BIOCHEMICAL STUDIES ON CERTAIN HELMINTH PARASITES

(SUMMARY)

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

in the Faculty of Science, Aligarh Muslim University, Aligarh

By

AFZAL AHMAD ANSARI
M. Sc., M. Phil.

Division of Biochemistry
Central Drug Research Institute
Lucknow
1971
Parasitic diseases are inherent ecological problems and their endemicity or suppression is dependent upon man's ability to control his environment. A variety of parasitic helminths are known to infest animals, birds or man. The ravages caused by them were recognized long ago; none the same, they continue to sap the vitality of our race. Undoubtedly, epidemiological measures to improve environmental sanitation have led to dramatic successes in the eradication of many infectious diseases in continental Europe and America. In countries like India, only in recent years have control and prevention programmes been initiated and as such, helminthiasis and filariasis continue to be among the biggest public health problems.

Due to the lack of effective immunotherapeutic remedies, the only available treatment of helminthic infections is by synthetic drugs designed on piperazines and other naturally occurring compounds. Chemotherapy of the ailments caused by the filarial worms or the intestinal round worms has not been successful so far on account of the fact that the meagre information on the biochemical activities of these parasites do not permit a rational design of new drugs.

Information available on the morphology, anatomy and nutritional requirements of many helminth species suggests that a specific biological interaction between the host and
the parasite is a pre-requisite for the manifestation of the pathological symptoms. These advances notwithstanding, in vitro cultivation of parasitic helminths in a medium independent of the host has not yet been achieved. Naturally, progress in the chemotherapy of helminthiasis has been slow.

The work presented in this dissertation was carried out with the aim of gaining some understanding of the carbohydrate metabolism of *Setaria cervi*, the filarial parasite of buffaloes and *Ascaridia galli*, the intestinal nematode of domestic fowls, and proteinase inhibitors present in *A. galli*. These test organisms were used as models in the absence of the human parasite *Brugia malayi*.

The investigations have indicated that glucose, the main energy source for the filarial parasite *S. cervi*, can conveniently be employed for its maintenance in vitro for studying the effect of antifilarial compounds acting particularly on the motility of the parasite. The worm is equipped with nearly all the enzymes mediating the Embden-Meyerhof pathway of glycolysis by which glucose is metabolized. The parasite is capable of synthesizing glycogen in an environment having surplus amount of glucose and the polysaccharide thus synthesized can be utilized for supplying energy under conditions of starvation. The parasite also has a full complement of enzymes of the Krebs cycle similar to those present in other animals. *A. galli*, the intestinal
parasite, contains most of the enzymes mediating TCA cycle indicating resemblance of the two nematodes. Presence of an alternate pathway branching off at PEP level has been detected in these two parasites and the production of some unusual end-products of glycolysis could be explained on the basis of the enzymes of PEP-oxaloacetate pathway.

A study with the purified aldolase of S. cervi revealed that the filarial enzyme resembled more closely the mammalian aldolase and hence can be classified as aldolase I. Among the various metabolic inhibitors and antifilarial drugs tried, cyanine-863 strongly inhibited the activity of this enzyme.

The intestinal parasite A. galli contained potent inhibitors for trypsin and chymotrypsin activities. The inhibitors separated by TCA and heat treatment followed by DEAE- and CM-cellulose chromatography were found to be low molecular weight proteins.

The work presented, is not claimed to be complete nor does it throw light on all unillumined areas of parasitic metabolism. The author is conscious of the fact that he has touched only the fringe of the vast uncharted area of helminth carbohydrate biochemistry. The questions that have been left unanswered are many; but the efforts have been fruitful in enabling the identification of some aspects of the energy metabolism of these parasites which need more intensive investigation.
BIOCHEMICAL STUDIES
ON
CERTAIN HELMINTH PARASITES

Thesis
submitted for the degree of

DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

in the Faculty of Science,
Aligarh Muslim University, Aligarh

By

AFZAL AHMAD ANSARI
M. Sc., M. Phil.

Division of Biochemistry
Central Drug Research Institute
Lucknow
1971
ACKNOWLEDGEMENTS

I am extremely grateful to Dr. M.L. Dhar, F.R.I.C., F.N.A., Director, Central Drug Research Institute (CDRI), Lucknow, for providing the facilities to carry out this work and to the Council of Scientific and Industrial Research, New Delhi, for the award of a Junior Research Fellowship during the tenure of which this work was completed.

I take the opportunity to express my deep feeling of gratitude to Dr. C.R. Krishna Murti, F.N.A., Scientist-in-Charge, Division of Biochemistry, CDRI, Lucknow, for his constructive suggestions and encouragement throughout the period of this investigation.

I am extremely indebted to Dr. S.N. Ghatak, Scientist, Division of Biochemistry, CDRI, Lucknow, and Dr. Abdul Majid Siddiqi, Head, Biochemistry Division, Department of Chemistry, Aligarh Muslim University (AMU), Aligarh, for their valuable guidance, supervision and healthy discussions at every phase of this work.

I am also grateful to Prof. Wasiur Rahman, Head, Department of Chemistry, AMU, Aligarh, for his encouragement and permission to submit the thesis.
I feel great pleasure in extending my thanks to the members of Biochemistry Division, CDRI, Lucknow, and especially to Drs. M.M. Husain, V.M.L. Srivastava and Miss Shanta Amaralal for their untiring help and cooperation.

My thanks are also due to Mr. J.F. Khare for typing the thesis and to Mr. S. Banerji for the illustrations.

My acknowledgements will, I feel, remain incomplete without expressing my most sincere gratitude to my elder brother, Mr. Iqbal A. Ansari who has been a perennial source of inspiration in pursuing this work.

( Afzal A. Ansari )
Parasitic diseases are inherent ecological problems and their endemicity or suppression is dependent upon man's ability to control his environment. A variety of parasitic helminths are known to infest animals, birds or man. The ravages caused by them were recognized long ago; none the same they continue to sap the vitality of our race. Undoubtedly, epidemiological measures to improve environmental sanitation have led to dramatic successes in the eradication of many infectious diseases in continental Europe and America. In countries like India, only in recent years have control and prevention programmes been initiated and as such, helminthiasis and filariasis continue to be among the biggest public health problems.

Due to the lack of effective immunotherapeutic remedies, the only available treatment of helminthic infections is by synthetic drugs designed on piperazines and other naturally occurring compounds. Chemotherapy of the ailments caused by the filarial worms or the intestinal round worms has not been successful so far on account of the fact that the meagre information on the biochemical activities of these parasites do not permit a rational design of new drugs.
Information available on the morphology, anatomy and nutritional requirements of many helminth species suggests that a specific biological interaction between the host and the parasite is a pre-requisite for the manifestation of the pathological symptoms. These advances notwithstanding, in vitro cultivation of parasitic helminths in a medium independent of the host has not yet been achieved. Naturally, progress in the chemotherapy of helminthiasis has been slow.

