STUDIES ON THE GLUTAMATE DEHYDROGENASE OF SOME DIGENETIC TREMATODES OF BUFFALO.

Dissertation
For
Master of Philosophy

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This is to certify that the work presented in this dissertation has been carried out by S.M. Abbas Abidi and is allowed to submit for the award of the Master of Philosophy degree in Zoology of the Aligarh Muslim University, Aligarh.

(WAIJH A. NIZAMI)
Supervisor
To

My Grand Father
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(S.M. Abbas Abidi)
CHAPTER I
**INTRODUCTION**

The scourge of parasitic diseases among ruminants in tropical and sub-tropical countries are responsible for great economic losses. In India the parasitic infections among cattle and particularly in buffaloes alone, are responsible for huge revenue losses. This affects the rural economy which mainly thrives on cattle and cattle products.

A number of cattle products like hide and skin, milk and milk products, horns and hoofs, cattle hairs etc. are a source of a quantum of foreign exchange. Despite their importance in the national economy, the net contribution made by India's animal wealth is not much, as is evident from the live-stock census of the year 1972.

Of the many reasons for meagre contribution of live-stock to the national income, poor animal health might be considered as one of the major factor. A number of viral, bacterial, protozoan and helminth infections are not only responsible for poor quality of our cattle products but are also responsible for heavy mortalities during epidemic break-outs. Therefore, there is an obvious need to check this parasitic menace in order to save the cattle industry.

Before embarking upon the chemothapeutic control
measures, it is of primary importance to understand the parasite metabolism, host-parasite relationship, various biochemical peculiarities and adaptations found among different groups of parasitic helminths. Such studies could be followed by an attempt to explore the avenues which can be exploited for control measures.

With the advent of modern techniques, study of biochemical phenomenon among parasites has revolutionised the knowledge and understanding of parasite metabolism. Inspite of this fact the branch of helminth physiology and biochemistry is still in its infancy and therefore a lot of study is yet to be done for better understanding of the host-parasite relationship and their metabolic activities. The metabolic adaptations and variations of the parasites at biochemical level, have always provoked the thought of investigators and this has led to unravelling the mysteries of parasitic adaptations.

Parasites live in microenvironments which are often difficult to precisely simulate in the laboratory conditions because their metabolic pathways are either very unusual or unique. These modified metabolic pathways in parasites offer the possibility of developing chemotherapeutics, which take advantage of metabolic idiosyncrasies (Isseroff, 1980).
Because of diversified microenvironments inhabited by helminths belonging to different taxonomic groups, it is very difficult or rather not possible to generalise the results of any biochemical study. According to their respective needs, the parasites have not only adapted morphologically but have also changed or modified metabolically as well. The helminth parasites possess metabolic shunts, secrete anti-enzymes and also have a remarkable capacity to regulate their metabolic activities, suitable to their respective habitats through which they have to pass. The development of new strains of zoonotically important parasites also pose a challenge to the investigators working on physiology and biochemistry, as in vitro culture and iso-electric focussing studies of different strains of *Echinococcus granulosus* show biochemical variations (McManus and Smyth, 1979). Thus, it is necessary to study the basic biochemical principles operating in helminths with respect to their habitats.

Among the various metabolic activities, carbohydrate metabolism is the most widely studied aspect of helminth physiology. Therefore, most of the anthelmintics available today, have been designed to interfere or inhibit the energy production processes of the parasites. However, due to lack of sufficient knowledge of protein metabolism, which is vital for the parasitic mode of life, development of anthelmintics affecting
this metabolism has hardly been exploited for the control measures.

Proteins are ubiquitous in their distribution and have many biological functions. Besides being building blocks of tissues, proteins are also involved in contractile and transport system. They also act as protective agents, hormones, toxins, amino acid reserves and a source of energy when primary energy source (carbohydrates) is not available. The immunoglobulins are nothing but protein molecules which function as specific antibodies.

The rapid multiplication of many parasitic protozoa, the immense fecundity of most parasitic worms, rapid strobilisation in cestodes and asexual multiplication of larval trematodes etc., obviously prove that parasites synthesize proteins rapidly. The nitrogenous end products of protein metabolism and the presence of metabolically utilisable compounds like amino acids in the excretory fluid of helminths also show the importance of protein catabolism. Proteins can serve to differentiate parasite and host enzymes that have identical catalytic properties, an approach having obvious implications for chemotherapeutic measures (von Brand, 1973).

In spite of their diversified functional significance, proteins and their metabolism has remained a neglected aspect of parasite physiology. It has been mainly studied in protozoa
for the control purpose. Whereas in helminths, the study has been restricted to a limited group of parasites. Among the trematodes, *Fasciola* sp. and schistosomes have been extensively used for the study of protein metabolism. Although amphistomes are quite important from epidemiological as well as pathophysiological point of view but they have escaped the attention of parasitologists. Therefore, helminth physiology in general and amphistome physiology in particular, provide an ample opportunity for various investigations in this field.

To understand the phenomenon of protein metabolism, it is a prime requisite to work out the presence and nature of their key enzymes. Among the various enzymes like transaminases, dehydrogenases, oxidases and carboxylases taking part in anabolic and catabolic activities of proteins, glutamate dehydrogenase is of great importance. It is a ubiquitous enzyme which stands at the cross-roads to carbohydrate and protein metabolism.

Besides being an important enzyme of nitrogen metabolism, glutamate dehydrogenase also serves as a marker enzyme because its increased levels in the serum of the host, is a manifestation of various pathological disorders. But the functional significance of this enzyme in the protein metabolism of amphistomes is yet to be known.
Glutamate dehydrogenase (GLDH) (E.C. 1.4.1.3) is a pyridine linked enzyme responsible for catalysing the reversible deamination of glutamate to \(-\text{keto-glutarate}\) and ammonium ions as shown in Fig. 1.

The redox reaction catalysed by GLDH, plays an important role in amino acid metabolism as it has been established that glutamate, a GLDH catalysed reaction product, is a major donor of amino group for the synthesis of various non-essential amino acids. The catabolic activity of the enzyme results in liberation of ammonia which could be incorporated into urea, Fig. 2.

The major mechanism of ammonia fixation is carried out by GLDH, thereby reducing the intracellular toxic levels of ammonia. The high level of intracellular ammonia not only alters pH but also influences membrane permeability and also tends to reverse the GLDH reaction. This last effect tends to suppress tricarboxylic acid cycle by depletion of \(-\text{keto-glutarate}\) (Prosser, 1973).

The amphistomes infect the ruminants in the agrarian tropics and sub-tropics. The prevalence rate of these parasites in Aligarh is staggeringly high. Regarding the possible role of GLDH in the helminth metabolism in general and in amphistome in particular, no information is available till to date.
Therefore, it was decided to use these parasites as models for the present study in order to understand the nature of GLDH which plays a central role in protein metabolism.
Fig. 1. Glutamate dehydrogenase reaction.

- Reductive amination of α-KGA.
- Reversible deamination of glutamate.
\[ \alpha\text{-Ketoglutarate} + \text{NH}_4^+ + \text{NADH} \rightleftharpoons \text{Glutamate} + \text{H}_2\text{O} + \text{NAD}^+ \]

\[ \text{Glutamate} + \text{NAD}^+ \rightleftharpoons \text{Imminoglutaric Acid} + \text{H}^+ + \text{NADH} \]

\[ \text{Imminoglutaric Acid} + \text{H}_2\text{O} \rightleftharpoons \alpha\text{-Ketoglutarate} + \text{NH}_3 \]
Fig. 2. Pathway showing the mitochondrial GLDH involved in providing \( \text{NH}_3 \) for carbamoyl-phosphate formation.
MITOCHONDRIAL GLDH INVOLVED IN PROVIDING NH₃ FOR CARBAMOYL-PHOSPHATE FORMATION.
CHAPTER II
HISTORICAL REVIEW AND STATEMENT OF PROBLEM

The last two decades have witnessed a changed attitude of parasitologists to switch over from taxonomic and morphological studies to more applied aspects in order to understand the basis of host parasite relationship at physiological, biochemical and molecular levels. The available literature of past several years on parasite physiology, clearly reveals that extensive studies have been restricted to a limited group of parasites. Among these, the parasitic protozoa have been extensively studied. However, among helminths, only schistosomes, Fasciola sp., and Hymenolepis sp., have been used as the best experimental models for biochemical investigations. The members belonging to the family paramphistomatidae (Fischoeder, 1901) have remained more or less neglected inspite of their high prevalence rate.

