GENERAL DISCUSSION
Enzymes influence the chemical reactions in the body from the very beginning of the embryonic state. In animal body the primary control of cellular functions and metabolisms are accomplished and controlled by the regulation of secretion of the enzymes. Changes in the physiological and pathological conditions produce alteration in the activity of enzymes and it is expected that important enzymes alteration may play an important role in the development of neoplastic condition. As enzymes are protein in nature, their activities are dependent not only on the synthesis of protein in the cellular level but also on the growth of the cells as well. The cancer cells are characterized by the loss of growth control and altered rate of proliferation and thus they differ from the normal cells. However, the rate of proliferation in tumour cells is not necessarily to be rapid and due to loss of control over growth, invasion and metastasis results which ultimately changes to malignancy.

A great variety of enzymes and isoenzymes have been observed to alter in the tumours themselves and in the body fluids of cancer-bearing subjects. Often a revision towards foetal isoenzyme patterns is noted (Schwartz, 1973; Shapira, 1973). However, it is to be noted that specificity of enzymes in malignancy is yet be determined and hence except a very instances enzymes are as a whole considered to be rarely
FIG. 29: Alkaline phosphatase activity in whole embryo, brain and liver (Control and Test) at different stages of development.
pathogenetic directly, even then enzymes like acid phosphatase, alkaline phosphatase, prolyl hydroxylase, 5'-nucleotide phosphodiesterase, sialyltransferase, lysozyme, γ-glutamyl transpeptidase etc. are considered to be of great importance in monitoring the progress of the malignancy indicating disturbance in the enzyme system at some uncertain stage, and it is yet to be determined if the disturbance observed is the genesis of malignancy or not.

In the present investigation, the alteration in the two enzyme systems namely alkaline phosphatase and lactate dehydrogenase during the period of embryogenesis have been studied by injecting 0.07 ml. of 4-DAB (0.1 mg/1 ml.) in a single dose on the 4th day of development.

An enhanced alkaline phosphatase enzyme activity was observed in the whole embryo treated with 0.07 ml. of 4-DAB on 4th day of development (Fig. 15) and the findings were in conformity with the findings of Reger et al. (1960) in chick embryos, of Chiquione (1954) in mouse and of Mulnard (1955) in rat embryos. Elevation of alkaline phosphatase activity in the brain tissues of the treated embryos, the maximum elevation being on the 12th day of incubation. The sharp increase activity of the alkaline phosphatase activities in the test group was observed from 8th to 12th day of incubation (Fig. 15). A higher alkaline phosphatase activity was observed in the liver of the chick embryos in all the stages of development, but the
FIG. 30: Lactate dehydrogenase activity in whole embryo, brain and liver of chick embryo (Control and Test) at different stages of development.
maximal activity of alkaline phosphatase was found on 12th day followed by a lowering activity gradually upto 16th day of development (Table 14) an ascending type of enhanced alkaline phosphatase activity was noted upto 12th day and then the activity declined on 16th day of development (Fig. 15). In case of whole embryo the highest activity however was noted on 8th day and was ascending type upto 8th day followed by declined activity towards the 12th day. It is to be noted that the alkaline phosphatase activity in the liver of the growing chick embryo treated with 4-DAB on all the phases development was definitely higher than that of observed in the untreated embryos (Table 38).

Elevation of alkaline phosphatase has been observed in malignancy of liver and bone, however, primary hepatomas, unlike metastatic tumours of the liver tend to have normal alkaline phosphatase osteogenic sarcoma especially of the osteoclastic type (Maggin's et al. 1941, 1951) showed a high alkaline phosphatase due to release of the enzyme from the bone, similarly high alkaline phosphatase was observed in the bone metastasis from prostatic cancer and greatly elevated levels of the enzyme were noted in the prostatic cancer, however, no alteration in the alkaline phosphatase was noted in case of Ewing's Sarcoma, and giant cell tumour (Bodansky, 1975).

