compton saline.

**Stage of the embryo**: 3* or 4

**Concentration of urethane**: 0.168 M

**Duration of treatment**: 4 hours

**Number of embryos**: 5 in each set

**Number of sets**: 8.
### TABLE 7
TOTAL RNA CONTENT OF UNTREATED (CONTROL) AND TREATED (EXPERIMENTAL) CHICK EMBRYOS.

<table>
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<tr>
<th>Set No.</th>
<th>Wet weight of embryos (mgm)</th>
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<th>ρgRNA/gm of protein</th>
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</table>

**Control**: Untreated embryos grown in Pannett Compton saline.

**Experimental**: Urethane treated embryos grown in Pannett Compton saline.

**Stage of the embryo**: 3rd or 4th

**Concentration of urethane**: 0.168 M

**Duration of treatment**: 4 hours

**Number of embryos**: 5 in each set

**Number of sets**: 8
DISCUSSION
The problem of mechanism of action of any carcinogen or teratogen in an animal is always closely linked with a question as to what happens to the critical macromolecular components, namely, the proteins and nucleic acids.

The first evidence for any interaction of urethane and nucleoproteins was given by Cornman et al (1951), when they studied the uptake of labelled urethane in sea urchin eggs and sperms. A possible effect of urethane on nucleic acid metabolism in general was suggested by Haddow and Sexton (1946), Cowen (1949) and many others. Later on, urethane was shown to affect the nucleic acid levels of mice tissue (Bhide & Ranadive, 1966), and also the incorporation of labelled phosphate $^{32}$P into nucleic acids (Giri & Bhide, 1969). This undoubtedly supported the view that urethane has an inhibitory influence on the biosynthesis of nucleic acids. There have been various reports linking the mechanism of biological action of urethane with a disturbance in nucleic acid metabolism and more specially the pyrimidine metabolism (Bresnick, 1960; Edinoff et al, 1961, Handschumacher & Welch, 1960 and Rogers, 1957).

A direct interference with DNA synthesis after urethane treatment was shown by Rocchi et al (1974) in regenerating rat
liver. Lombardi (1974) in his studies concluded that urethane is a chemical which inhibits both DNA and RNA synthesis. Joshi (1975) in his studies concluded that urethane probably brings about a reduction in the nucleic acid contents.

In the present investigation we have seen that urethane brings about a significant decrease in all the three macromolecular components, namely, DNA, RNA and proteins. It appears that the suppression of DNA synthesis has occurred to a greater extent as compared to depression in RNA and protein.

In addition to the significant role played by one carbon units in the metabolic processes mediated by folic acid, as discussed earlier, the one carbon units are also known to be involved in the methylation of proteins like histones (Tidwell et al., 1968; Borun et al., 1972 and Stein & Borun, 1972) which regulates DNA replication. Similarly, one carbon units are known to play a significant role in the methylation of t-RNA (Marcker & Sanger, 1964 and Revel & Litteuer, 1965) and certain DNAs. The above mentioned processes constitute key reactions in the protein biosynthesis and also in the formation of N-formyl methionine which is involved in the initiation and formation of new proteins, and nucleic acid metabolism.

The decrease in the DNA, RNA and protein content of
the urethane treated chick embryos may presumably be due to interference of urethane with the biogenesis of folic acid. The inhibition of nucleic acid and protein metabolism may thus be attributed to a reduction in the level of folic acid coenzymes essential for their synthesis.
<table>
<thead>
<tr>
<th>Plate No.</th>
<th>Text Figs.</th>
<th>Particulars</th>
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<tr>
<td>XXVI</td>
<td>86</td>
<td>Histogram showing the wet weight of untreated (control) and urethane treated (experimental) chick embryos.</td>
</tr>
<tr>
<td>XXVI</td>
<td>87</td>
<td>Histogram showing the protein content of untreated (control) and urethane treated (experimental) chick embryos.</td>
</tr>
<tr>
<td>XXVII</td>
<td>88</td>
<td>Histogram showing the DNA content of untreated (control) and urethane treated (experimental) chick embryos.</td>
</tr>
<tr>
<td>XXVII</td>
<td>89</td>
<td>Histogram showing the RNA content of untreated (control) and urethane treated (experimental) chick embryos.</td>
</tr>
</tbody>
</table>
Text Fig. A

A Histogram showing the wet weight of control and experimental chick embryos cultured in vitro.