Realising the importance timely, von Brand (1973, 1979), exhaustively reviewed the literature on parasite biochemistry and physiology. Barrett (1981) and Chappell (1980) have also given comprehensive accounts of the parasite metabolism and their metabolic peculiarities. But little is known about protein synthesis in parasitic helminths (Cox, 1982).
Glutamate dehydrogenase is known to play a pivotal role in protein metabolism, but only a few scattered reports regarding the role of this enzyme are available on protozoa, helminths and other group of parasites. The GLDH has been shown to be present in several species of parasitic protozoa viz: Plasmodium berghei (Langer et al., 1970), in Plasmodium lophurae (Sherman et al., 1971). The glutamate catabolism by GLDH yields the α-ketoglutarate which is an important intermediate of the TCA cycle and therefore this reaction helps in the energy metabolism of parasitic protozoa (Gutteridge and Coombs, 1977).

Among the helminths the GLDH activity in Fasciola hepatica, Dicrocoelium lanceolatum, Paramphistomum cervi and liver tissue of ruminants, has been estimated by Kravavica et al. (1967). They reported that the enzyme activity in F. hepatica was maximum, followed by liver tissue, P. cervi and D. lanceolatum. These results were contrary to the expectations because in P. cervi, enzyme level was not higher than in F. hepatica, inspite of the fact that P. cervi inhabits the rumen which has a high concentration of free ammonia. The GLDH activity has also been reported from the daughter sporocysts of Microphallus pygmaeus (Pascoe, 1970).

Daugherty (1954) reported reductive amination of keto acids in Hymenolepis diminuta while Mustafa et al. (1978)
have studied the activity of cytosolic GLDH in the anterior proglottids of this cestode.

Oxidative deamination by GLDH has also been demonstrated in a number of nematode species. Pollak and Fairbairn (1955) reported transdeamination in the ovaries of *A. lumbricoides* in which a weak GLDH system linked with alanine and aspartate transaminases was found. Langer (1972) reported activity of this enzyme from female *A. suum*, while Barrett and Beis (1973) have shown that ovaries of *A. lumbricoides* possess a weak GLDH but their muscles have higher activity of this enzyme which may indicate a greater potential rate of deamination in this tissue. The activity of GLDH has also been reported from the *Panaqueparus redivivus* (Wright, 1975) and *Haemonchus contortus* (Croll, 1976).

Since ketoacids are common intermediates of carbohydrate metabolism, therefore reductive amination has been postulated on the basis of appearance of the labelled glucose (14C) carbons in amino acids and proteins (von Brand, 1973). This has been investigated in *Ancylostoma caninum* in which the relevant enzyme, GLDH has also been reported by Perez-Gimenez *et al.* (1967).

According to Rasero *et al.* (1968) the presence of GLDH, glutamate decarboxylase and aminotransferase and the excretion
of 4-aminobutyrate in *A. lumbricoides* and *Moniezia expansa* under anaerobiosis, suggests that a 4-aminobutyrate bypass which exists in the mammalian brain may also occur in helminths. However, Kerkut (1976) has also suggested that glutamate (a dicarboxylic amino acid) may function as the excitatory transmitter at the nerve-muscular junctions of crustaceans, insects and molluscs.

McCivan and Chappell (1975) have not only reported high activity of this enzyme from rat liver tissue but they have also inferred a possible physiological role of GLDH. According to these investigators (loc. cit.) this enzyme is involved mainly in glutamate synthesis, which is the first reaction on the pathway to glutamine synthesis that ultimately helps in nitrogen storage. However, according to Barrett (1981) the activity of GLDH in pig liver mitochondria was approximately 50 times higher than in muscle mitochondria and inspite of the fact that GLDH is widely distributed in helminths, its regulatory properties have not been studied in detail so far.

Mere estimation of an enzyme activity does not suffice to understand their molecular behaviour unless some kinetic parameters are also studied. Therefore it is necessary to know the optimum substrate concentration, pH maxima, effect of various stimulators as well as inhibitors which influence the enzyme activity and also help to understand the nature of the enzyme molecules.
In *Hartmanella culbertsoni*, the Michaelis constant (Km) value for the substrate of GLDH catalysed reaction has been reported as $2.6 \times 10^{-4}$ M by Rao and Hussain (1979), while in *H. diminuta* as $0.20$ mM for $\alpha$-ketoglutarate (Mustafa *et al.*, 1978).

The optimum pH for the activity of this enzyme from human serum has been reported to be 8.0 (Schmidt, 1965). Rao and Hussain (1979) using triethanolamine (TRA) buffer and phosphate buffer, found higher activity of GLDH at pH 8.0 in *H. culbertsoni* but relatively the activity was higher in TRA-buffer than phosphate buffer. Mustafa *et al.* (1978) have reported highest activity at pH 7.4 in $0.05$ M tris-maleate buffer for $\alpha$-ketoglutarate ($\alpha$-KGA) at $1.0$ mM concentration. These investigators have further suggested that the pH of the assay mixture substantially modifies $\alpha$-ketoglutarate binding. In fact, decrease in the pH of assay mixture from 7.4 to 6.5 lowered the apparent Km value for $\alpha$-KGA almost ten folds. Values at pH 6.5 were atleast 90 times higher for NADH oxidation than NAD reduction.

The GLDH is a pyridine linked enzyme, therefore, it utilises either NAD or NADP as coenzymes. The NADP specific GLDH (E.C. 1.4.1.4) has been reported from *P. lophurae* by Sherman *et al.* (1971), from *P. chabaudi* by Walter *et al.* (1974). The NAD-linked GLDH has been reported from *Trypanosoma cruzi* by
Cazzulo et al. (1979). According to Rao and Husain (1979), GLDH of *H. culbertsoni* oxidised NADH and not NADPH, while in a sea anemone, *Metridium senile*, the GLDH was found to be specific for NADP (Stephen et al., 1978). Mustafa et al. (1978) have reported the NAD/NADH dependent GLDH activity in *H. diminuta*. Langer (1972) and Barrett and Beis (1973) have reported NAD-specific enzyme from *A. suum* and *A. lumbricoides* respectively. The NAD-specific GLDH has also been found in *H. contortus* (Rhodes and Ferguson, 1973) and in *Dirofilaria immitis* (McNeil and Hutchinson, 1971).

Besides coenzyme specificity, the nucleotides also play an important role in protein metabolism. The pyrimidine as well as purine nucleotides influence the catalytic activity of this enzyme. The nucleotides participate in nearly all biochemical processes. They are activated precursors of DNA and RNA. Nucleotides are metabolic regulators (Stryer, 1975) and therefore, GLDH being an allosteric enzyme is markedly influenced by allosteric modifiers, viz: AMP, ADP, ATP, GMP, UMP and GTP etc. Of the various nucleotides tested at 1.6 x 10^{-3} M concentration, only AMP produced 400 percent activation of GLDH which was partially purified from *H. culbertsoni* (Rao and Husain, 1979). According to Mustafa et al. (1978), the GLDH of *H. diminuta* was not affected by 1 mM AMP, ADP, ATP, IDP, GDP or GTP. The nucleotide concentrations appeared to be physiologically significant as Barrett and Beis
(1973) have calculated nucleotide concentrations for *H. diminuta* to be in the order of 0.29 - 1.57 μ moles/g fresh weight. However, Barrett (1981) has pointed out that the mammalian GLDH is activated by ADP and GDP while it is inhibited by ATP and GTP. Rhodes and Ferguson (1973) have found that GLDH in *H. contortus* was not activated by either AMP or ATP, although GLDH plays an important role in regulating protein catabolism and anabolism of nematodes.

Besides metabolic activators or inhibitors, the ionic concentrations and different osmolarities also influence the activity of many dehydrogenases, specifically of LDH and GLDH of some invertebrates (Schoffeniels, 1964). The osmotic pressure of the environment determines the amount of osmotic work that an organism has to do. During its life cycle, a helminth parasite has to pass through a wide range of osmotic pressures, from mammalian tissue to the tissues of fresh water or marine invertebrates. The effect of changing osmotic pressures and ionic concentrations have never been studied earlier in relation to the enzyme activities. However, a number of investigators have used weight changes and other parameters as an index of osmotic and ionic activity of parasites. The regulation of osmotic and ionic balance in relation to weight alterations in *F. gigantica* and other digenetic trematodes has been extensively studied by Siddiqi and Lutz (1966) and Siddiqi *et al.* (1975), whereas Knox and
Pantelouris (1966) have studied the osmotic behaviour of *E. hepatica* in modified Hedon-Fleig media. The osmo-regulation studies have also been made on the trematode, *Haematoloechus medioplexus* by Bair and Peters (1971).