The increased activity of the alkaline phosphatase in the growing embryo may be due to the fact during the process
of development, the enzyme plays an important role through some mechanisms yet to be explained - as it was earlier also observed in case of sea-urchin and Ascidians during the early stages of development (Ellis, 1966), and in the nervous system during the foetal development of chick (Rogers et al., 1960; Kallen and Velmin, 1960). If that be so, the higher alkaline phosphatase level noted in the 4-DAB treated whole embryos, brain and liver tissues of the same group of embryos is the expected observation as malignancy is a process of uncontrolled growth. This hypothesis is also acceptable on the basis of the findings of Rubini et al. (1964) who suggested that the nucleus and/or nuclear membrane alkaline phosphatase may influence DNA synthesis, histochemical investigation showed a decreased activity of alkaline phosphatase in the preneoplastic stage of brain tissue and in tumour produced by 3-methylcholangthrene (Kirsch, 1963).

Fishman et al. (1968) stressed the importance of studying the isoenzymes of alkaline phosphatase; of the different phenotypes of the enzymes, Inglish et al. (1973) noted only one type in each patient and a further closely related isoenzyme has been noted to be a product of hepatocarcinoma (Higashino et al. 1974). Though Nathanson and Fishman (1971), Belliveau et al. (1974) observed the different isoenzymes of the alkaline phosphatase of serum in the patients with wide variety of different tumours, other workers like Jacoby and Bagshawe (1972), Usategul-Gomes et al. (1973), Cadeau et al. (1974) all failed to
confirm any significant number of elevated isoenzymes of alkaline phosphatase in neoplasms and even the suggestion of Stelbach et al. (1969) that the elevated levels of the isoenzymes related to the tumour mass has also been doubted very much. Considering all these views, in the present investigation it has not been attempted to study the isoenzymes of the alkaline phosphatase in the growing chick embryos either untreated or treated with 4-DAB.

It is to be noted that the kinetic behaviour of the enzyme, alkaline phosphatase varies from tissues to tissues (Graham, 1979), heterogeneity is even seen between different cell strains from the same tissue and different clones from the same cell strain (Parayannopoulos and Martin, 1967) and hence the observations of different workers on variable activity of the enzyme in different neoplasms have complicated to come to a definite conclusion. A progressive increase of the alkaline phosphatase in the early stages of development as observed in the present investigation has also been noted by Emeleot and Bos (1969a) in hepatoma 484. It may be mentioned that an alteration in the cellular architectures leading to preneoplastic changes after single dose administration of 4-DAB into growing chick embryos (Sarma, 1982) and the increase in alkaline phosphatase activity in the early stages of development (are in conformity with the findings of Emeleot and Bos (1969b). Lumb and Deoll (1970) observed that the alkaline phosphatase in
mouse tumours seemed to be related to that of embryothymus or normal spleen and concluded that the appearance of the enzyme is dependent on the cell and may arise from depression of a cellular gene. Graham (1979) also suggested that the activity of the enzyme is to be made in different components namely the membrane, the whole cells, in tissues as the changes one compartment may mask the changes in another compartment and the changes in distribution is more important than the specific activity of alkaline phosphatase.

The elevation of alkaline phosphatase activity in the hepatomas induced by 4-DAB or azodyes ingested or injected rat liver was reported earlier by Woodward (1943), Pearson et al. (1949, 1950) and Robertson et al. (1949) and explained as due to obstruction of extrahepatic biliary tract by carcinomas (Gutman, 1959).

Lactate dehydrogenase (LDH) activity in neoplasm since the initial report of Warburg on metabolic differences between normal and neoplastic tissue is as conflicting as the alkaline phosphatase activity. Groups of workers (Green et al. 1958, Wroblewski et al. 1957, Wroblewski, 1959, Buckell, 1965, Wroblewski et al. 1958) observed elevated lactate dehydrogenase in tumours specially brain tumours; others (Jakoby and Jakoby, 1958; Fleisher et al. 1957, Meister, 1950, Weinhouse and his collaborators, 1955, 1951, 1953, 1952, 1953) did not notice any change in the lactate dehydrogenase activity between the normal
and the neoplastic growth. Even then, measurement of LDH activity for screening and as an adjunct in the diagnosis of carcinoma of different organs has been stressed upon (Figus et al. 1972, Faulk et al. 1972, Piper et al. 1963, Simon and Figus, 1972). In case of the tumours of the central nervous system, Seidenfeld and Marton (1981) noted a predictive value for a positive result of 37 percent and an efficiency of 63.4 percent though the authors agreed that LDH activity may not be a very reliable marker for use in diagnosis of CNS tumours.