The work presented in this dissertation was carried out with the aim of gaining some understanding of the carbohydrate metabolism of *Setaria cervi*, the filarial parasite of buffaloes and *Ascaridia galli*, the intestinal nematode of domestic fowls, and proteinase inhibitors present in *A. galli*. These test organisms were used as models in the absence of the human parasite *Brugia malayi*. The work presented, is not claimed to be complete nor does it throw light on all unillumined areas of parasitic metabolism. The author is conscious of the fact that he has touched only the fringe of the vast uncharted area of helminth carbohydrate biochemistry. The questions that have been left unanswered are many; but the efforts have been fruitful in enabling the identification of some aspects of the energy metabolism of these parasites which need more intensive investigation.
ABBREVIATIONS USED

AMP  Adenosine 5'-monophosphate
ADP  Adenosine 5'-diphosphate
ATP  Adenosine 5'-triphosphate
cpm  Counts per minute
CM-cellulose  Carboxymethyl cellulose
DEAE-cellulose  Diethyl aminoethyl cellulose
EDTA  Ethylene diamine tetraacetate
F-6-P  Fructose-6-phosphate
FDP  Fructose-1,6-diphosphate
G-1-P  Glucose-1-phosphate
G-6-P  Glucose-6-phosphate
G-6-P DH  Glucose-6-phosphate dehydrogenase
KRB  Krebs-Ringer bicarbonate
LDH  Lactate dehydrogenase
MDH  Malate dehydrogenase
$\mu$ Ci  Micro Curie
m Ci  Milli Curie
NAD  Nicotinamide adenine dinucleotide
NADP  NAD phosphate
NADH$_2$  Reduced NAD
(iv)

NEM  N-ethyl maleimide
nm  Nanometre
PCA  Perchloric acid
pCMB  p-Chloromercuribenzoate
PEP  Phosphoenol pyruvate
PGA  Phosphoglyceric acid
P\textsubscript{i}  Inorganic phosphate
SE  Standard error
TCA  Trichloroacetic acid
TCA cycle  Tricarboxylic acid cycle
Tris  Tris - (hydroxymethyl) - aminomethane
CHAPTER I

INTRODUCTION
Parasitic organisms are ubiquitously distributed in nature and there is hardly any organ or tissue of plants or animals which is not a victim of parasitic invasion. From time immemorial there has been a constant fight for mutual survival between parasites and human beings, and man has never ceased to devise ways of escaping from parasitic invasions. Chemotherapy of parasitic diseases would involve interference with the life processes of the parasites and as such it is imperative to explore their life cycle and metabolic activities and host-parasite relationships.

Problem of helminth infestations in man and animals

Helminths are a group of parasites abundantly distributed in nature. They infest all types of animals including humans. Helminths are sub-divided into platyhelminthes (flatworms) and Aschhelminthes (nemathelminthes) (roundworms) and the details of the classification with examples are given in Fig. 1. Among this group the class 'nematoda' is of particular public health interest because they give rise to a variety of endemic diseases in man. 'Filariasis' and 'ascariasis' have become public health problems and attempts to control them have been assigned highest priority in our national health programmes. In humans, filariasis is caused by
Fig. 1 - Classification of Helminth Parasites.
**Wuchereria bancrofti** and **Brugia malayi**, while in animals and birds the causative agents are **Setaria cervi** (cattle), **Dirofilaria immitis** (dog), **Dirofilaria uniformis** (rabbit), **Litomosoides carinii** (cotton rat) and **Chandlerella hawkingi** (crow). Similarly ascariasis in humans and pigs is caused by **Ascaris lumbricoides** and the counterpart of this disease in poultry is **Ascaridia galli**.

**Filariasis**

Filariasis is observed in many parts of the world and is well known with its typical pathological symptoms, namely, eosinophilia and elephantiasis. This disease has been reported from many Indian States and Uttar Pradesh is one of the most heavily infected areas. According to Ramakrishnan et al. (1960) more than 40 million people live in filarial area and hence are constantly exposed to the risk of contracting this disease. In the absence of effective methods for the treatment of carrier cases and also due to the lack of potent insecticides for eradicating **Culex fatigans**, the vector of **W. bancrofti**, the control of filarial infection has become very difficult. The expert committee on filariasis of the World Health Organization has repeatedly stressed the urgent need for carrying out biochemical investigations on the parasites (Edeson, 1967).
Ascarasis

Ascarasis, a disease prevalent in human and animal population is widely distributed in tropical and sub-tropical regions of the world and *A. lumbricoides*, the causative organism in human beings, is known to cause mechanical injury and obstruction in the intestine. The worm enters the appendix causing appendicitis and on migration into the upper portion of the small intestine, it produces nausea and vomiting. Sometimes, it may die within the host's body and the toxic products released consequently may be absorbed by the host tissue and cause the appearance of unremitting symptoms. *Ascaris* worms have been also reported to pass through the umbilus and to migrate into the biliary tract (Wilson, 1968). Recently 'hemobilia' with multiple liver abscesses and intestinal haemorrhage have been described in patients suffering from ascariasis. A survey of the Pediatric Ward of K.G. Medical College, Lucknow, has revealed that about 40-50% of children admitted to the hospital for various ailments suffer from this disease. As many as 150 worms have been recovered from one child by the administration of a single dose of piperazine.

Chemotherapy of helminthic infections

Drugs effective against parasitic organisms probably
exert their action by interfering with the metabolic machinery which maintains the structural integrity or mediates reproduction of the parasites (Saz and Bueding, 1966; Bueding, 1969). Inhibition of carbohydrate metabolism of the helminths has been shown to result from the action of some drugs. Carbohydrates are known to constitute a major portion of the body of the parasites and the drugs may interfere with the absorption of carbohydrates or by altering the activity of the enzyme systems involved in carbohydrate metabolism. Bueding (1962a) has reported that the motility of Schistosomes is decreased due to the inhibition of glucose uptake by alkyl dibenzylamines. A similar mechanism of action has been suggested for cyanine dye in Trichuris vulpis (Bueding et al., 1961). Recently Srivastava et al. (1971) have suggested that cyanine-363 exerts its chemotherapeutic action by inhibiting glycolysis at the aldolase step in the filarial parasites, C. hawkingi and L. carinii. The chemotherapeutic effect of trivalent organic antimonials on Schistosomes has been explained on the basis of inhibition of phosphofructokinase activity with a depression of glycolysis in the parasite (Bueding, 1959). Likewise, tetramisole and its optical isomers have been shown to inhibit the succinic dehydrogenase activity of Ascaris muscle. Deprived of energy, the parasite is paralysed and expelled from the body of the host (Bossche and Janssen, 1967).
According to Norton and De Beer (1957) and Bueding et al. (1959), the paralysis of *Ascaris* muscle by piperazine was due to the inhibition of acetylcholine activity and decreased succinate production by the parasite. $\beta$-Hydroxy-naphthoic acid at $2.5 \times 10^{-3}$ M has been shown to act on *Ascaris* by inhibiting the synthesis of volatile fatty acids (Pushkarev, 1966), while sodium palasonin, the anthelmintic principle isolated from *Butea frondosa* seeds was shown to decrease the glycogen content in *A lumbricoides* due to the inhibition of glycogen synthetase.

An ideal chemotherapeutic agent for treatment of helminthic infections should be highly toxic to the parasite but comparatively non-toxic to the host and in practice some compromise has to be affected between the toxicity of the drug towards the host and the parasite by carefully controlling the dosage of the administered drug.

Since the present study is mainly concerned with the carbohydrate metabolism and the proteinase inhibitors of helminths, the survey of literature presented in this dissertation is limited to some aspects of the degradation and synthesis of carbohydrates and proteolytic inhibitors in the parasites.
CARBOHYDRATE METABOLISM OF HELMINTH PARASITES

Presence of glycogen in tapeworms, ascarides and flukes has been known for long (Claude Bernard, 1859) and in 1865 Sir Michael Foster performed the first quantitative analysis of glycogen in an ascarid. Weinland in 1901 surveyed the glycogen content in various helminth parasites.

The fermentation of carbohydrates resulting in the formation of acids as end-products is a characteristic feature of the intestinal worms. It is interesting to note that almost all the parasites studied so far are incapable of completely oxidising sugars to CO₂ and water. This is also true of parasites living in an oxygen rich habitat. Thus most endoparasites are characterized by the prevalence of anaerobic or aerobic fermentations. No single mechanism can explain all facts known about the fermentations of the parasites. According to McKee et al. (1943) parasites like plasmodia have a full complement of enzymes but a differential activity of various enzymes of glycolytic chain perhaps leads to an accumulation and final excretion of partially oxidised compounds. In Trypanosoma cruzi which excretes large amounts of succinic acid (Ryley, 1956), "despite possessing mechanisms for metabolizing succinic acid, the existence of
regulatory mechanism may be prevailing which restrict for unknown reasons the activity of certain enzymes" (von Brand, 1963).