However, studies in relation to the effect of osmotic and ionic stress on the activity of GLDH have been investigated on crustaceans, but no report is available from the helminth parasites. The GLDH extracted from the tissues of spiny lobster (*Panulirus vulgaris*), cray fish (*Astacus fluviatilis*) and lobster (*Homarus vulgaris*) is dependent on the ionic composition of the incubating medium (Schoffeniels and Gilles, 1963; Schoffeniels, 1964 a,b, 1965, 1966) and further observed that there is an inverse relationship as far as the salt effect is concerned. In the case of a euryhaline species, the activity of GLDH is maximal in concentrated media, while that of the other dehydrogenases (LDH etc.) is minimal. If the animal goes into a diluted medium the GLDH activity decreases (Florkin and Schoffeniels, 1969). Thus the synthesis of amino acids should decrease while the oxygen consumption and ammonia excretion should increase. This is indeed observed with most of the euryhaline species by Florkin and Schoffeniels (1969), Schoffeniels (1966). According to Gilles and Schoffeniels (1965), addition of 10 mM ammonium chloride to the incubating media of the surviving isolated tissue, increases the intra-cellular pool of free amino acids. Therefore,
it is evident from the fact that osmotic and ionic concentrations influence the metabolism through altering the enzyme activity.

In many invertebrates and possibly in helminths as well, amino acids are involved in the regulation of intra-cellular osmotic pressure. The disruption of the osmotic balance when helminths are removed from their hosts and when incubated in vitro results into an increased amino acid leakage. _H. diminuta_, which had previously absorbed labelled methionine, lost the compound slowly when incubated subsequently in saline, but very rapidly when incubated in saline containing amino acids (Hopkins and Callow, 1965; von Brand, 1973).

In free living invertebrates, free amino acids are important regulators of intra-cellular osmotic pressure. According to Kasschau (1975) proline is an osmotic effector in the larval stages of _Himasthla quissetensia_. Amino acid transport may also be involved in osmoregulation as observed in _H. diminuta_ (Lusier et al., 1978). The other osmotically important amino acids are: glycine, arginine, alanine, glutamine and glutamic acid (Florkin and Schoffeniels, 1969). Proline, an endproduct of nitrogen metabolism in _F. hepatica_, is released by the worm in large amounts (20-50 μ moles/day/worm). The excess proline excretion has at least three pathophysiological effects on the host, viz: bile duct hyperplasia, depressed
growth rate and weight gain and anaemia (Isseroff, 1980).
The release of large amounts of free proline has also been reported in Schistosoma sp. by Senft (1963) whereas Lutz and Siddiqi (1971) have reported unequal distribution of ninhydrin positive substances like proline, alanine, histidine, phenylalanine, serine and lysine in the protonephridial fluid of F. gigantica. The most common ones were proline, alanine and histidine.

The proline excretion has been found to stimulate collagen synthesis which may be related to fibrosis. Therefore, proline release may be considered as a strategy by which the parasites improve their definitive habitat.

However, McGivan and Chappell (1975) have found that rat liver GLDH is stimulated by leucine, isoleucine and methionine, while Mustafa et al. (1978) reported that these amino acids have no effect on the enzyme isolated from H. diminuta. Various metabolites are also known to influence the activity of GLDH. The glycolytic intermediates at 1 mM concentration were without any effect on the GLDH activity of H. diminuta. The TCA cycle intermediates inhibited the enzyme activity at relatively higher concentrations (10 mM malate and fumarate, 50 mM succinate). This concentration was necessary to achieve 50% inhibition, hence, their physiological significance is questionable (Mustafa et al., 1978).
Glutamate dehydrogenase isolated from different sources are affected by an almost bewildering array of compounds. But hardly any report is available on the inhibitory or stimulatory effect of various chemical agents on the activity of GLDH in amphi stomes, although reports are available on other parasites. A number of compounds viz: copper sulphate, mercuric chloride, zinc sulphate, sodium cyanide and hydrazine sulphate appreciably influence the activity of GLDH in *H. culbertsoni* (Rao and Husain, 1979). The cytoplasmic protein synthesis of trypanosomes is inhibited by cycloheximide. This metabolic inhibitor inhibits the protein synthesis in cell free extracts of intra-erythrocytic stages of malarial parasite *P. knowlesi*, but it has not been known that what enzymes of protein metabolism are sensitive to this compound by which blocking of the metabolic processes occur in parasites (Gutteridge and Coombs, 1977).

Previous studies reveal that the catalytic activity of GLDH differ in different organs and tissues, showing their relative variations in metabolic activities. This fact may very well be demonstrated by using histochemical techniques, localizing the differential distribution of enzyme in parasites and by enzyme polymorphism studies through electrophoresis. No report is available on histochemical localization and isoenzyme study of GLDH of amphi stome parasites. Thuman et al. (1965) found upto seven bands of activity in the radicles,
shoots and cotyledons of the broad bean (*Vicia faba*) and pea (*Pisum sativum*), while Grimes and Fottrell (1966) described three bands in the root nodules of *P. sativum*. According to Wilkinson (1970), it is uncertain as to how far these should be regarded as isoenzymes, and it may be possible that the various forms are separate adaptive enzymes produced in response to changes in environment. Florkin and Schoffeniels (1969) have reported that the GLDH extracted from muscle, does not behave exactly like the enzyme extracted from the gills of *H. vulgaris*. They further elaborated that cationic specificity as well as the anionic efficiency are different according to the origin of enzyme. This corresponds to the differences in the structure of the enzyme protein and offers a new example of isoenzymes. Van der Helm (1962) has resolved GLDH from human tissue by agar-gel electrophoresis into five separate isoenzyme which are detected by tetrazolium staining technique.

The metabolic changes during the life cycle of a parasite may include alterations in the intra-cellular distribution of enzymes, the example is of *A. lumbricoides* whose different developmental stages show enzyme polymorphism. In other parasites also, the enzymes of the free-living phase may be adapted for the parasitic stages and converse happens when free living stages are shed from the host. Barrett (1981) has
pointed out that the different properties of the separate isoenzymes are masked, when using the whole homogenates or whole animal extracts, as is often the case with helminths.

From the foregoing survey of literature the importance of protein metabolism and the functions of its key enzyme like GLDH, may easily be realised and therefore, the present study was designed considering the above fact. In Aligarh, buffaloes slaughtered at the local abattoir have been found to harbour enormous number of trematodes, particularly amphistomes. The two amphistomes viz: Gigantocotyle explanatum (Näsmark, 1937) and Gastrothylax crumenifer (Creplin, 1847), infecting two different organs viz: bile duct and rumen respectively, of the same host Bubalus bubalis, were chosen.

The present study has been carried out in a moderately equipped laboratory where facilities available to the author were limited. Therefore, it was decided to investigate the kinetic behaviour, effect of certain inhibitors and stimulators, histochemical localisation and enzyme polymorphism of GLDH of both the parasites. The purification of enzyme was not possible due to the lack of cold room facilities and ultracentrifuge.

In helminths, no generalisation can be made on the basis of results obtained with one member of the group, even though they may be related to each other morphologically or taxonomically, thus it is necessary to study each member separately
in order to see the influence of microenvironment on the helminth physiology. It is interesting to find that *G. explanatum* is found in bile duct where environment is more or less constant. Whereas *G. crumenifer* inhabits rumen which is rich in CO₂ concentration and free ammonia, and also the parasite experiences more or less continuously changing osmotic and ionic balance due to dietary habits of the host.

The ultimate aim of any physiological or biochemical study is to provide sufficient knowledge of parasite metabolism that would ultimately help in designing better and more effective drugs as well as specific drug formulations which might help in eradicating the parasite infections.
CHAPTER III
MATERIALS AND METHODS

A. Collection of parasites:

Gigantocotyle explanatum and Gastrothylax crumenifer were collected from the bile duct and rumen respectively, of the freshly slaughtered buffaloes (Bubalus bubalis) at the local abattoir.

The worms attached to the host tissue were expeditiously brought to the laboratory in thermosflasks and they were immediately transferred to the Hank's medium containing 136.89 mM NaCl, 5.36 mM KCl, 1.66 mM CaCl₂ (dehydrated), 0.81 mM MgSO₄, 0.33 mM anhydrous Na₂HPO₄, 0.44 mM KH₂PO₄ and 2.61 mM NaHCO₃ maintained at 37 ± 1°C. This medium was used without glucose. The worms were rinsed gently in the Hank's medium thrice and immediately after, carefully blotted on Whatman filter paper and frozen at -15°C or freshly used for homogenate preparation.

B. Homogenate preparation:

Homogenates of different concentrations were prepared as per requirements.