Control: Untreated embryos grown in Pannett Compton saline.

Experimental: Urethane treated embryos grown in Pannett Compton saline.

Stage of the embryo: 3rd or 4th.

Concentration of Urethane: 0.168 M.

Duration of treatment: 4 hours.

Number of embryos: 5 in each set.

Number of sets: 8.

The value depicted in the histogram is the mean of all the eight sets.

Text Fig. B

A Histogram showing the total protein content/100 mg wet weight of control and experimental chick embryos cultured in vitro.

Control: Urethane embryos grown in Pannett Compton saline.

Experimental: Urethane treated embryos grown in Pannett Compton saline.

Stage of the embryo: 3rd or 4th.

Concentration of Urethane: 0.168 M.

Duration of treatment: 4 hours.

Number of embryos: 5 in each set.

Number of sets: 8.

The value depicted in the histogram is the mean of all the eight sets.
Text Fig. 88  A histogram showing the total DNA content/gm of protein of control and experimental chick embryos cultured in vitro.

Control: Untreated embryos grown in Pannett Compton saline.

Experimental: Urethane treated embryos grown in Pannett Compton saline.

Stage of the embryo: 3+ or 4.

Concentration of urethane: 0.168 M.

Duration of treatment: 4 hours.

Number of embryos: 5 in each set.

Number of sets: 8.

The value depicted in the histogram is the mean of all the eight sets.

Text Fig. 89  A histogram showing the total RNA content/gm of protein of control and experimental chick embryos cultured in vitro.

Control: Untreated embryos grown in Pannett Compton saline.

Experimental: Urethane treated embryos grown in Pannett Compton saline.

Stage of the embryo: 3+ or 4.

Concentration of urethane: 0.168 M.

Duration of treatment: 4 hours.

Number of embryos: 5 in each set.

The value depicted in the histogram is the mean of all the eight sets.
PLATE XXVII

C = CONTROL
E = EXPERIMENTAL

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RNA/GM PROTEIN

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DNA/GM PROTEIN

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1. Effect of urethane treatment on total protein, deoxyribonucleic acid and ribonucleic acid contents of chick embryos was studied.

2. Fresh, fertilized eggs of white leghorn hens were used in the present investigation. The eggs incubated at 37.5° ± 1° C to get mid-primitive streak stage (stage 3\textsuperscript{a}) or definitive primitive streak stage (stage 4) and cultured \textit{in vitro} by New's method. Half of these embryos were treated with 0.168 M urethane for 4 hours which served as experimentals. The other half of embryos were grown in Pannett Compton saline which served as controls.

3. The nucleic acids were extracted by the method of Schmidt-Thannhauser(1945) in combination with that of Schneider (1946). Protein was extracted by the method used by Chaube and Swinyard(1975).

4. DNA was estimated by diphenylamine method (Burton, 1956), RNA by the orcinol reaction (Schneider, 1957) and protein by the method of Lowry \textit{et al}(1951).

5. The colorimetric estimations and histograms of the total protein content/100 mgm net weight and DNA, RNA content/gm of protein of control and experimental embryos show significant differences.
6. The overall results are discussed in view of probable interference of urethane with the biogenesis of folic acid which is manifested as a folic acid deficiency.
PART I (C)

SDS ACRYLAMIDE GEL ELECTROPHORESIS.
INTRODUCTION
Separation of proteins can be very effectively achieved by electrophoresis. Used primarily as an analytical tool, its application to the preparative separation of various proteins is very well known. It can also be employed to ascertain the purity of a protein or the impurities in a protein preparation. Polyacrylamide gel electrophoresis is more commonly used in this regard.