**Distribution of carbohydrates**

Parasitic worms contain significant amounts of glucose (Fairbairn, 1958). Reducing sugars (customarily expressed as glucose) occur in the body fluid of the worms and their concentrations are usually low in nematodes. The non-reducing disaccharide trehalose is widely distributed in helminths and is found in greater amounts than glucose in several species (Hymenolepis diminuta, A. lumbricoides hemolymph, L. carinii, Trichinella spiralis larvae and Trichuris ovis - Fairbairn, 1958).

Simple sugars occur in the parasites not only as free sugars but also as polysaccharides or as constituents of glycoproteins or glycolipids. Glycogen is the most widely distributed reserve polysaccharide of parasites and in adult nematodes by far the greatest part of the stored glycogen is found in the tissues of the body wall. The intracellular localization of glycogen has been investigated by using electron microscopy. The cuticle is generally glycogen-free but the lateral lines are often very rich in polysaccharide. The main storage areas, however, are the muscular layers.
Utilization and synthesis of carbohydrates

Carbohydrates are often used as constituents of media employed to culture or to maintain helminths in vitro and most of the worms become immotile when they are kept in a medium devoid of sugars.

Despite their great need for energy, the spectrum of utilizable carbohydrates is quite narrow in many cestodes and nematodes and consists essentially of glucose and galactose (Read and Rothman, 1958). All the helminths studied so far utilize glucose in varying rates. In Schistosoma mansoni (Bueding et al., 1947), Litomosoides carinii (Bueding, 1949), Dracunculus insignis (Bueding and Oliver-Gonzalez, 1950), Dirofilaria uniformis (von Brand et al., 1963) or Chandlerella hawkingi (Srivastava et al., 1968), the consumption rate in 24 hours is very high and account for 50-80% of the worm’s fresh body weight. In contrast, the larvae of Hystrostrongylides ignotus consume less than one per cent glucose. Fructose and mannose are also utilized by a few species but slowly. Litomosoides carinii (Bueding, 1949) and Moniliformis dubius (Laurie, 1957; 1959) were reported to utilize readily glucose, mannose, galactose and fructose; while Srivastava et al. have recently observed that C. hawkingi (1968) and L. carinii (1970c) metabolize glucose and mannose only. Ready utilization of glucose, fructose, maltose and saccharose
by *A. lumbricoides* has been reported by Cavier and Savel (1952). The utilization of pentoses and sugar alcohols by helminths has not been studied in detail.

A question that remains unanswered is whether the absorption of soluble sugars involves an active transport mechanism. There is good evidence to suggest that active transport mechanisms are employed by cestodes, and that the sites of entry may not be identical for all hexoses as separate systems for the permeation of glucose and galactose into *H. diminuta* have been observed by Rothman (1959). It is evident that the absorption of soluble sugars takes place through the surface in cestodes and Acanthocephala. However, in nematodes, the site of absorption is undoubtedly the intestinal canal (von Brand, 1966). Cavier and Savel (1952) have reported that the cuticle of *A. lumbricoides* is impermeable to soluble sugars.

Glycogen is the most important energy source in helminths and the worms can survive in a sugar free medium at the expense of their body glycogen. *A. galli* consumes 3.6 g and 2.8 g of glycogen per 100 g body weight in the first 48 hours when incubated at 37-41°C under anaerobic and aerobic conditions respectively (Reid, 1945). Under the same conditions *S. cervi* has been reported to utilize glycogen aerobically at the rate of 13.7 and 8.8 g for female and male respectively (Pandaya, 1961).
Polysaccharides stored in endoparasites seem to be derived in most cases from exogenous carbohydrates and several helminths have been shown to possess a rapid rate of glycogen synthesis. Experiments with aseptically isolated worms or with worms sterilized by antibiotics have shown that exogenous carbohydrates are utilized for polysaccharide synthesis (von Brand, 1966). Bueding (1949) and recently Srivastava et al. (1970c) using uniformly labelled glucose observed rapid glycogen synthesis by L. carinii. Fernando and Wong (1964) also observed glycogen synthesis by Ancyclostoma caninum from glucose medium fortified with serum. In vitro synthesis of glycogen from glucose, fructose, sorbose, maltose and saccharose but not from mannose, galactose, lactose, glycerol and various sugar alcohols has been reported in A. lumbricoides (Cavier and Savel, 1952).

End-products

A number of substances, mainly acids are produced as the end-products of utilization of carbohydrates by the parasites while in case of vertebrate tissues almost exclusively lactic acid is formed. In D. uniformis, practically all the carbon of the metabolized glucose is converted to lactic acid (von Brand et al., 1963). Similarly L. carinii, D. insignis and S. mansoni (Bueding, 1949; 1950; Bueding and Oliver-Gonzales, 1950) and C. hawkingi.
(Srivastava et al., 1968) also convert nearly all the carbon of the sugar utilized into lactic acid. While traces of this acid have been reported in *Heterakis gallinae* (Laurie, 1959). von Brand and Bowman (1961) have shown pyruvic acid to be the aerobic and anaerobic end-product of *Taenia taeniaeformis*. Pyruvic acid has also been identified as the aerobic end-product in the larvae of *Echinococcus granulosus* (Agosin, 1957) and *Ancylostoma caninum* (Warren and Guevarra, 1962; Crowley and Warren, 1963). Several intestinal nematodes have been shown to excrete a variety of lower fatty acids, both aerobically and anaerobically. *Ascaris lumbriscoides* excretes \(\alpha\)-methylbutyric acid (Bueding and Yale, 1951), n-valeric and \(\alpha\)-methyl crotonic (tiglic) acids (Bueding, 1953). The only \(C_6\) acid regularly excreted by *Ascaris* is \(\alpha\)-methyl valeric acid (Saz and Gerzon, 1962; Whitlock and Strong, 1963). This organism in addition produces succinic acid (Bueding and Farrow, 1956). Succinic acid is also the major end-product of both aerobic and anaerobic fermentations of many other helminth parasites, and is frequently excreted in large amounts by tapeworms, anaerobically than aerobically (Agosin, 1957; von Brand and Bowman, 1961).

In addition to the acidic end-products, parasites also have been shown to excrete non-acidic organic metabolic end-products. Thus, appreciable amounts of acetoin (acetyl methyl-carbinol) have been identified in the incubates
Main pathways of carbohydrate metabolism.

Enzymes of pentose phosphate pathway:

1. G-6-P dehydrogenase
2. 6-PG dehydrogenase
3. Pentose phosphate isomerase
4. Pentose phosphate epimerase
5. Transketolase
6. Transaldolase

Enzymes of glycolytic pathway:

1. Glycogen phosphorylase
2. Phosphoglucomutase
3. Phosphoglucoisomerase
4. Phosphofructokinase
5. FDP Aldolase
6. Glyceraldehyde phosphate dehydrogenase
7. Phosphoglycerokinase
8. Phosphoglyceromutase
9. Enolase
10. Pyruvate kinase
11. Lactate dehydrogenase
12. Glucokinase
13. Fructokinase
14. Triosephosphate isomerase
15. Glyceroephosphate dehydrogenase
16. Glycerokinase

Enzymes of tricarboxylic acid cycle:

1. Pyruvate decarboxylase
2. Pyruvate carboxylase
3. Condensing enzyme
4. Aconitase
5. Isocitrate dehydrogenase
6. Carboxylase
7. ß-Ketoglutaric dehydrogenase
8. Succinate dehydrogenase
9. Fumarase
10. Malate dehydrogenase
11. Malic enzyme
MAIN PATHWAYS OF CARBOHYDRATE METABOLISM

GLYCOLYTIC PATHWAY
PENTOSE PHOSPHATE PATHWAY
TRICARBOXYLIC ACID CYCLE
ALTERNATIVE PATHWAY IN ASCARIS

GLYCOGEN → G-1-P → G-6-P → F-6-P → FDP → DIOXYGlycerol → 3-PGA

Ribulose-5-phosphate
Xylose-5-phosphate
Erythrose-4-phosphate
Sedoheptulose-7-phosphate
3-Phosphoglycerate
1-3-Diphosphoglycerate
Phosphoglycerate
Glucose
Fructose
NADH

PEP
Acetyl-CoA
Methylmalonyl-CoA
Succinyl-CoA
Malate
Fumarate
Pyruvate
Lactate
Glutamate

CO2
of *L. carinii* (Bueding, 1951), *Setaria* (Yonesawa, 1953) and *A. lumbricoides* (Saz et al., 1958). A small amount of glycerol has also been identified as the end-product of aerobic and anaerobic fermentation of *T. taeniaeformis* (von Brand and Bowman, 1961).