(i) For enzyme assay:

Freshly blotted or frozen 1 gm worms were homogenized in
Potter-Elvehjrn glass tissue homogenizer in 10 volumes of 70 mM phosphate buffer saline, pH - 7.4, containing 0.25 M sucrose, and left to stand for an hour at 0-4°C. Since the enzyme activity was assayed in crude homogenate therefore care was taken that maximum enzyme is released in order to obtain maximum possible activity in the homogenate. The homogenate was centrifuged at 6000 r.p.m. for fifteen minutes at 0-4°C, in a cold rotar head of centrifuge (Remi: R-24). The supernatant fluid was gently collected with the help of a pipette and used for enzymatic assay. Where ever it was found necessary, the enzyme preparation was diluted and the dilution factor was taken into account in calculations.

(ii) For electrophoretic study:

The fresh adult flukes of Gigantocotyle explanatum, Gastrothylax crumenifer, and host liver and rumen tissue, each weighing 2.5 gm, were homogenized separately in 5.0 ml of ice cold double distilled water in Potter-Elvehjrn glass teflon tissue grinder, maintained at 0-4°C, and left to stand for an hour so that maximum enzyme is released from the cytosol as well as mitochondrial particles. Before centrifugation at 10,000 r.p.m. for 15 minutes at 0-4°C, 5 ml of 70 mM phosphate buffer saline containing 0.5 M sucrose was added. The supernatant, thus obtained, was used for electrophoretic mobility of the isoenzymes of GLDH.
C. GLDH assay:

(i) Normal activity of the enzyme:

GLDH activity was assayed spectrophotometrically at 340 nm, using the method of Schmidt (1974) in the direction of glutamate formation. The principle of the reaction is based on the oxidation of NADH which leads to decrease in extinction at 340 nm, on Spectronic 21D (Bausch and Lomb, USA). All the assay experiments were carried out in a moderately equipped lab., at room temperature. The standard assay mixture of total volume 3 ml, in a quartz cell of 1 cm pathlength contained: 0.2 mM/ml NADH, 50 mM/ml triethanol-amine buffer (pH 8.0), 2.5 mM/ml EDTA, 100 mM/ml ammonium acetate, 2 U LDH, 7 mM/ml 2-oxoglutarate, and enzyme preparation of both the amphistomes. The enzyme activity was assayed for ten minutes and a change in extinction has been recorded at 1 minute interval.

(ii) Effect of substrate concentration:

For the kinetic study different (2-oxoglutarate) substrate concentrations ranging from: 0.5 mM, 1.0 mM, 2.0 mM, 4.0 mM, 6.0 mM, 6.5 mM, 7.0 mM, 7.5 mM, 8.0 mM and 10 mM were prepared. Michaelis constant values for -KGA has been determined in both the parasites, using double reciprocal plots of Lineweaver-Burk (1934).
(iii) Effect of pH:

To study the influence of pH on the activity of GLDH, following buffers (70 mM conc.) of different pH range were used:

- **a. Phosphate buffer**  
  pH 6.0 - 8.0
- **b. Triethanolamine**  
  pH 7.0 - 8.5
- **c. Glycine-NaOH**  
  pH 8.0 - 9.5
- **d. Phosphate-NaOH**  
  pH 9.5 - 10.0

The enzyme activity, thus obtained was plotted against different pH. All the pH were adjusted using a Systronics digital pH meter.

(iv) Effect of osmotic and ionic stress:

The effect of osmotic and ionic stress on the activity of GLDH was studied after incubating the worms in Tyrode saline of different ionic concentrations and osmolarities. One gramme parasites were weighed on a single pan electrical balance and then transferred to 50 ml beaker, each one containing: 0% (double distilled water), 25%, 50%, 75%, 100% (normal), 150% and 200% Tyrode, maintained at 37 ± 2°C in a metabolic shaker. 100% Tyrode containing 136 mM NaCl, 2.6 mM KCl, 1.8 mM CaCl₂, 1.1 mM NaHCO₃, 0.32 mM NaH₂PO₄, 0.9 mM MgCl₂ was considered as isotonic saline. The osmolarity of different concentrations of Tyrode salines were determined by using Osmomat-030. The
instrument was calibrated by using double distilled water. The incubation of worms were carried out for a fixed period of two hours, and immediately after, worms were removed, and homogenized for the assay of enzyme activity as described earlier.

(v) Effect of amino acids:

A number of amino acids have been found to influence the intra-cellular osmotic balance. Therefore, various amino-acids were chosen for the present study to investigate whether they have some influence on the activity of GLODH. The following amino acids at 1 mM concentration each, were added to the assay mixture:

1. L-leucine
2. DL-alanine
3. L-proline
4. L-arginine
5. L-glycine
6. L-glutamine

(vi) Effect of nucleotides:

Various nucleotides act either as allosteric activator or inhibitor of enzyme activity. The effect of following nucleotides at 1 mM concentration each, was studied by adding to the assay mixture in order to find out their effect on
GLDH activity.

1. Adenosine 5'-monophosphate (AMP).
2. Adenosine 5'-diphosphate (ADP).
3. Adenosine 5'-triphosphate (ATP).
4. Guanosine 5'-monophosphate (GMP).
5. Cytidine 5'-monophosphate (CMP).
6. Uridine 5'-monophosphate (UMP).

(vii) Effect of chemicals:

Enzyme molecules respond differently to different chemicals. Some chemical agents cause reversible while others result in irreversible inhibition of enzyme activity, still others may behave as specific inhibitors. A number of inhibitors were selected to see their effect on the GLDH activity and the following were used in the present study.

<table>
<thead>
<tr>
<th>Concentrations - M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cycloheximide</td>
</tr>
<tr>
<td>2. Diethyldithiocarbamate (DDC)</td>
</tr>
<tr>
<td>3. Semicarbazide hydrochloride</td>
</tr>
<tr>
<td>4. Iodoacetate</td>
</tr>
<tr>
<td>5. Mercuric chloride</td>
</tr>
<tr>
<td>6. Potassium cyanide</td>
</tr>
<tr>
<td>7. Ammonium sulphate</td>
</tr>
<tr>
<td>8. Sodium sulphate</td>
</tr>
<tr>
<td>9. Copper sulphate</td>
</tr>
</tbody>
</table>
D. **Histo-enzymological study:**

In order to demonstrate the differential distribution of GLDH in various regions of the parasites, *G. explanatum* and *G. crumenifer* were cryo-stretched and embedded in Tissue-Tek II (Lab-Tek Products, USA). The worms were sliced at 10-12 μm on a cryostat (American Optical Corporation, USA) at -25°C. The frozen sections were taken on clean glass slides which were immediately transferred to coupling jars containing test medium preincubated at 37 ± 1°C. Controls were also run simultaneously and prepared either by omitting the substrate from the medium or by treating the sections at 70°C. The test medium contained:

1. NAD⁺ 60 mgm.
2. Nitro-BT 30 mgm.
3. PMS 2 mgm.
4. 0.5 M phosphate buffer (pH 7.0) 25 ml.
5. 0.5 mM Na-glutamate (pH 7.0) 5 ml.
6. Distilled water 70 ml.

E. **Polyacrylamide disc gel electrophoresis:**

Polyacrylamide gel electrophoresis was carried out according to the method of Davis (1964). The small pore gel contained 7% acrylamide and N,N-methylene-bis-acrylamide. The gels were loaded with about 50 ulitre aliquotes of the
homogenate containing 50–80 µg of proteins. Electrophoresis was conducted at 4°C in Shandon Electrophoresis assembly, using 0.1 M tris-HCl, pH 7.4, as bridge buffer. A current of 3 mA/gel column (6 x 75 mm.) was maintained until the tracking dye (0.05% bromophenol blue) had moved about 60 mm in the gel. This usually takes about two and a half hour.

Following electrophoresis, the gels were immediately removed from the glass tubes and a black nylon bristle was carefully inserted in the middle of the bromophenol blue band to mark the tracking dye, which usually disappears during staining process. The gels were placed in the test tubes containing the test media as described for histoenzymological study, for 30 minutes at 37 ± 1°C. Controls were incubated without the substrate. After incubation gels were rinsed in distilled water and stored in 7% acetic acid. The gels with their cathodal end at the top were photographed under transillumination.

Densitometry:

For the quantitative estimation of the isoenzymes, gels were scanned in the horizontal position on a Systronics Densitometer. The unstained portion of the gel below the marker was used as a blank to set the instrument for 100% transmission. The isoenzymes were numbered as described by Webb (1964).
Enzyme unit:

An enzyme unit is defined as that amount which oxidised one μmole of NADH per minute under the experimental conditions. An extinction coefficient value of 6.22 at 340 nm for NADH was employed for the calculation of the amount of NADH oxidised. Specific activity is expressed as the number of enzyme units per mg. protein per hr.