Numerous gel systems have been used for polyacrylamide gel electrophoresis such as the 7.5% acidic gel system at pH 4.0 using 3-alanine buffer, 7.5% alkaline gel system at pH 8.3 using tris-glycine buffer with and without urea and 10% SDS gel system at pH 7.2 using sodium phosphate buffer. Earlier, in this laboratory, Katdare (1978) found that of all these methods, 10% SDS gel system at pH 7.2 using sodium phosphate buffer was best suited for the separation of proteins from the chick embryo. The disc electrophoretic patterns with SDS Polyacrylamide gel electrophoresis were more distinct and therefore this procedure was employed in further work.

Polyacrylamide gel electrophoresis in the presence of Sodium Dodecyl Sulphate (SDS) separates polypeptides according to their molecular weights. Sodium Dodecyl Sulphate (SDS) is a highly anionic detergent that binds and dissociates protein into its constituent polypeptide chains. As stated earlier, the
separation of polypeptide chains is essentially according to their molecular weights, because in the presence of SDS, the resulting protein - SDS complex, irrespective of the initial charge on the protein molecule, is anionic in character.
MATERIALS AND METHODS
In the present investigation, the method of Weber and Osborn (1969) was used. The apparatus was very simple and adapted from that described by Davis (1964). It essentially consists of two vessels forming the upper and lower chambers. Out of these two vessels, the upper one consists of holes, into which fit the gel tubes with the help of rubber stoppers.

(A) Preparation of solutions

1. Acrylamide solution - 22.2 g acrylamide (E. Merck, Germany) and 0.6 g of N, N', methylene-di-acrylamide or methylene bis acrylamide (BDH) were dissolved in 100 ml of distilled water. The resulting solution was filtered with Whatman filter No.1 and stored at 4°C in a dark bottle.

2. Gel buffer - 0.2 M pH 7.2. 8.81 g of NaH₂PO₄·2H₂O (E. Merck, Germany); 51.56 g of Na₂HPO₄·12H₂O (E. Merck, Germany) and 2.0 g of Sodium Dodecyl Sulphate (SDS) were dissolved in distilled water and volume made upto one litre.

3. Ammonium per sulphate - (BDH) (15 mg/ml) was dissolved in water. The solution is prepared just prior to actual use as it is unstable.

4. TEMED - N,N',N', N' tetraethylene methylene diamine - It was used directly.
5. Reservoir buffer - This was made by mixing 1 part of gel buffer with 1 part of distilled water.

6. Sample buffer - It is 0.01 M Sodium phosphate buffer of 7.2 pH containing 0.1% SDS and 0.1% \( \beta \)-mercaptoethanol.

(B) Preparation of Gels

To start with, all the constituent solutions were brought to room temperature. The mixing of the various solutions was done as follows: 13.5 ml\((1)\) + 15 ml\((2)\) + 1.5 ml\((3)\) + 0.045 ml\((4)\). Of these four solutions, 1 and 2 were deaerated after mixing for 2 minutes to ensure complete polymerization. Solution no. 3 was the last to be added. With the help of a hypodermic syringe, fitted with thin polythene tubing to its needle, the solutions were thoroughly mixed. This mixture was quickly added into gel tubes upto 6 cm mark. Care was taken to avoid any air bubbles as far as possible. A small quantity of distilled water was added on the top of each gel to prevent oxidation due to the formation of air gel meniscus and to ensure a flat gel surface. The whole set up was kept at room temperature for half an hour to ensure complete polymerization.

(C) Preparation of Protein Samples

The preparation of protein sample should ensure complete denaturation and/or reduction of sulphide bonds. Proteolysis
due to impurities in the sample or due to inherent proteolytic activity should be carefully avoided.