**Pathways and Enzymes**

Different techniques have been employed to demonstrate the operation of the main metabolic pathways in the parasites. One way is the demonstration of the individual enzymes of the pathway, another one, identification of the intermediates within the tissues of the worms. Yet another technique usually used is the administration of labelled glucose and then examining the distribution of radioactivity in the end-products. The map (facing page) summarizes the existing knowledge regarding the individual steps of the main pathways of carbohydrate metabolism (Fig. 2).

**Glycolysis**

In vertebrate tissues, glycolysis leads to the formation of lactic acid from carbohydrates, the individual steps of the sequence being catalyzed by specific enzymes. Phosphorylative glycolysis is apparently widely distributed among parasites as typical phosphorylated intermediates, viz. G-1-P, G-6-P, F-6-P, FDP, glyceraldehyde-3-phosphate
and phosphoglyceric acid have been shown in extracts of *Hymenolepis diminuta* (except G-1-P) (Read, 1951) and *Strongyloides ratti* (Jones et al., 1955).

All the enzymes of the glycolytic pathway have been demonstrated in *Haemonchus contortus* larvae (Ward and Schofield, 1967a; Ward et al., 1968a,b), *C. hawkingi* (Srivastava et al., 1968; Srivastava and Ghatak, 1971), *A. galli* (Srivastava et al., 1970a) and *L. carinii* (Srivastava et al., 1970b). Furthermore, most of the enzymes mediating glycolysis have been shown to be functioning in *A. lumbricoides* (Rogers and Lazarus, 1949; Cavier and Savel, 1952; Bueding and Most, 1953; Rathbone and Rees, 1954; Bueding and Saz, 1963), *H. diminuta* (Read, 1951), *S. mansoni* (Bueding and Mackinnon, 1955; Bueding and Mansour, 1957; Bueding and Saz, 1968), *T. spiralis* larvae (Goldberg, 1958; Agosin and Aravena, 1959a; Ward et al., 1969), *E. granulosus* (Agosin and Aravena, 1959b; 1960) and *T. taeniaeformis* (Waltz, 1963).

However, in many cases only a few enzymes have been studied and hence the evidence for the operation of the complete glycolytic sequence is incomplete. Among the enzymes studied in some detail are lactic dehydrogenase and phosphotrioseisomerase, enolase and aldolase. The best documented data with regard to purification and properties is on aldolase, e.g. in *Taenia crassiceps* (Ryley, 1955; Phifer, 1958), *Trichinella spiralis* (Agosin and Aravena, 1959a) and *A. galli* (Srivastava *et al.*, 1970a). Inhibition, activation studies have been performed on phosphofructokinase in *Schistosoma mansoni* (Mansour and Bueding, 1954) and *Fascola hepatica* (Mansour, 1962).

**Pentose-phosphate pathway**

Relatively little information is available on the pentose-phosphate pathway. Some evidence for the operation of a functional oxidative cycle is available for helminth parasites. All the enzymes of the pathway have been demonstrated in *E. granulosus* (Agosin and Aravena, 1960), while few enzymes viz. G-6-P dehydrogenase, 6-phosphogluconate dehydrogenase, transketolase and phosphopentoseisomerase have been detected in *A. lumbricoides* (De Ley and Vercruysse, 1955; Entner, 1957). The presence of G-6-P dehydrogenase and 6-P-G dehydrogenase has been shown in a number of parasites e.g. *A. galli*, *T. taeniaeformis* and *F. hepatica*.
(De Ley and Vercruysse, 1955). G-6-P dehydrogenase of *A. lumbricoides* (Entner, 1957) and *A. galli* (Srivastava et al., 1970a) and ribose-5-phosphate isomerase of *A. lumbricoides* were partially purified and the properties of the purified enzymes were studied in detail.

**Tricarboxylic acid cycle (Krebs cycle)**

Almost all the enzymes of the cycle have been demonstrated in *Trichinella spiralis* larvae (Goldberg, 1957), *Strongyloides papillosus* larvae (Costello and Grollman, 1959), unembryonated eggs of *A. lumbricoides* (Costello and Brown, 1962; Smith et al., 1963; Oya et al., 1963), scolices of *E.granulosus* (Agosin and Repetto, 1963), *A. lumbricoides* muscle (Oya et al., 1965) and *H. contortus* larvae (Ward and Schofield, 1967b). The utilization of the intermediates of the cycle have been studied in *A.galli*, *Nematodirus* species, *N.glaseri* (Massey and Rogers, 1949; 1950) and *E.granulosus* (Agosin and Repetto, 1963). Some of the intermediates have also been shown to be utilized by *A. lumbricoides* (Rathbone, 1955) and *Nippostrongylus muris* larvae (Schwabe, 1957). The presence of Krebs cycle intermediates has been established in the bodies of *F.hepatica* (Bryant and Williams, 1962; Bryant and Smith, 1963; Thorsell, 1963) and Scolices of *E.granulosus* (Agosin and Repetto, 1963). α-Ketoglutaric and succinic acids have also
been detected in *A. lumbricoides* (Ueno et al., 1960). The pathway for the formation of ω-methyl valerate and ω-methyl butyrate in *A. lumbricoides* has been traced by Saz and Weil (1960; 1962).

Succinoxidase system of *A. lumbricoides* has been studied intensively by Bueding (1962b; 1963). The success in elucidating the details of the succinoxidase system of *Ascaris* was due largely to the fact that succinic dehydrogenase could be dissociated from the electron transport system by acetone treatment (Kmetec and Bueding, 1961) or by the action of lipase. The NADH and succinate system could be dissociated by heating at 55°C for 10 min. inactivating the NADH system without affecting the succinate system (Kmetec and Bueding, 1961). Bueding and Charms (1962) have shown that adult *Ascaris* does not contain appreciable amounts of either cytochrome C or cytochrome oxidase indicating the absence of cytochrome oxidase as a terminal oxidase. The system reacts with atmospheric oxygen by means of a flavin containing terminal oxidase which is activated by Mn²⁺ since purified preparations contain flavin adenine dinucleotide as a major component and very little flavin mononucleotide or riboflavin (Bueding, 1963).

Although not strictly a Krebs cycle enzyme, the enzyme catalyzing the oxidative decarboxylation of malate in *Ascaris* (Saz and Hubbarad, 1957) may be mentioned here.
since by virtue of its reverse reaction, fixation of CO₂ into pyruvate it is closely related to the succinoxidase system. The malic enzyme preparations so far studied in systems other than parasites decarboxylate oxaloacetic acid between pH 4 and 5. In contrast, Ascaris malic enzyme does not catalyze the decarboxylation of oxaloacetic acid at any pH in the absence or presence of ATP.