Protein estimation:

Proteins of the parasites and host tissues were estimated according to the method of Lowry et al. (1951) using bovine serum albumin as standard.
CHAPTER IV
RESULTS

A. Normal enzyme activity:

The normal values of GLDH estimated in G. explanatum, liver; G. crumenifer and rumen tissue were found to be: 1.906 ± 0.024, 0.813 ± 0.031, 0.586 ± 0.006 and 0.600 ± 0.018 µmole/mg-protein/hour, respectively. Therefore the highest enzyme level has been found in G. explanatum and the liver tissue, Table - 1.

B. Kinetic studies:

The results of the kinetic parameters reveal that the enzyme GLDH of the two parasites differ markedly from one another. In G. explanatum, the apparent Km value for \( \alpha \)-KGA was found to be 2.12 mM while the Vmax was 9.09. However, in G. crumenifer the Km value for \( \alpha \)-KGA was 4.16 mM and Vmax was 1.90. It is interesting to find that the Km value in G. crumenifer is much higher as compared to G. explanatum. Whereas the value of Vmax/Km in G. crumenifer is quite low (0.46) as compared to G. explanatum (4.28) as given in Table - II. The double-reciprocal plots of activity Vs \( \alpha \)-ketoglutarate concentration exhibited a hyperbolic (Michaelis-Menton) kinetics in both the parasites (Fig. 3, 4).
**TABLE - I**

Normal activity of GLDH in parasites and host tissues.

<table>
<thead>
<tr>
<th>PARASITES/HOST TISSUE</th>
<th>SPECIFIC ACTIVITY*</th>
<th>μmoles NADH oxidised/mg. protein/hr. ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gigantocotyle explanatum</td>
<td>1.906 ± 0.024</td>
<td>(5)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.810 ± 0.031</td>
<td>(4)</td>
</tr>
<tr>
<td>Gastrothylax crumenifer</td>
<td>0.586 ± 0.008</td>
<td>(4)</td>
</tr>
<tr>
<td>Rumen</td>
<td>0.600 ± 0.018</td>
<td>(3)</td>
</tr>
</tbody>
</table>

*The enzyme activity was determined with 7 mM α-KGA at pH 8.0 for 10 minutes.

Figures in parentheses show number of replicates.
**TABLE - II**

Kinetic parameters of GLDH from trematodes of *Bubalus bubalis*.

<table>
<thead>
<tr>
<th>PARASITES</th>
<th>Vmax *</th>
<th>Km * (mM)</th>
<th>Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gigantocotyle explanatum</em></td>
<td>9.09</td>
<td>2.12</td>
<td>4.28</td>
</tr>
<tr>
<td><em>Gastrothylax crumenifer</em></td>
<td>1.90</td>
<td>4.16</td>
<td>0.46</td>
</tr>
</tbody>
</table>

*Values were estimated from Lineweaver-Burk double reciprocal plots of saturation curves.*
Fig. 3 (A). Enzyme activity as a function of substrate concentration.

(B). Double reciprocal plot of Lineweaver-Burk in G. expleanatum.
Fig. 4 (A). Enzyme activity as a function of substrate concentration.

(B). Double reciprocal plot of Lineweaver-Burk in G. crumenifer.
C. **Effect of pH:**

The enzyme reactions are highly pH dependent, therefore GLDH activity of both the amphistomes was assayed in buffers of different pH range in order to determine the pH optima.

The results presented in the Table - III and Fig. 5, clearly explain that maximum activity of GLDH in both, *G. explanatum* as well as in *G. crumenifer*, was found to be at pH 8.0. Triethanol amine hydrochloride buffer (pH 8.0), was found to be the most suitable for enzyme activity.

D. **Effect of nucleotides:**

Nucleotides are the allosteric modulators which also influence the GLDH activity in both the trematodes understudy.

The results of the present investigations are summarized in Fig. 6, which shows that the enzyme of the two amphistomes respond differently to different nucleotides. The ADP caused slight enhancement, about 2.8 percent, of the enzyme activity in *G. explanatum* whereas ATP was responsible for 98.1 percent inhibition in the enzyme activity of the liver parasite. In the presence of other nucleotides, the GLDH activity was found to be at a lower level as compared to the normal. According to their degree of inhibition, the various nucleotides were in the order of:

\[ \text{AMP} < \text{UMP} < \text{GMP} < \text{CMP} < \text{ATP} \]
**TABLE - III**

Effect of pH on the GLDH activity.

<table>
<thead>
<tr>
<th>PARASITES</th>
<th>pH</th>
<th>SPECIFIC ACTIVITY*</th>
<th>µmoles/NADH oxidised/mg.-protein/hr. ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gigantocotyle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>explanatum</td>
<td>6.0</td>
<td>0.590 ± 0.039</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>0.711 ± 0.010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>1.045 ± 0.103</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>1.069 ± 0.081</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>1.313 ± 0.045</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>0.958 ± 0.066</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>0.945 ± 0.113</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>0.790 ± 0.216</td>
<td></td>
</tr>
<tr>
<td>Gastrothylax</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crumenifer</td>
<td>6.0</td>
<td>0.071 ± 0.034</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>0.085 ± 0.026</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>0.320 ± 0.063</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>0.450 ± 0.112</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.536 ± 0.131</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>0.442 ± 0.098</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>0.388 ± 0.164</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>0.241 ± 0.079</td>
<td></td>
</tr>
</tbody>
</table>

*Five replicates were assayed to determine the specific activity.*
The GLDH of *G. crumenifer* was activated by AMP at 1.0 mM conc. and the activity was enhanced by about two folds. The pyrimidine nucleotides, CMP and UMP, also caused slight stimulation whereas ADP, ATP and GMP inhibited the enzyme but not to a significant level. The relative influence of these nucleotides on the GLDH activity was found in this order:

$$AMP > CMP > UMP > ATP > GMP > ADP$$

E. Effect of osmotic and ionic stress:

In the present study, the two amphistomes were subjected to the osmotic and ionic stresses (hypotonic and hypertonic salines) in order to see the influence on the GLDH activity of *G. explanatum* and *G. crumenifer*. The enzyme activity obtained in the isotonic saline (100% Tyrode) was considered as normal or control. It has been found that the enzyme level in the two parasites after two hours of incubation in normal saline show decreased activity as compared to the fresh parasites.

*G. explanatum* was found to be very sensitive to zero percent saline (double distilled water). These worms were water logged and died within an hour after incubation. The enzyme activity determined thereafter, was found to be enhanced by 56 percent, whereas in *G. crumenifer* no water logging was observed and the parasites survived even after two hours of
Fig. 7. Effect of osmotic and ionic stress on the GLDH of *G. explanatum* and *G. crumenifer*. 
incubation, but the enzyme activity decreased slightly. In *G. explanatum*, the hypotonic saline decreased in the level of GLDH activity, whereas in *G. crumenifer* the catalytic activity of GLDH was not influenced significantly, Fig. 7. It has been found that the GLDH of liver trematode is also sensitive to hypertonic salines in which the catalytic activity increases, while the rumen trematode shows slightly increased activity only in 200 percent saline. Therefore, it is evident from the present results that the hypo- as well as hypertonicities cause a change in the GLDH activity of *G. explanatum*, inhabiting liver, which maintains a more or less constant environment. Whereas in *G. crumenifer* the enzyme activity fluctuates within a narrow range, thereby indicating that the GLDH of this parasite is adapted to changes in the ionic concentrations and osmotic pressures of the incubating media.

F. Effect of amino acids:

Certain amino acids have been found to be osmotically important. It was decided to investigate the effect of amino acids on GLDH activity which is also influenced by the osmotic and ionic changes in the incubating media. Among the various amino acids, leucine, alanine, proline, arginine, glycine and glutamine; only leucine (1 mM) increases the catalytic activity of GLDH to 3.69 μmoles/mg-protein/hr. in *G. explanatum*. Thus, it is observed that the enzyme activity is increased about
Fig. 8. Effect of amino acids on the GLDH of *G. explanatum* and *G. crumenifer*.

LEU = Leucine, ALA = Alanine, PRL = Proline,
ARG = Arginine, GLY = Glycine, GLU = Glutamine.
two to three folds. The other amino acids, in 1 mM concentration, do not alter the enzyme activity significantly, Fig. 8. In *G. crumenifer* the effect of amino acids, glutamine, glycine, arginine (1 mM), hardly produced any change in the enzyme activity, but leucine, alanine and proline cause 187.7 percent, 170.6 percent and 143.3 percent increase in GLDH activity, respectively.