In the present investigation the measurement of LDH activity and the pattern of the LDH isoenzymes were carried out in the growing chick embryos with i.e. the Test group and without i.e. the Control group, treatment with 4-DAB at different stages of development. With the progress of the development, the LDH activity also increased both in the control and the test groups, the maximum enhancement being noted on 12th day of development. An altered LDH activity in the liver of the growing chick embryos was noted in the present investigation. The LDH activity in the liver of the control groups was noted to increase from 8th to 12th day and then the activity declined towards the 16th day of incubation, whereas in the test group the maximal activity was observed on the 8th day with subsequent decrease of the activity on the 12th day followed by enhancement of the activity again on the 16th day of development.
A progressive increase in the LDH activity in the brain and skeletal muscles of the growing chick embryo was noted earlier by different groups of workers (Solomon, 1958; Flexner et al. 1960; Himwich et al. 1942, 1949; Tyler and von Harreveld 1942; Chasler and Himwich, 1944; Reiner, 1947; Muir et al., 1959). A five fold increase of LDH activity in the liver of developing embryos during 7-13 days of incubation was noted by Solomon (1958). It was also noted that in the mouse brain embryo the LDH activity was relatively consistent until 12th day and there was three fold increase reaching the adult level at the end of 19th day (Flexner et al. 1960). The study of LDH activity in the normal and tumors of the numbers of tissues including brain. Meister (1950) reported that values for the tumors were higher or the same as the corresponding tissues of origin and concluded that the tumors possess high LDH activity and that the level of activity lies in about the middle of the range for normal tissues. Significantly higher level of LDH activity was also reported by Wenner et al. (1952), Mechi et al. (1955).

However Lenta and Reihl (1949) pointed out that mice bearing transplanted tumors were not influenced with significant alteration of LDH activity or malic dehydrogenase by the growing neoplasm though Meister (1950) noted that the transplanted hepatoma 112 B exhibited somewhat more LDH activity than the normal liver and hepatoma 13/8 possessed about the same activity as the liver.
In the present investigation, no detectable qualitative changes in the LDH isoenzyme pattern was noted. Markert (1964) reported no change in the isoenzyme pattern of LDH during the development of all animals. In the chick embryos LDH-1 rather than LDH-5 was the prominent isoenzyme and the development of skeletal muscle leads to shift towards LDH-5. Only LDH-1 and LDH-2 were detected in tissues like heart muscle, leg muscle and breast muscle during the process of development and other bands were usually absent (Lindsay, 1963) and no difference in LDH isoenzyme pattern between the embryo and adult heart tissue was noted (Markert, 1964). A number of studies of LDH isoenzymes in the brain tumour were carried out; Goldman et al. (1964) suggested that the shift of isoenzyme distribution from LDH-1 to LDH-5 in tumour tissues occurs because LDH-5 more actively converts pyruvate to lactate at elevated concentrations of pyruvate with the result that more NAD is produced by LDH-5 than LDH-1. However, this observation has not been universally accepted.

Experimental evidences of the present investigation as presented in Fig. 31 revealed that in the control groups both the alkaline phosphatase and LDH activities had a tendency for elevation up to 12th day of incubation in the liver tissues as the development proceeded. In case of brain tissue of the control group the LDH activity decreased on the 12th day and then increased again gradually. However, alkaline phosphatase activity
increased from 8th to 12th day and from 12th to 16th day of incubation in brain tissues. In contrast a very much altered activity of both the enzymes was observed in the embryos treated with a single dose 0.07 ml. of 4-DAB (0.1 mg/ml.) on 4th day of development. In case of brain tissues of treated group alkaline phosphatase increases gradually from 8th to 12th day and 12th to 16th day. In case of liver the maximal level of alkaline phosphatase was found on the 12th day followed by decreased activity on the 16th day of incubation. LDH activity also behaved more or less in the same manner though the LDH activity was noted to decrease on 12th day of incubation and subsequent increase on 16th day of incubation both in case of brain and liver tissues of the treated group of embryos.