PEP-oxaloacetate pathway

As mentioned earlier, a number of parasitic helminths have been shown to produce succinic and volatile fatty acids, rather than lactic acid as their major fermentation end-products (von Brand, 1966). Ward and Schofield (1967b), Ward et al. (1968a, b; 1969) demonstrated that in H. contortus and T. spiralis there was a major modification to the glycolytic sequence at the level of PEP and suggested that carboxylation of PEP rather than pyruvate may be involved in succinate production by the operation of a PEP-oxaloacetate pathway (Fig. 3). Recent investigations on the glycolytic and CO₂ fixing enzymes in parasitic helminths indicate that the succinate/volatile fatty acid fermenters are characterized by low pyruvate kinase and lactic dehydrogenase and high PEP-carboxykinase and malic dehydrogenase activities. Examples of this group are H. contortus larvae (Ward and Schofield, 1967a, b; Ward et al., 1968a, b), A. lumbricoides,
Fig. 3 - PEP-Oxaloacetate Pathway observed in Helminth Parasites.
H. diminuta (Bueding and Saz, 1968) and T. siralis (Ward et al., 1969). In contrast, S. mansoni, a typical lactic acid producer, resembles vertebrate tissues in possessing high pyruvate kinase and lactic dehydrogenase and negligible PEP-carboxykinase and malic dehydrogenase activities (Bueding and Saz, 1968). The filarial parasites L. carinii and C. hawkingi produce significant amounts of lactic acid with the ratio of pyruvate kinase to PEP-carboxykinase around three, and the parasites had significantly high activity of lactic dehydrogenase and resemble more closely S. mansoni than the succinate/volatile fatty acid fermenters (Srivastava et al., 1970b; Srivastava and Ghatak, 1971).

**PROTEINASE INHIBITORS IN PARASITES**

Inhibitors for proteolytic enzymes are distributed both in plant and animal kingdom and investigations made thus far have revealed the polypeptide character of these substances. The first proteolytic inhibitor from vegetable source was discovered from soybean in 1944 (Bowman, 1944; Ham and Sandstedt, 1944), followed by the discovery of another inhibitor from lima beans (Borchers and Ackerson, 1947) as well as from other leguminous plants (Borchers and Ackerson, 1947). Now all the members of leguminosae family are considered to contain proteinase inhibitors.
The first report on the inhibition of proteolytic enzymes by substances derived from animal tissues dates back to the beginning of this century. Previously these substances were known as "antienzymes". The modern approach to the problem of inhibitors commences in 1931 when Northrop and Kunitz crystallized trypsin (Northrop and Kunitz, 1931; 1932), chymotrypsinogen (Kunitz and Northrop, 1933), trypsinogen (Northrop and Kunitz, 1934) and chymotrypsin (Kunitz and Northrop, 1935; Kunitz, 1938). At about the same time a crystalline compound of trypsin and trypsin inhibitor as well as a free crystalline pancreatic trypsin inhibitor were isolated (Kunitz and Northrop, 1936). Besides mammalian species, the presence of proteinase inhibitors has only been reported in nematodes and the eggs of certain birds.

As early as 1903, Weinland considered that the resistance of nematodes towards the enzymes of the digestive tract might be due to the presence of an "antienzyme". Dastre and Stassano (1903), Hamill (1906), Fetterolf (1907), Tallqvist (1907), Mendel and Blood (1910), Harned and Nash (1932), Sang (1938), von Bonsdorff (1939; 1948) have reported the presence of these inhibitors in ascarides and tapeworms. Stewart (1933), Bushnell and Erwin (1949) have also located these inhibitors in smaller nematode species.
Isolation of *Ascaris* inhibitors

Various methods have been used for obtaining these inhibitors in pure form. Northrop and Kunitz (1932) developed a method for the concentration of pancreas inhibitor and employing a similar technique Collier (1941) succeeded in preparing an amorphous trypsin inhibitor. Hot 2.5% trichloroacetic acid treatment followed by salt fractionation helped in the resolution of specific inhibitor components leading to the preparation of a trypsin inhibiting and a pepsin inhibiting fraction. According to Green (1957) heat treatment in acid solution as employed by Collier (1941) resulted in the destruction of chymotrypsin inhibitor. Peanasky and coworkers (Peanasky and Laskowski, 1960; Peanasky, 1963; Peanasky and Szucs, 1964) succeeded in isolating the chymotrypsin inhibitor in crystalline form by salt fractionation, electrophoresis and column chromatography, using a modification of Green’s method. Pudles and Rola (Pudles, 1962; Pudles and Rola, 1964; Rola and Pudles, 1966) have described methods for the purification and separation of various proteolytic inhibitors obtaining three active zones while Rhodes et al. (1963) and Peanasky (1963) obtained two and one fraction respectively. Employing DEAE-cellulose Pudles et al. (1967) obtained a band which specifically inhibited trypsin activity and ultracentrifugation and electrophoretic studies
revealed the homogeneous property of the preparation. Rhodes et al. (1963) have shown that in *Ascaris* besides the body walls, intestines, ovaries, uteri and perienteric fluids also contained trypsin and chymotrypsin inhibitors.

**Properties of *Ascaris* trypsin inhibitor**

The trypsin inhibitor is stable to acid and heat treatment (Green, 1957) and has a low molecular weight (8200; Pudles et al., 1967). It is similar to the chymotrypsin inhibitor with respect to reduction and reoxidation properties (Pudles et al., 1967). One microgram of the inhibitor inhibits 100% activity of 0.5 mg of trypsin (Green, 1957). According to Collier (1941) the complex formation between trypsin and the inhibitor was not reversible in the acidic range. However, Green (1957) believes that the complex formation is reversible and is competitive in the presence of substrates. The dissociation constant of the complex is $3 \times 10^{-9}$ M. Recent works of Kucich and Peanasky (1970) have shown that the bodywalls of *Ascaris* contain two trypsin inhibiting fractions differing with respect to $K_d$ values 90 nM for peak I and 13 nM for peak II respectively. In the presence of 10 mM p-toluene sulphonyl-L-arginine methyl ester, the complex formation between pork trypsin and I and II inhibitor peaks is dissociated to 65-75 and 19-22% respectively. The molecular weight has been estimated to be 5520 comprising
of 50 amino acid units. The presence of leucine, histidine, methionine and tyrosine could not be detected in the inhibitors.

**Properties of *Ascaris* chymotrypsin inhibitor**

The molecular weight of chymotrypsin inhibitor as found by molecular Sieve method was 8600 (Rola and Pudles, 1966). Later Rola and Pudles (1966) have reported that chymotrypsin inhibitor was stable to heat and 8 M urea. Reduction of the inhibitor with mercaptoethanol in the presence of 8 M urea causes inactivation of the inhibitor which is partially reversible but becomes irreversible after further treatment with iodoacetic acid thus leading to the conclusion that S-S bridges are present in the molecule. The crystalline preparation has one N-terminal group namely, arginine and is devoid of tyrosine, phenylalanine and isoleucine (Peanasky, 1963). The isoelectric point is above 10.75. Like trypsin inhibitor, chymotrypsin inhibitor is also competitive and the inhibition is strongly retarded particularly by the synthetic substrates (Werle and Boetsch, 1959). The dissociation constant of the complex with chymotrypsin A is $6.9 \times 10^{-9}$M (Peanasky and Szucs, 1964) or $4.7 \times 10^{-8}$M (Rola and Pudles, 1966) and with chymotrypsin B is $3.2 \times 10^{-8}$M (Peanasky and Szucs, 1964). The dissociation constant of the complex changes only slightly between pH 5.0 and 9.0.
Rola and Pudles (1964) obtained chymotrypsin inhibitor complex in electrophoretically pure state and the molecular weight of the complex was estimated to be 26,000 (Rola and Pudles, 1966) thereby showing that the enzyme and the inhibitor have combined in a molar ratio of 1:1 in the complex. The enzyme molecule, in the complex state, is protected by heat treatment (50 min. at 50°C) and against denaturation by urea (Rola and Pudles, 1966).