G. Effect of inhibitors:

The results of the present study have been presented in Table IV-V. A number of chemical agents like iodoacetate, semicarbazide hydrochloride, copper sulphate and mercuric chloride are well known for their catabolic inhibitory effects in a variety of organisms.

In *G. explanatum*, the GLDH was completely inhibited by iodoacetate (1 mM), semicarbazide hydrochloride (1 mM) and copper sulphate (1 × 10⁻² M). Mercuric chloride inhibited the enzyme activity by 99.16 percent, Table - IV. Cycloheximide (1 × 10⁻³ M), a known protein inhibitor did not inhibit the enzyme activity appreciably. Diethylidithiocarbamate (1 × 10⁻³ M) inhibited 36% enzyme activity. Sodium sulphate (1 × 10⁻² M) and ammonium sulphate (1 × 10⁻² M) also caused pronounced inhibition in the GLDH activity by 37.6% and 17.68% respectively. Potassium cyanide (1 × 10⁻² M) was also found to inhibit the enzyme activity by 40.55 percent (Table - IV).
### TABLE - IV

Effect of inhibitors on the activity of cLDH in *G. explanatum*.

<table>
<thead>
<tr>
<th>INHIBITORS</th>
<th>SPECIFIC ACTIVITY (μmoles/mg-protein/hr. ± SEM)</th>
<th>PERCENT INHIBITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cycloheximide</td>
<td>1.866 ± 0.318 (4)</td>
<td>- 002.10</td>
</tr>
<tr>
<td>2. Iodoacetate</td>
<td>---</td>
<td>- 100.00</td>
</tr>
<tr>
<td>3. Diethyldithiocarbamate (DDC)</td>
<td>1.217 ± 0.163 (4)</td>
<td>- 36.02</td>
</tr>
<tr>
<td>4. Potassium cyanide</td>
<td>0.773 ± 0.112 (4)</td>
<td>- 40.55</td>
</tr>
<tr>
<td>5. Semicarbazide hydrochloride</td>
<td>---</td>
<td>- 100.00</td>
</tr>
<tr>
<td>6. Ammonium sulphate</td>
<td>1.569 ± 0.098 (4)</td>
<td>- 17.68</td>
</tr>
<tr>
<td>7. Sodium sulphate</td>
<td>1.189 ± 0.201 (4)</td>
<td>- 37.62</td>
</tr>
<tr>
<td>8. Mercuric chloride</td>
<td>0.016 ± 0.003 (4)</td>
<td>- 99.16</td>
</tr>
<tr>
<td>9. Copper sulphate</td>
<td>---</td>
<td>- 100.00</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the number of replicates.
TABLE - V

Effect of inhibitors on the activity of GLDH in *G. crumenifer*.

<table>
<thead>
<tr>
<th>INHIBITORS</th>
<th>SPECIFIC ACTIVITY μmoles/mg-protein/hr. ± SEM</th>
<th>PERCENT INHIBITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cycloheximide</td>
<td>0.350 ± 0.111 (4)</td>
<td>- 06.15</td>
</tr>
<tr>
<td>2. Iodoacetate</td>
<td>0.014 ± 0.009 (4)</td>
<td>- 97.60</td>
</tr>
<tr>
<td>3. Diethyldithiocarbamate (DDC)</td>
<td>0.326 ± 0.039 (4)</td>
<td>- 54.40</td>
</tr>
<tr>
<td>4. Potassium cyanide</td>
<td>0.225 ± 0.102 (4)</td>
<td>- 61.61</td>
</tr>
<tr>
<td>5. Semicarbazide hydrochloride</td>
<td>0.014 ± 0.10 (3)</td>
<td>- 57.61</td>
</tr>
<tr>
<td>6. Ammonium sulphate</td>
<td>0.350 ± 0.379 (4)</td>
<td>- 40.27</td>
</tr>
<tr>
<td>7. Sodium sulphate</td>
<td>0.536 ± 0.137 (4)</td>
<td>- 08.53</td>
</tr>
<tr>
<td>8. Mercuric chloride</td>
<td>---</td>
<td>100.00</td>
</tr>
<tr>
<td>9. Copper sulphate</td>
<td>---</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the number of replicates.
The same concentration (1 mM) of iodoacetate which inhibited the enzyme activity completely in *G. explanatum*, caused 97.6 percent inhibition in GLDH of *G. crumenifer*. Whereas mercuric chloride (1 x 10^{-3} M) and copper sulphate (1 x 10^{-2} M) inhibited the enzyme completely (Table - V). Addition of semicarbazide hydrochloride (1 x 10^{-3} M) to the assay mixture stopped the enzyme activity by 97.61 percent.

It was found to be interesting that the sulphhydryl reagent, diethyldithiocarbamate (1 x 10^{-3} M) caused only 54.4 percent loss in enzyme activity. Cycloheximide (1 x 10^{-3} M) and sodium sulphate (1 x 10^{-2} M) caused least inhibition, 6.15 percent and 8.53 percent respectively, whereas the inhibition due to ammonium sulphate was only 40.0 percent. Potassium cyanide also inhibited 61.0 percent of the enzyme activity (Table - V).

From this study it is evident that the enzyme molecules respond differently to different chemical agents and also the similar anions of different salts produce varying degree of inhibition in the enzyme activity.

H. Histochemical distribution of GLDH:

Histochemical distribution of this enzyme was studied in different regions of the trematodes under study. The formazan deposits have been found in different concentrations
**TABLE - VI**

Histochemical localisation of GLDH.

<table>
<thead>
<tr>
<th>ORGANS</th>
<th><strong>G. explanatum</strong></th>
<th><strong>G. crumenifer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tegument</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Sub-tegument</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Doral sucker</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Intestinal caeca</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Vitellaria</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Ventral pouch a)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>b) Outer lining</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Testes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ovaries</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Uterine tube</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Egg-shell</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Acetabulum</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

+ : Low;  ++: Moderate;  +++: Intense;  - : Negative.
in different regions of the parasites showing the differential activity of this enzyme. The results of this study have been presented in Plate - 1; Fig. 1-4; Plate - 2; Fig. 1-4; and Table - VI.

In *G. explanatum*, maximum enzyme activity was found in vitellaria followed by oral sucker and egg-shell. In other regions the enzyme activity was moderately present except in the intestinal caeca where formazan deposits were totally absent. In ventral sucker, the stain was only present in the outer acetabular lining showing weak activity of GLDH in this region (Plate - 1: Fig. 1-4).

The enzyme in *G. crumenifer* exhibited strong activity in vitellaria, intestinal caeca, egg-shell, oral sucker and sub-tegumental region, whereas the enzyme activity is weak in posterior sucker and uterine tube. Only the inner lining of the acetabulum shows moderate localization of the GLDH (Plate - 2: Fig. 1-4).

I. Electrophoretic mobility of GLDH:

Variation in the electrophoretic mobility of various isoenzymes of GLDH have been found in both, the parasites as well as the host tissues (Fig. 9: a-d). The scanning of poly-acrylamide gels reveal that the GLDH of *G. explanatum* clearly shows 6 components of varying mobility whereas in *G. crumenifer*
Plate 1. Histochemical localisation of GLDH in *G. explanatum* (Fig. 1-4).

Os. = Oral sucker, Gp. = Gonopore, Tg. = Tegument,
Ut. = Uterus, Vit. = Vitellaria, Ts. = Testis,
Ic. = Intestinal caeca, Es. = Egg-shell,
Plate 2. Histochemical localisation of GLDH in
G. crumenifer (Fig. 1-4).

Os. = Oral sucker, Tg. = Tegument, S.Tg. = Sub-
tegment, Pa. = Parenchyma, Es. = Egg-shell,
Ut. = Uterine tube, Ov. = Ovary, Ic. = Intestinal
cæcae, Vit. = Vitellaria, Ts. = Testis,
Vs. = Ventral sucker, Al. = Acetabular lining,
there were only 5 bands. The rumen and liver GLDH was resolved in five components each with varying degree of mobility and stain intensity. Thus the phenomenon of polymorphism is exhibited by the GLDH of both the parasites as well as their host tissues under study.
Fig. 9. Electrophoretic mobility of GLDH of *G. explanatum* (A), liver (B), *G. crumenifer* (C) and *numen* (D).
CHAPTER V
DISCUSSION

The results of the present study reveal that the glutamate dehydrogenase is present in both the amphistome species under study and also catalyze the reductive amination of \( \alpha \)-ketoglutarate (\( \alpha \)-KGA) actively in the presence of ammonium ions and NADH.