Cancer is a process of growth - a process of initiation followed by stimulation of tumour growth (Farber, 1973) and the neoplastic cell induced by initiation may remain dormant either for a short or for a longer period (Berenblum, 1954). Alteration of the enzymic activities of both the alkaline phosphatase and the LDH are altered with the progress of the growth and the proliferation of the cells and as such the alteration of both the enzymatic activities in carcinoma seems to be quite natural, however, one cannot ascertain if this alteration is during the period of initiation or during the period of stimulation.
FIG. 31: Showing the comparison of alkaline phosphatase and lactate dehydrogenase pattern in whole embryo, brain and liver (Control and Test) at different stages of development.
### Table - Showing the comparative value of alkaline phosphatase and LDH in whole embryo, brain and liver tissues at the different stages of development (Control and Test).

<table>
<thead>
<tr>
<th>Group</th>
<th>4th day</th>
<th>6th day</th>
<th>8th day</th>
<th>12th day</th>
<th>16th day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WHOLE EMBRYO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alk. Phosp. mg. PO₄/gm.</td>
<td>2.88±</td>
<td>3.21±</td>
<td>4.07±</td>
<td>3.89±</td>
<td>5.03±</td>
</tr>
<tr>
<td>LDH IU/gm. whole embryo</td>
<td>101.98±</td>
<td>100.21±</td>
<td>108.78±</td>
<td>107.07±</td>
<td>139.07±</td>
</tr>
<tr>
<td><strong>BRAIN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alk. Phosp. mg. PO₄/gm.</td>
<td></td>
<td></td>
<td>5.48±</td>
<td>10.84±</td>
<td>7.66±</td>
</tr>
<tr>
<td>LDH IU/gm. brain</td>
<td></td>
<td></td>
<td>315.66±</td>
<td>352.33±</td>
<td>157.0±</td>
</tr>
<tr>
<td><strong>LIVER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alk. Phosp. mg. PO₄/gm.</td>
<td></td>
<td></td>
<td>8.96±</td>
<td>17.26±</td>
<td>16.24±</td>
</tr>
<tr>
<td>LDH IU/gm. liver</td>
<td></td>
<td></td>
<td>653.2±</td>
<td>698.0±</td>
<td>666.0±</td>
</tr>
</tbody>
</table>

LDH = Lactate dehydrogenase.
The altered pattern of both the enzymes indicated that in 4-DAB treated embryos the metabolic processes influenced either jointly or independently by the two enzymes were disturbed.

The altered activities of both the enzymes also suggested that somehow or otherwise 4-DAB may affect the synthetic pattern of the enzymes during carcinogenesis in the tissues of growing chick embryos. This may be due to the toxic action in the cellular level while changes take place from normal to preneoplasia or neoplasia condition. Reports are also available in support of such toxic changes of various carcinogens in organs and at cellular level. Himsworth (1950), Law (1941), Morogenskya (1939), Turner et al. (1942) described the changes brought about by e-aminooazotoluene, induction of tumours by 4-DAB, 1-azo-2-naphthalenes and 1(2 tolylazo) 2-naphthal (oil orange Tx). Marshall reported the effect of prolonged use of 'Evan blue' and observed a variety of proliferation of reticular tissues varying from reversible changes to malignant tumours. Development abnormalities using Trypan blue was also reported by Weddington (1953). Cohira et al. (1981) reported on injection of p-nitrophenyl-β-D-xyloside in a single dose 1.0 mg/egg in developing embryos on 3rd day of development, the protein and DNA content began to increase 12 hours after the treatment and reaches maximum level (about 140%) within 3 days.
It was suggested that the carcinogen, 4-DAB covalently binds to the macromolecular structures like protein, DNA and RNA (Miller, 1970). It was observed that the nucleic acid bound derivatives formed from MAB in rat liver in vivo are also derived from the esters of N-hydroxy MAB or derivatives like DAB with similar activity. Sarma and Goswami (1984) noted the alteration of DNA content in 4-DAB inoculated embryonic liver and suggested as the possible resultant effect of the binding of 4-DAB to the macromolecules stimulating the cyclic activation of DNA synthesis of embryonic liver cells. The toxin like aflatoxin which is also a carcinogen was found to inhibit the cellular synthesis by binding with DNA and produced a delayed decrease in protein synthesis (Clifford et al. 1967, Mesbitt et al. 1962). In higher organisms, the substance produced DNA synthesis in regenerating liver cells and reduction in synthesis but not the nuclear RNA and explained due to blockage of m-RNA production after the administration of carcinogen (Clifford, Rees and Stevens, 1967). The trend of LDH level observed in the present investigation might thus be due to interference of the carcinogen with the protein metabolism at the stage of initiation most probably by binding structurally with DNA and thereby deviating the normal process of synthesis.