The proteolytic inhibitors have been assumed to play an important role in protecting the intestinal parasites from being digested by the digestive juices of the host. Both intestinal nematodes and cestodes have been observed to escape trypsin digestion in vitro (Fredericq, 1878; Burge and Burge, 1915; De Waele, 1933) as long as they are living and intact, but they are digested when dead or when their cuticle is injured. Protection against trypsic digestion may be an important role but not the only property. Resistance to trypsin is not confined to helminths. Mucosa of the intestinal tract itself and many free living organisms also show resistance to the digestive enzymes (Fermi, 1910; Northrop, 1926). According to Symons and Fairbairn (1963), *Nippostrongylus*, an intestinal nematode does not produce antienzymes. The amino acid sequence of trypsin inhibitor of *Ascaris* has been worked out (Fraefel and Acher, 1968).
SCOPE AND PLAN OF THE PRESENT WORK

The above survey of literature indicates that despite rapid strides made recently in carbohydrate biochemistry, the pathways by which carbohydrates are utilized by helminths still remain unelucidated. The evidence regarding the functioning of metabolic pathways of carbohydrate utilization in many worms is incomplete and only one or a few of the required enzymes have been demonstrated. This is particularly true with filarial parasites where in spite of their being a public health problem, the knowledge about their biochemistry and specially carbohydrate metabolism is very meagre. According to Bueding and Most (1953) no generalization concerning helminth metabolism can be made on the basis of results obtained with one member of a group, even though they may be related to each other morphologically and taxonomically, thus pointing out the necessity of studying each member separately. It is apparent that studies pertaining to the metabolism of carbohydrates and the enzymes mediating the individual reactions can also help in the design of new drugs.

Further, similar studies may also be of use in answering certain questions concerning the mode of action
of anthelmintic drugs; acquisition of resistance by the parasites to the digestive enzymes of the host; preponderance of anaerobic fermentations in parasites and the ultimate fate of the host due to the constant depletion of food reserves during these infections.

In recent years interest in the field of natural proteinase inhibitors has increased considerably mainly due to the multifaceted problems concerning their structure and the possibilities of their therapeutic applications. Necrosis of the pancreas in humans has for a long time been attributed to premature activation of proteolytic proenzymes in the pancreas. Hence attempts were made to prevent, or at least to reduce, the autolysis of the pancreas by the administration of proteinase inhibitors (Vogel et al., 1963). These inhibitors are also useful in the treatment of shock, pancreatitis and in the control of blood clotting. With the development of improved techniques and use of modern methods in the separation of proteins many important results have been obtained which have widened the area and increased the depth of our knowledge concerning proteolytic inhibitors.

Studies made so far with proteinase inhibitors of parasitic helminths have mainly been confined to the easily available large worm *A. lumbricoides* found in pigs and no
attention seems to have been paid towards the isolation, purification and characterization of these inhibitors from other intestinal parasites. Detailed investigations on the proteinase inhibitors from helminth parasites may also be expected to lead to the development of newer approaches in the treatment of parasitic diseases.

During the last decade the Central Drug Research Institute, Lucknow, has been engaged in the study of the chemotherapy of filarial and other helminthic infections. In the absence of human filarial parasite *W. bancrofti* as an experimental test system, other related filarial parasites like *C. hawkingi*, *L. carinii* and *S. cervi* are being used in the screening of antifilarial compounds and also for the elucidation of the intricate mechanisms of this disease.

*Setaria cervi* is a nematode parasite found in the peritoneal cavity of cattle. This parasite has a high rate of incidence and has been associated with 'lumbar paralysis' and 'cerebrospinal nematodiasis' in cattle. The parasite like the human strain *W. bancrofti* releases microfilariae in the host's blood. However, no concerted efforts have so far been made in studying the biochemistry of this parasite.
Ascaridia galli, the fowl intestinal nematode, is a constant source of infection for poultry causing loss of life and also affecting their egg laying capacity. Experimental infection of fowls with this parasitic nematode is currently used in this Institute for the evaluation of ascaricidal drugs.

The ready availability of these helminth parasites prompted the present investigations and the experiments described in the thesis were carried out with the object of studying:

1. The breakdown of endogenous carbohydrates during in vitro starvation condition of the bovine filarial parasite (S. cervi); the nature and fate of monosaccharides utilized by the parasite, and in vitro synthesis of glycogen and other macromolecules from hexoses utilized by this worm.

2. The operation of glycolytic pathway in S. cervi as evidenced by the presence of the required enzymes mediating the pathway; to see whether cyclic oxidation similar to those of the TCA cycle occur in the tissues of the two different nematode parasites and to have a comparative idea about the functioning of this pathway in a filarial (S. cervi) and an intestinal (A. galli) parasite; to examine the presence of alternate pathway in these parasites to account for the formation of some unusual end-products of anaerobic glycolysis and significance of the production of these end-products.
3. Purification of *S. cervi* aldolase, a key enzyme of the glycolytic pathway, by ammonium sulphate fractionation and DEAE-cellulose chromatography, and to see the effect of certain antifilarial compounds and metabolic inhibitors on the purified enzyme system.

4. The mechanism of host-parasite interactions with reference to the survival of the intestinal parasite *A. galli* in the presence of high concentrations of proteolytic enzymes in its habitat; localization of the proteolytic inhibitors in whole worms and different anatomical parts of *A. galli*; purification of these inhibitors by TCA and heat treatment followed by DEAE-cellulose and CM-cellulose chromatography and characterization of the inhibitors.

The investigations have indicated that glucose, the main energy source for the filarial parasite *S. cervi*, can conveniently be employed for its maintenance in vitro for studying the effect of antifilarial compounds acting particularly on the motility of the parasite. The worm is equipped with nearly all the enzymes mediating the Embden-Meyerhof pathway of glycolysis by which glucose is metabolized. The parasite is capable of synthesizing glycogen in an environment having surplus amount of glucose and the polysaccharide thus synthesized can be utilized for supplying energy under conditions of starvation. The
parasite also has a full complement of enzymes of the Krebs cycle similar to those present in other animals. *A.galli*, the intestinal parasite, contains most of the enzymes mediating TCA cycle indicating resemblance of the two nematodes. Presence of an alternate pathway branching off at PEP level has been detected in these two parasites and the production of some unusual end-products of glycolysis could be explained on the basis of the enzymes of PEP-oxaloacetate pathway.

A study with the purified aldolase of *S.cervi* revealed that the filarial enzyme resembled more closely the mammalian aldolase and hence can be classified as aldolase I. Among the various metabolic inhibitors and antifilarial drugs tried, cyanine-863 strongly inhibited the activity of this enzyme.

The intestinal parasite *A.galli* contained potent inhibitors for trypsin and chymotrypsin activities. The inhibitors separated by TCA and heat treatment followed by DEAE- and CM-cellulose chromatography were found to be low molecular weight proteins.
CHAPTER II

MATERIALS AND METHODS
Chemicals and enzymes used

Barium salts of G-6-P and FDP; NADP, AMP, ADP. ATP (disodium salt), pCMB, β-mercaptoethanol, DL-isocitrate, crystalline trypsin and rabbit muscle LDH were obtained from Sigma Chemical Co., U.S.A., while α-oxoglutarate, sodium succinate, sodium fumarate, lithium DL-lactate, iodoacetate, EDTA, 8-hydroxyquinoline, α-α-dipyridyl, hydrazine sulphate, hydroxylamine hydrochloride, semicarbazide, DL-serine, L-alanine, DL-aspartic acid, crystalline chymotrypsin, Tris, and ammonium sulphate (enzyme grade) were from British Drug House, London. Sodium pyruvate, malic acid, L-glutamic acid, cysteine hydrochloride, glutathione (reduced), veronal and o-phenanthroline were procured from E. Merck, A.G. Darmstadt, while DEAE-cellulose, CM-cellulose, benzoyl-L-arginine ethylester hydrochloride were purchased from Mann Research Lab.Inc., New York. NEM, 2-PGA (barium salt) and L-tyrosine ethylester were the products of Schwarz Bio-Research Inc., New York, and GDP and MDH were obtained from Boehringer, Germany. NADH₂, glycogen and oxaloacetate were supplied by V.P. Chest Institute, New Delhi, and NAD and G-1-P (sodium salt) by Nutritional Biochemicals Corporation, U.S.A. Sephadex G-75, PEP (trisodium salt), BPA and dihydroxyacetone phosphate were purchased from Pharmacia, Uppsala, Sweden,
Calbiochem., U.S.A., Armour Laboratories, U.S.A. and Kochlight Lab., Colnbrook, Bucks, England, respectively. Glucose-U-C\(^{14}\) and fructose-U-C\(^{14}\) were procured from the Isotope Division, The Atomic Energy Establishment, Trombay, Bombay. Rest of the chemicals used were of analytical grade. Barium salts were converted to sodium salts prior to use.