Chick embryos were cultured in vitro by New's method and processed further for controls and experimentals as described in Part 1(A). In all six sets were used. The embryos from both the control and experimental groups were homogenized separately in 1 M saline. The homogenate was centrifuged at 10,000 r.p.m. for 15-20 minutes. The supernatant was used for further experiments. Its protein content was determined by Lowry's method (1951), using bovine serum albumin as the standard protein. To one part of protein, nine parts of buffer (0.01 M sodium phosphate pH 7.0) was added and this was boiled in a water bath at 100°C for 3-5 minutes along with 1% SDS and 1% β-mercaptoethanol. Similarly five standard proteins namely Crystalline bovine serum albumin, Ovalbumin, Pepsin, Papain and Cytochrome C were dissolved directly in this buffer so as to get a concentration of 1 mg/ml and processed in the same way as the control and experimental samples described above. All these solutions were cooled down to room temperature and then used for loading the gels.

(D) Loading of gels and electrophoresis

The water layer on the top of the gels was sucked out before loading the sample mixture. Each sample mixture was
prepared as follows:

25 - 50 μl of protein sample + 5 μl tracking dye (0.05% Bromophenol blue) + 1 drop glycerol + 5 μl β-mercaptoethanol. The gel tubes were fitted in the reservoir chamber. The samples were carefully loaded and reservoir buffer was then loaded on the top of the mixture. Both the compartments were filled with reservoir buffer. Positive electrode was connected to the lower compartment and negative to the upper one. Electrophoresis was performed at room temperature since SDS is less soluble below 15°C. Current of 8 mA/tube was used. The migration of the tracking dye to the opposite end of the tube took about 3 1/2 to 4 hours.

(E) Removal of the gel

After electrophoresis was complete, length of the gel in each tube and distance travelled by the marker protein was noted down. Gels were removed carefully by squirting water between the gel and the wall of the tube with the help of a syringe fitted with a long needle.

(F) Staining and Destaining

Staining solution - 1.25 g of Coomassie Brilliant Blue R-250 (Sigma, U.S.A.) is dissolved in 227 ml of methanol and 46 ml of glacial acetic acid and the total volume made upto
500 ml with distilled water. It was filtered through Whatman No. 1 and stored at room temperature.

The gels were stained for 2-3 hours at room temperature. After the staining was complete all the gels were rinsed with distilled water and then put into the destaining solution.

Destaining solution - It is prepared by adding 50 ml methanol (E. Merck) and 75 ml of glacial acetic acid (E. Merck) and 875 ml of distilled water.

The diffusion method was employed in the present work. After the destaining was over, the length of the gel as well as the distance of each band of protein from the top was measured. The gels were preserved in 75% acetic acid at 4°C.

(6) Calculations

The mobility of each band was calculated directly as follows:

\[ M = \frac{\text{Distance of protein migrated}}{\text{Distance of tracking dye migrated}} \times \frac{\text{Gel length before staining}}{\text{Gel length after destaining}} \]

The mobilities were calculated relative to the tracking dye. The mobilities were plotted against the known molecular
weights of standard proteins expressed on a semilogarithmic scale. Thus the molecular weights were calculated. The mobilities and the molecular weights thus calculated are represented in tables 8, 9 and 10 respectively.
RESULTS AND DISCUSSION
SDS gel electrophoretic patterns of proteins in the control and the experimental embryos are shown in Plate XXVII. It is obvious that there is a considerable difference in the disc-electrophoresis patterns of the control (Text Fig.90; Plate XXVIII) and the experimental (Text Fig.91; Plate XXVIII). The total number of bands that could be easily counted in the controls is 16 while in the experimental only 15 are seen.

In the experimental set, the band corresponding to band number eight in the control is seen to be missing. These results suggest that the missing band may be corresponding to a protein which must have been affected by treatment with urethane.

The mobilities of experimental and control are given in table no.8. From the graph, molecular weights of proteins in control and experimental embryos were calculated and are shown in table no. 9.

From tables 8 and 9, it is clearly seen that there is a difference in the mobilities and molecular weights of different proteins in the chick embryos from the control and the experimental series.