The results presented in the Table - 1 indicates that the activity of GLDH varies greatly from each other in different parasites as well as in host tissues. In G. explanatum, the enzyme activity was found to be the highest, even higher than the liver of the host. These results are in agreement with the findings reported by Krvavica et al. (1967) for F. hepatica. However, the enzyme activity was found much lower in G. crumenifer which inhabits rumen although rumen has been reported to contain very large quantities of free ammonia due to the activity of intra-rumenal flora and high concentration of CO\(_2\). The present results are also compatible with the findings of Krvavica et al. (1967) with Paramphistomum cervi which also inhabits the rumen. Therefore, the parasites found in different organs of the same host show different metabolic activities.

Liver is a habitat where bile and bile salts play an important role in maintaining the hydrogen ion concentration
of the medium and the pH is found to be alkaline whereas in rumen the pH is slightly acidic. However, the pH optima of this enzyme in both the parasites was found to be 8.0, as reported earlier for another parasite, *H. culbertsoni* (Rao and Hussain, 1979). Schmidt (1974) also reported maximum enzyme activity at pH 8.0 in human serum. Mustafa *et al.* (1978) have emphasised that the GLDH activity in *H. diminuta* at different assay pH substantially modify α-ketoglutarate binding and decreasing the assay pH favours NADH oxidation by lowering the Michaelis constant (Km) values. Thus, the difference in the pH of the habitat may be attributed as one of the factor influencing the enzyme activity in different parasites of that microenvironment.

The low Km, high Vmax and Vmax/Km values for α-KGA in *G. explanatum* clearly show that the enzyme is highly active in the liver trematode whereas in *G. crumenifer* the Km value was found to be much higher, thereby showing lower Vmax and Vmax/Km values. The kinetics of GLDH of the two amphistomes differ markedly from GLDH purified from *H. diminuta* (Mustafa *et al.*, 1978) which is found, altogether, in a different habitat, i.e., intestine, where physiological conditions are also different from that of liver as well as rumen.

As far as coenzyme specificity is concerned, the amphistome GLDH show single coenzyme specificity for NAD(H)
as addition of 0.2 mM NADP(H) in assay mixture stops the enzyme activity completely. Therefore, NADH is the preferred coenzyme. The amphistome GLDH differ markedly from the enzyme isolated from mammalian sources (Frielen, 1959a, 1959b), for example, the rat liver GLDH shows little coenzyme specificity and functions with either NAD(H) or NADP(H) whereas the GLDH of H. diminuta was found to be specific for NADH (Mustafa et al., 1978).

The influence of purine and pyrimidine nucleotides on the activity of GLDH of G. explanatum and G. crumenifer vary from one another remarkably. The allosteric modifier, ADP, increases the enzyme activity in G. explanatum, whereas AMP, UMP, GMP, CMP and ATP produced significant inhibitory effects on the enzyme under specified assay conditions. The ATP produces most pronounced inhibitory effects. The same concentration of these nucleotides does not produce significant changes except AMP which has caused about two fold increase in the GLDH of G. crumenifer. However, the other nucleotides, ADP, ATP and GMP have been found to produce slight inhibition of enzyme of the rumen trematode.

The rat liver GLDH is inhibited by GTP, ATP and activated by ADP (Smith et al., 1975). Thus the enzyme of liver trematode behaves like the mammalian liver GLDH in response to nucleotides. In contrast the H. diminuta GLDH was not affected by 1 mM AMP, ADP, ATP, IDP, GDP or GTP.
(Mustafa et al., 1978). The concentration of these nucleotides for *H. diminuta* appear to be physiologically significant as Barrett and Beis (1973) have calculated nucleotide concentrations for *H. diminuta* to be in the order of 0.29-1.57 μmoles/g-fresh weight. The enzyme of *H. contortus* is said to be inhibited by AMP, ADP, ATP, aspartate and thyroxine (Barrett, 1981). But contrary to this, AMP stimulated the enzyme activity in *G. crumenifer* whereas it produced inhibitory effects in *G. explanatum* in the present study.

The osmotic and ionic stress studies reveal that when parasites are removed from their respective microenvironments and incubated for a fixed period of two hours in different concentrations of salines of different osmolarities, the GLDH exhibit varying degree of activity. The two amphistomes do not behave in a similar manner as far as the effect of osmotic and ionic stress on GLDH activity is concerned. *G. explanatum* has been found to be sensitive to hypotonic medium in which enzyme activity was decreased whereas the catalytic activity increased in hypertonic saline. In double distilled water, the parasites are water-logged and died within an hour and the GLDH activity was found to be the highest when estimated in the dead parasites after one hour of incubation. The increased catalytic activity in hypertonic saline might increase the glutamate formation at the intracellular level and the glutamate, thus synthesized would ultimately serve as a precursor for various biosynthetic
processes. Since the flukes of *G. explanatum* inhabit the bile duct where they remain in a more or less constant environment, therefore, when they are exposed to any osmotic or ionic stress, the GLDH activity changes greatly.

The results of the osmotic and ionic stress studies presented in Fig. - 7 shows that the catalytic activity of GLDH of *G. crumenifer* is not influenced remarkably by hypotonic or hypertonic salines when compared with the activity in isotonic medium. In hypotonic medium (75%) the activity was inhibited by only 8% and slightly stimulated in 200% saline but no definite pattern of the enzyme activity is seen in various salinities. Infact, GLDH activity was observed to fluctuate in a very narrow range.

Since *G. crumenifer* in vivo is exposed to more or less constantly changing microenvironment as far as ionic concentrations and osmolarities are concerned in the rumen. The enormous quantities of food in the rumen, high activity of the intra-rumenal flora and intake of plenty of water causes more or less continuous change in the rumen environment. Therefore fluctuations of *G. crumenifer* GLDH activity in a narrow range due to osmotic and ionic stress may be considered as an example of parasitic adaptation at the enzymatic level and it may also be inferred that the microenvironment in which the parasites live, greatly influence the biochemical as well as physiological activity of the parasites.
No previous report is available on the effects of osmotic and ionic stress studies on the GLDH activity in helminths. However the results of the present study on GLDH of the two amphistomes resemble that of the results reported for an euryhaline crustacea (Florkin and Schoffeniels, 1969).

There are several amino acids which are osmotically and ionically important as they regulate intracellular osmotic and ionic balance. The most important from this aspect are leucine, alanine, proline, glycine, arginine and glutamine etc. Any increase in the concentration of glutamate due to enhanced activity of GLDH, would ultimately increase the other amino acids output as it has been observed in Crithidia oncopelti which converts glutamic acid for the synthesis of twelve amino acids, especially arginine and proline (von Brand, 1973).

In the present study various amino acids, most notably leucine greatly activates the GLDH activity in G. explanatum by 93.4 percent whereas other amino acids, viz: alanine, proline and glycine caused slight inhibition of enzyme activity which is still lower in presence of arginine and glutamine. The low alterations of the enzyme activity in presence of these amino acids may be attributed to the fact that the parasites live in a nutrient rich environment and therefore, their enzyme might be adapted for little variations in the
concentration of nutrients but the stimulatory role of leucine may also be considered as physiologically important and further studies are required on this aspect.

The presence of 1 mM leucine, alanine and proline in the assay mixtures also stimulates the GLDH activity in *G. crumenifer* in the following order: leucine alanine proline. The other three amino acids, arginine, glycine and glutamine, do not influence the enzyme activity significantly.