**PARASITIC MATERIALS**

*Sataria cervi*

The worms (female - average length 9 ± 0.2 cm, average weight 38 ± 0.5 mg) were collected from the peritoneal cavity of freshly slaughtered water-buffalo (*Bubalus bubalis* Linn.). They were transported to the laboratory from the slaughter house in normal saline (0.9% sodium chloride) containing glucose. The worms were washed repeatedly in normal saline for removing blood and other adhering substances. For enzymic studies, the worms were incubated for 10 hours in Krebs-Ringer bicarbonate buffer containing sufficient glucose in the presence of antibiotics.

*Ascaridia galli*

*A. galli* (average length 6 ± 2 cm, average weight 80 ± 20 mg) were collected from fowl's intestine, thoroughly washed with normal saline and left overnight at 37 ± 1°C in dextrose-NaCl (11 mM and 15 mM respectively) solution
containing per ml 1000 units penicillin G and 500 μg dihydrostreptomycin sulphate. The worms were then washed and separated into the cuticular layer (body wall), intestine, and the tubular testicular or ovarian structure. These were then immediately transferred to chilled normal saline and processed for making homogenates.

METHODS

Metabolic studies with *S. cervi*

Weighted quantities of female worms (about 300-400 mg) were suspended in 10 ml Krebs-Ringer bicarbonate buffer, pH 7.4 (DeLuca and Cohen, 1964) containing glucose, fructose, mannose or galactose (1 mg/ml) in Erlenmeyer flasks of 50 ml capacity. Three hundred units of penicillin G and 100 μg dihydrostreptomycin sulphate per ml were added to the medium (for overnight incubation twice the volume was used). The flasks were incubated aerobically at 37°C. Controls that contained all the constituents except the sugars were run simultaneously. The worms were removed after two hour intervals, washed twice by dipping in normal saline and then transferred to a fresh set of flasks containing identical media. Concentrations of reducing sugars (glucose, mannose and galactose) and that of fructose, pyruvic and lactic acids were estimated in each set of the medium.
Incorporation of glucose-U-\(^{14}\)C and fructose-U-\(^{14}\)C into glycogen and other macromolecules of \(S.\ cervi\)

The incubation medium and other relevant details were the same as described earlier except that one micro Curie of labelled hexose (specific activity: 17 and 20 m Curie per m mole respectively for glucose and fructose) per reaction mixture was added and after two hours incubation at 37\(^{\circ}\)C the worms were washed thrice with normal saline containing carrier hexose. For observing the breakdown of the \(^{14}\)C-incorporated glucose and the excretion of labelled metabolic products, the worms were transferred to flask containing sugar-free media and the contents were analysed after eight hours starvation period. The worms were homogenized in 0.15 M KCl and 20% homogenate thus prepared were used for the separation of glycogen, lipids, nucleic acids and proteins employing the following procedures:

Glycogen was isolated from an aliquot of the homogenate according to the method of Good \textit{et al.} (1933) and the remainder was treated with cold PCA (final conc. 5%), and lipid was extracted from PCA sediment by treatment with chloroform:methanol (2:1 v/v). The lipid-free residue was treated with 5% PCA and kept at 90\(^{\circ}\)C for 10 min., centrifuged and the sediment containing proteins was dissolved in 0.1N NaOH. The hot PCA supernatant contained nucleic acids. Appropriate volumes of all the fractions.
were applied on Whatmann No. 3 filter paper strips (2.0 x 2.5 cm) and counted in a Packard Tricarb Liquid Scintillation Spectrometer using scintillation fluid containing 0.4 % 2,5-diphenyloxazol (PPO) and 0.01 % dimethyl-1,4-bis[2 (5-phenyloxazol)-benzene] (POPOP).

**ENZYMIC STUDIES**

Preparation of tissue extracts

A ten per cent (w/v) homogenate of *S. cervi* and *A. galli* (whole worms or different anatomical parts) was prepared in ice-cold 0.15 M potassium chloride or M/15 phosphate buffer, pH 7.4 using Potter Elvehjem tissue homogenizer fitted with a Teflon grinder. The tissue extracts were chilled in an ice-bath throughout homogenization. The homogenate obtained was centrifuged at 800 g (15 min.) in a refrigerated Janetzki K24 centrifuge for removing cell debris and the supernatant was used for the assay of Krebs cycle enzymes.

The cell-free homogenate of *S. cervi* was further subjected to fractional centrifugation at 12,000 g (45 min.) and 105,000 g (60 min.) for the separation of mitochondria and ribosomes. The enzymes of glycolytic pathway were assayed in particulate-free supernatant, mitochondria and ribosomes.
Dehydrogenation of Krebs-cycle intermediates and some amino acids employing Thunberg technique

The modified Thunberg technique (Battelli and Stern, 1910; Szent-Gyorgyi, 1924) was employed for the dehydrogenation of Krebs-cycle intermediates using methylene blue as the electron acceptor. To the Thunberg tube were added 1 ml of 2.67 x 10^{-4} M methylene blue, 2 ml of M/50 substrate (40 \mu moles), 2 ml of M/15 phosphate buffer, pH 7.4 (133 \mu moles), 1.0 ml (9-10 mg) enzyme protein and volume made up to 6.0 ml. The enzyme preparation was placed in the sidecap and the joint of the cap was greased with high vacuum grease and firmly placed on the tube. The tube was then evacuated completely using high vacuum oil-pump. The tube was then placed in a water bath at 37^\circ C for 10 min. for equilibration. The contents were then mixed carefully and the reduction of the dye was observed visually. Comparison was made against a tube containing one-tenth the dye concentration without any substrate which represents 90 % reduction of the dye in the assay. For each substrate a separate control was also run which contained heat-killed enzyme and the respective substrate.

Definition of activity

The activity is expressed in terms of Q_{MB} (Corren et al., 1939). The Q_{MB} was defined as microlitres of H_{2} transferred.
to methylene blue per hour per mg protein. 1 ml of $1 \times 10^{-3}M$ methylene blue requires 22.4 µl of $H_2$; hence 1 ml of $2.67 \times 10^{-4}M$ methylene blue used in the assay requires 5.98 µl of $H_2$ or at 90% reduction, 5.44 µl of $H_2$.

ASSAY OF ENZYMES

Spectrophotometric estimations

Measurements of extinction changes were carried out in a Beckman DU Spectrophotometer with silica cuvettes having 1-cm light path. Except when otherwise stated, all assay mixture were incubated for 3 min. at the appropriate temperature before starting the reaction by the addition of the substrate. Rates of changes in the extinction values were noted both for the period prior to the addition of the substrate as well as for the period of actual enzymic reaction following the addition of the substrate. The net reaction rate was calculated from the difference between the two changes in extinction. All assays were performed at $28 \pm 2^\circ C$ (room temperature) in a reaction mixture of 3.0 ml volume unless otherwise stated.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase; EC 1.1.1.49)

It was assayed according to the procedure of Shonk and Boxer (1964). Reaction mixture contained: Tris-HCl buffer,
pH 7.4, 200 μ moles; MgCl₂, 10 μ moles; glucose-6-phosphate, 10 μ moles; NADP, 0.24 μ mole; and 600-800 μg enzyme protein. Increase in absorbancy at 340 nm was followed for 5 min.

**Phosphopyruvate hydratase** (2-phospho-D-glycerate hydro-lyase; EC 4.2.1.11)

It was assayed according to the method of Warburg and Christian (1941). The reaction mixture contained:
- Tris-HCl buffer, pH 7.4, 200 μ moles; MgCl₂, 10 μ moles;
- 2-phosphoglycerate, 5 μ moles; and enzyme protein 150-300 μg.
Increase in absorbancy at 240 nm was followed for 5 min.