Since Warburg's initial report (1924) about the metabolic differences between the normal and the tumour cells with high glycolysis of the tumour slices it has been attempted
repeatedly to explain the carcinogenesis in terms of changes of the enzymatic process or processes. Kensler et al. (1942) demonstrated that substances which were isolated from the urine of rats fed with P-DAB depressed the activity of the NAD enzyme system. Further, Potter (1943) advanced a theory that cancer may be the result of a competition between the hypothetical enzyme (x) and a derivatives there of arising out from it by the action of carcinogenic agents. Siebert et al. (1965) observed that alkaline phosphatase has high affinity for and considerable activity towards phosphoenol pyruvate and therefore might compete with pyruvate kinase for the common substrate phosphoenol pyruvate. Beck (1955, 1958) also suggested that the rate limiting enzymic activities appeared to be lactic dehydrogenase and 3 phosphoglyceraldehyde dehydrogenase. It is therefore likely the alkaline phosphatase, by competing with the pyruvate kinase and lactic dehydrogenase working as the rate limiting enzyme bring some change in the carbohydrate metabolism facilitating change of normal cells to malignant cells and altered activities of both the enzymes are brought by the carcinogen. However, this hypothesis is far fetching at this stage and further detail works including Le Page's hypothesis (1950) of existence of a hormonally relieved block in the hexokinase reaction are required to test the hypothesis.

Malformation in chick embryo though not a very rare and unusual occurrences (Ridgway and Karnefsky, 1952; Shibke et al.
1968; Kaplan and Johnson, 1970), is more commonly induced by different physical factors like temperature, nutritional, genetic strains as well as mutant (Daresti, 1891; Landauer, 1954; Romanoff, 1972). Various chemical compounds are also known to cause development of embryonic malformations. Reports of such malformations in the chick embryo induced by various chemical agents in the form of growth inhibition, abdominal defects, defects in brain, liver, heart limb buds etc. are available (Dessi et al. 1959; Kirman, 1961, Kurby and Crosby, 1967; Kurby and Craig, 1967; Martin, 1975, Jacob, 1978). In the present investigation, malformations were observed in the form of hematoma, liver defect, and malformation of abdominal wall on the 12th and 16th day of incubation in the test groups after administration of 0.07 ml. of 4-DAB (0.1 mg/ml.) on the 4th day of development. The observed abnormalities is likely to be due to the abnormalities in genetic material. As 4-DAB binds with the proteins which ultimately attacks hereditary materials (Miller, 1970; Sarma and Goswami, 1984) and thus may likely cause for the development of malformation (Plate. 3, 4, 5, 6). Reports are also available for the development of malformation by the action of carcinogens, as 3-methylcholanthrene causes retardation of growth and effects the brain (Beskrovni, 1941). Armstrong and Hae (1947) noted that the liver and heart of tumour bearing chick embryos are found to be larger than those of the controls.
In the present study abnormal development observed in a few embryos in the 12th and 16th day of incubation treated with 4-DAB certainly seems to emphasize the importance of chemicals, as no such abnormality was observed in the control groups of the same stage. However, it would be far reaching conclusion to infer the influence of chemicals from the few cases of the abnormalities encountered during the period of investigation. However, it was not designed to study of malformation in the chick embryo after treatment with 4-DAB. More than one factor may be involved to manifest for such abnormalities and 4-DAB could be one of such factors.

From the experimental evidences of the present investigation and also from the review of the literatures as cited in the thesis one can postulate that 4-DAB interact with the macromolecules more specifically with DNA and thereby a change or changes in the enzymatic system is/are brought about in the stage of initiation and the altered enzymatic activities influence the metabolic processes in this particular instance in carbohydrate metabolism and thereby malformation and transfer of normal cell to malignancy are the resultant effect. However, as it has been stressed earlier this hypothesis is to be tested with more detail work.