The end product of nitrogen metabolism, i.e., proline, for which glutamate also acts as a precursor, is excreted by liver fluke, *F. hepatica* in large amounts (20-50 μmoles/day/worm) and in *F. gigantica* as reported by Isseroff (1980) and Lutz and Siddiqi (1971) respectively. The increased proline in the infected liver/bile may be due to the high activity of GLDH of the parasites. Thus it may also be assumed that GLDH indirectly helps the parasite to establish the host parasite relationship as this amino acid has been found to cause bile duct hyperplasia, thereby making ample room for the developing flukes. The leucine has been found as a potent activator of the enzyme in both the parasites. Similarly leucine activates the GLDH activity of sonicated rat liver mitochondria (McGivan et al., 1973), but detailed physiological study is required to understand the effect of leucine in relation to the role of GLDH.
It has been found that the GLDH activity is influenced by a bewildering array of chemical compounds. The GLDH of both the amphistomes appear to be sensitive to different chemical agents used in the present study. Iodoacetate, semicarbazide hydrochloride and copper sulphate inhibited 100 percent enzyme activity of _G. explanatum_ where as in _G. crumenifer_, complete inhibition of the enzyme activity was observed in the presence of copper and mercury ions only and iodoacetate and semicarbazide bring about 97 percent inhibition of the enzyme activity. Inhibition of the GLDH in both the parasites may be due to the presence of -SH groups in the enzyme protein as suggested by Olson and Anfinson (1963) in the case of crystalline beef liver enzyme. Another compound, diethylthiocarbamate, containing sulpho-hydryl group, also inhibited the enzyme activity of both the parasites but the inhibition is slightly more pronounced in rumen trematode. Cycloheximide, causes very insignificant decrease in GLDH activity of _G. explanatum_ as well as _G. crumenifer_. Potassium cyanide at 1 x 10^{-2} M concentration has been found more inimical to the rumen trematode than the liver trematode. Ammonium sulphate and sodium sulphate also inhibit the enzyme activity in both the amphistomes. Therefore, it can be summarized that the enzyme molecules from different parasitic species belonging to the same group, respond differently to various chemical reagents and the results of this study show
that cationic specificity and anionic efficiency are different according to the origin of the enzyme. This is most likely corresponds to the differences in structure of the enzyme proteins and suggests the possible existance of isoenzymes.

The differential distribution of enzyme activity in the two parasites has been found to show variation. Vitellaria are the site of active protein metabolism where the GLDH activity is strongly present in both the amphistomes. The other regions which show intense to moderate enzyme activity in *G. explanatum* are egg-shell, oral sucker and gonopore whereas the acetabular lining, tegument and parenchymatous tissue exhibit low enzyme activity while the enzyme is totally absent in the intestinal caecae. However in *G. crumenifer*, the intense enzyme reaction was observed in sub-tegmental region, intestinal caecae and vitellaria, indicating high rate of catalytic activity of GLDH in these organs. Oral sucker also shows strong staining whereas the inner acetabular lining shows the moderate reaction. The high activity in oral sucker and sub-tegment may be due to the fact that the parasites are in direct contact with the high concentration of ammonia, thus ammonium ions are freely available to the parasites for the synthesis of various important nitrogenous compounds. Therefore, the catalytic activity of GLDH in the above tissues is also high. The present study also makes it clear that an enzyme may be ubiquitously distributed but its activity vary in different regions.
The difference in the activity of GLDH of the two trematodes in the present study may also be due to the presence of different isoenzymes. The electrophoretic study has confirmed this assumption and the isoenzyme profile of the parasites and host tissues show difference in their electrophoretic mobility. In *G. explanatum*, the GLDH has been resolved into six components of varying degree of electrophoretic mobility whereas in *G. crumenifer*, five isoenzymes have been found. The host tissue, liver and rumen, GLDH has been found to have five isoenzymes, each of which exhibits difference in the relative mobility on polyacrylamide gel.

Therefore, it can be concluded from the present study that the GLDH is an active enzyme in the parasites of buffalo. The enzyme response to different activators or inhibitors also vary according to the origin of enzyme. This offers an interesting inference that the parasites of different habitat possess different isoenzymes of GLDH. The difference in the activity and the isoenzyme pattern is a clear indication that the enzymes are also adapted to their microenvironment upto a certain extent and this offers an example of parasitic adaptation at the biochemical level.
CHAPTER VI
The present dissertation deals with the studies on the glutamate dehydrogenase of some digenetic trematodes. *Gigantocotyle explanatum* from liver and *Gastrothylax crumenifer* from rumen, belonging to the family paramphistomatidae (Fischoeder, 1901) were selected in order to characterize the nature of this enzyme by studying different kinetic properties and its possible role in the metabolism of trematodes.

It has been found that the level of enzyme activity in the parasite and host tissue occur in the order of:  
*G. explanatum* > liver tissue > rumen tissue > *G. crumenifer*

The higher activity of GLDH in *G. explanatum* clearly indicates that the enzyme is responsible for the reductive amination of α-ketoglutarate actively and thus synthesizes glutamate at a faster rate than in *G. crumenifer*.

The kinetic behaviour of GLDH of the two amphistome parasites show some differences as well as some similarities. The NAD(H) specific GLDH of the two parasites show variation in their Michaelis constant values for α-ketoglutarate (α-KGA). The Km values for α-KGA in *G. explanatum* and *G. crumenifer* were found to be in the order of 2.12 mM and 4.16 mM respectively.
The pH optima for maximum activity of GLDH in both the amphistomes was determined. It was observed that the enzyme exhibits maximum activity at pH 8.0 in both the trematodes.

Nucleotides, the allosteric modifiers also influence the GLDH of the amphistomes. Similar to the mammalian enzyme, the GLDH of *G. explanatum* is slightly stimulated by ADP whereas AMP, GMP, CMP and UMP produce inhibitory effects.

In *G. crumenifer* only AMP stimulates the enzyme activity by two folds whereas other nucleotides do not produce significant alteration in the enzyme activity.

The effect of osmotic and ionic stress on the GLDH activity has also been studied in the two parasites. It was observed that the enzyme in both the parasites respond differently to the changing ionic concentrations and osmolarities of the incubating media. In hypotonic media except 0% saline (double distilled water), the enzyme activity is retarded in *G. explanatum* whereas in *G. crumenifer* no remarkable change was observed when compared with the activity in isotonic saline (100% Tyrode). However, in hypertonic media, the catalytic activity of GLDH is accelerated in *G. explanatum* but slight stimulation was observed in *G. crumenifer*, only in 200 percent saline. The difference in GLDH level during osmotic and ionic stress may be attributed to the fact that these trematodes occupy two different habitats which entirely differ from one
another in their ecophysiological conditions. Therefore, it may also be concluded that the microhabitats exert an influence on the parasite metabolism. In *G. crumenifer* the narrow range of fluctuation in the enzyme activity exhibits an enormous adaptive flexibility in the continuously changing osmotic conditions.

The nutritional conditions of the habitat may also influence the metabolic activities of the parasites. Therefore, various amino acids have also been found to exert a change in the GLDH activity of the two amphistomes under study. Among the various amino acids, leucine activates the GLDH in both the parasites. Whereas in *G. explanatum*, the enzyme was insignificantly influenced by alanine, proline, arginine and glycine. However, glutamine was found to produce 40 percent inhibition of the enzyme activity in this parasite. The low alteration in the GLDH activity may be due to the fact that the liver parasites occupy a nutritionally rich habitat and therefore, the enzyme may be adapted for little variations in the nutritional status of their surrounding microenvironment.

Besides leucine, proline and alanine also stimulated the GLDH activity in *G. crumenifer*. However, arginine, glycine and glutamine do not influence the enzyme activity appreciably. Therefore, it can be concluded from the present study that the role of these amino acids and more particularly of leucine, is
physiologically important in relation to GLDH activity in the parasites and need further studies.

Various metabolic inhibitors have been found to produce different degree of inhibition in the GLDH activity of the two amphistomes. The differences in sensitivity towards the metabolic inhibitors may be correlated to the origin of enzyme from two different species. Iodoacetate, semicarbazide hydrochloride and copper sulphate inhibited the enzyme activity completely in *G. explanatum* whereas in *G. crumenifer* the complete inhibition was observed only in presence of mercury and copper ions. Other compounds like potassium cyanide, sodium sulphate and ammonium sulphate produce inhibitory effects on the GLDH activity in both the parasites. Cycloheximide is a well known inhibitor of protein synthesis but this compound does not produce any significant inhibition.

The results of histochemical localisation of GLDH also indicate the differential distribution of the catalytic activity of this enzyme in various regions of the two parasites. High GLDH activity was observed in vitellaria and egg-shell in both the parasites. In *G. explanatum*, the enzyme activity is totally absent in intestinal caeca while in rumen trematode intense activity of the enzyme was observed. The tegumental and sub-tegumental regions of *G. crumenifer* show high activity of GLDH whereas in liver trematode low enzyme activity was observed.
Such differences may also be attributed to the consequences of living in two different habitats.

The electrophoretic studies have shown that the phenomenon of polymorphism exists in the GLDH of the parasites. The enzyme from *G. explanatum* has been resolved into six components whereas the enzyme of *G. crumenifer* shows five zones, each identified as a separate isoenzyme. The isoenzymes of the GLDH of parasites also differ from that of the host enzyme in their electrophoretic mobility. Therefore, it can be concluded that the GLDH of the two trematodes which belong to the same taxonomic group show intraspecific variation as well as from the host.

Therefore, the similarities found in the nature of GLDH of the two parasites may be due to the fact that the trematodes under study belong to the same taxonomic group but variation in the kinetic properties may be because of the differences in the physico-chemical nature of the micro-environments.
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