Particularly band ‘8’ is totally missing in the treated
### TABLE 8

**MOBILITIES OF PROTEINS IN CONTROL AND EXPERIMENTAL EMBRYOS**

<table>
<thead>
<tr>
<th>No. of Band</th>
<th>Distance travelled in cms</th>
<th>Mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E</td>
</tr>
<tr>
<td>1</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>7</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>missing</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>10</td>
<td>2.6</td>
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<tr>
<td>14</td>
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<tr>
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<td>4.2</td>
<td>4.0</td>
</tr>
<tr>
<td>16</td>
<td>4.9</td>
<td>4.7</td>
</tr>
</tbody>
</table>

C = Control: untreated embryos grown in Pannett Compton saline.

E = Experimental: Urethane treated embryos grown in Pannett Compton saline.
## TABLE 9

**MOLECULAR WEIGHTS OF DIFFERENT PROTEINS IN CONTROL AND EXPERIMENTAL EMBRYOS**

<table>
<thead>
<tr>
<th>No. of band</th>
<th>Log mol. wt. × 10^4</th>
<th>Approximate molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E</td>
</tr>
<tr>
<td>1</td>
<td>1.18</td>
<td>1.13</td>
</tr>
<tr>
<td>2</td>
<td>1.11</td>
<td>0.99</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0.96</td>
</tr>
<tr>
<td>4</td>
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<td>0.92</td>
</tr>
<tr>
<td>5</td>
<td>0.89</td>
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<tr>
<td>6</td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>7</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>8</td>
<td>0.71 (missing)</td>
<td>51280 (missing)</td>
</tr>
<tr>
<td>9</td>
<td>0.65</td>
<td>0.67</td>
</tr>
<tr>
<td>10</td>
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<tr>
<td>15</td>
<td>0.20</td>
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<tr>
<td>16</td>
<td>0.03</td>
<td>0.09</td>
</tr>
</tbody>
</table>

C = Control: Untreated embryos grown in Pannett Compton saline.

E = Experimental: Embryos treated with urethane grown in Pannett Compton saline.
samples.

From our results in the present investigation, it is clear that one of the proteins is missing. In our earlier studies we have seen that urethane interferes in various folic acid mediated reactions and brings about a significant decrease in the protein, DNA and RNA content in chick embryos treated with urethane. We had suspected this decrease due to a folic acid deficiency.

The observations in the present studies further support our earlier view that urethane interferes with the synthesis of proteins (see page 99). It has been shown that growing chick embryo produces folic acid (Landi et al., 1972). Folic acid deficiency, possibly on account of a lack of a protein involved in its biosynthesis, occurs. It would be of interest to know whether the band '8' corresponds to a particular enzyme involved in folate biosynthesis. The absence of a protein suggests the possible mutagenic action of urethane.

These dislocations or disturbances mediated by urethane may be responsible for the embryotoxic action of urethane and the malformations observed.
ILLUSTRATIONS
## LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Plate No.</th>
<th>Text Fig.</th>
<th>Particulars</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXVIII</td>
<td>90</td>
<td>SDS-polyacrylamide gel electrophoretic pattern of untreated (control) chick embryos.</td>
</tr>
<tr>
<td>XXVIII</td>
<td>91</td>
<td>SDS-polyacrylamide gel electrophoretic pattern of urethane treated (experimental) chick embryos.</td>
</tr>
</tbody>
</table>
Text Fig. 90 SDS-polyacrylamide gel electrophoretic pattern of proteins of untreated (Control) chick embryos. The bands are seen from top to bottom. Total number of bands that can be easily seen is 16.

Text Fig. 91 SDS-polyacrylamide gel electrophoretic pattern of proteins of urethane treated (Experimental) chick embryos. The bands are seen from top to bottom. Total number of bands that can be easily seen is 15.
1. SDS-acrylamide gel electrophoresis patterns of control (untreated) and experimental (treated) embryos was studied.

2. Electrophoresis was carried out by the method of Weber and Osborn (1969).

3. In the control embryos 16 bands could be seen and in the experimental only 15 could be clearly seen. In the experimental, band corresponding to band number '8' in the controls was missing.

4. A difference exists in the mobilities and hence in the molecular weights of proteins from the control and experimental set.

5. In the light of these observations, interference of urethane with the synthesis of proteins and therefore indirectly with the biosynthesis of folic acid is contemplated.