**Pyruvate kinase** (ATP:pyruvate phosphotransferase; EC 2.7.1.40)

It was assayed by a method based on that described by Shonk and Boxer (1964). The reaction mixture contained:
- Tris-HCl buffer, pH 7.4, 200 μ moles; MgCl₂, 10 μ moles;
- ADP, 5 μ moles, PEP, 5 μ moles; rabbit muscle LDH, 9.0 U;
- NADH₂ 0.24 μ mole; and enzyme protein 2.5 - 3.5 mg. Decrease in absorbancy at 340 nm was followed for 5 min.

**Lactate dehydrogenase** (L-lactate:NAD oxidoreductase; EC 1.1.1.27)

It was assayed according to Kornberg (1955). The reaction mixture contained: Tris-HCl buffer, pH 7.4, 200 μ moles; MgCl₂, 10 μ moles; sodium pyruvate, 5 μ moles; NADH₂, 0.24 μ mole; and enzyme protein 20-40 μg. Decrease in absorbancy at 340 nm was followed for 5 min.
Phosphoenol pyruvate carboxykinase (GTP:oxaloacetate carboxylyase (transphosphorylating); EC 4.1.1.32)

It was assayed by the method of Utter and Kurahashi (1954) as adapted by Ward et al. (1969). The reaction mixture contained: Tris-HCl buffer, pH 7.4, 200 μ moles; MnCl₂, 10 μ moles; NaHCO₃, 20 μ moles; guanosine diphosphate, 1 μ mole; MDH 8.0 U; PEP, 5 μ moles; NADH₂ 0.24 μ mole; and enzyme protein 300-500 μg. Decrease in absorbancy at 340 nm was followed for 5 min.

Aconitate hydratase (citrate (isocitrate) hydro-lyase; EC 4.2.1.3)

It was assayed according to the method described by Racker (1950). Reaction mixture contained: phosphate buffer, pH 7.4, 146 μ moles; citric acid, 75 μ moles; and 1.0-1.5 mg (A. galii) or 0.5-1.0 mg (S. cervi) enzyme protein. Increase in absorbancy at 240 nm was followed for 5 min.

Isocitrate dehydrogenase (Ls-isocitrate:NADP oxidoreductase (decarboxylating); EC 1.1.1.42)

It was assayed according to the procedure of Ochoa (1955). Reaction mixture contained: Tris-HCl buffer, pH 7.4, 200 μ moles; MgCl₂, 10 μ moles; MnCl₂ 10 μ moles; isocitrate, 10 μ moles; NADP, 0.24 μ mole; and 0.8-1.2 mg (A. galii) or 0.5-1.0 mg (S. cervi) enzyme protein. Increase in absorbancy at 340 nm was followed for 5 min.
Fumarate hydratase (L-malate hydro-lyase; EC 4.2.1.2)

It was assayed according to the method described by Racker (1950).

**Forward Reaction**

\[ \text{Fumarate} + \text{H}_2\text{O} \rightarrow \text{Malate} \]

Reaction mixture contained: phosphate buffer, pH 7.4, 150 μ moles; sodium fumarate, 6.8 μ moles; and 1.0-1.5 mg (A. galli) or 0.5-1.0 mg (S. cervi) enzyme protein. Decrease in absorbancy at 240 nm was followed for 5 min.

**Backward Reaction**

\[ \text{Malate} \rightarrow \text{Fumarate} + \text{H}_2\text{O} \]

Reaction mixture used was of the following composition: phosphate buffer, pH 7.4, 150 μ moles; L-malate, 30 μ moles; and 20-40 μg enzyme protein. Increase in absorbancy at 240 nm was followed for 5 min.

Malate dehydrogenase (L-malate: NAD oxidoreductase; EC 1.1.1.37)

It was assayed according to the method of Ochoa (1948a). Reaction mixture contained: phosphate buffer, pH 7.4, 75 μ moles; oxaloacetate, 0.76 μ mole; NADH₂, 0.24 μ mole; and 1.2-1.6 μg (A. galli) or 0.5-1.1 μg (S. cervi) enzyme protein. Decrease in absorbancy at 340 nm was followed for 5 min.
Malate dehydrogenase (decarboxylating) (Malic enzyme) (L-malate: NADP oxidoreductase (decarboxylating); EC 1.1.1.40)

It was assayed according to the method described by Ochoa (1948b). Reaction mixture contained: Tris-HCl buffer, pH 7.4, 75 µ moles; MnCl₂, 5 µ moles; L-malic acid, 3 µ moles; NADP, 0.24 µ mole; and 0.8-1.3 mg (A. galli) or 0.5-1.0 mg (S. cervi) enzyme protein. Increase in absorbancy at 340 nm was recorded for 5 min.

Trypsin and chymotrypsin

Solutions of trypsin and chymotrypsin were made in 1 x 10⁻³ N HCl. Trypsin and chymotrypsin (BDH - crystalline preparations) esterase activities were determined by the spectrophotometric procedure of Schwert and Takenaka (1955) using benzoyl-L-arginine ethyl ester and L-tyrosine ethyl ester respectively as substrates.

Trypsin (EC 3.4.4.4)

In a total volume of 3.2 ml the assay system contained: Tris-HCl buffer, pH 8.0, 150 µ moles; benzoyl-L-arginine ethyl ester, 3 µ moles; calcium chloride, 64 µ moles; and 10 µg enzyme. The increase in absorbancy was followed at 254 nm for 5 min.
Chymotrypsin (EC 3.4.4.5)

The assay system in total volume of 3.2 ml contained: Tris-HCl buffer, pH 7.0, 150 μ moles; L-tyrosine ethyl ester, 6 μ moles; CaCl₂, 64 μ moles; and 100 μg enzyme. The decrease in absorbancy was followed at 234 nm for 5 min.

Trypsin and chymotrypsin inhibitors

Activity of the inhibitor was determined by incubating the desired quantity of the inhibitor with trypsin or chymotrypsin in 0.05 M Tris-HCl buffer, (pH 8.0 in case of trypsin and pH 7.0 for chymotrypsin) in a final volume of 1.7 ml at 37°C for 10 min.

The residual activity for trypsin or chymotrypsin was determined by adding 1.5 ml of substrate (containing 3 and 6 μ moles for trypsin and chymotrypsin respectively) and recording the optical density change for 3 min. at intervals of 30 seconds.

Colorimetric estimations

The volume of each reaction mixture was 1.0 ml unless otherwise specified. Appropriate blanks, where the substrates were added after stopping the reaction, were run simultaneously. The reaction mixtures were preincubated for 5 min. to bring the contents to the temperature of the reaction i.e. 37°C before starting the enzyme reaction.
Glycogen phosphorylase ($\alpha$-1,4-glucan:orthophosphate glucosyltransferase; EC 2.4.1.1)

It was assayed according to the method described by Cavier and Savel (1952). The reaction mixture contained:
citrate buffer, pH 5.8, 50 μ moles; cysteine, 12 μ moles;
sodium fluoride, 25 μ moles; AMP, 0.14 μ mole; glycogen, 20 mg; and enzyme protein 600-800 μg. The reaction was stopped at 0 and 60 min. incubation at 47°C with 1.0 ml ice cold TCA (10 %) and phosphorus estimated in the protein free supernatant fluid.

Hexokinase (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1)

It was assayed by the method of Crane and Sols (1953).
For allowing hexose disappearance, the reaction mixture contained: Tris-HCl buffer, pH 7.4, 50 μ moles; KH$_2$PO$_4$, 2 μ moles; MgCl$_2$, 10 μ moles; ATP, 5 μ moles; NaF, 50 μ moles; hexoses, 2 μ moles; and enzyme protein 1.0-1.5 mg.
After 60 min. incubation at 37°C, proteins and phosphorylated derivatives were removed by precipitation with Na$_2$SO$_4$ and ZnSO$_4$ and the residual hexoses were estimated colorimetrically.

Phosphoglucomutase (D-glucose-1,6-diphosphate:D-glucose-1-phosphate:phosphotransferase; EC 2.7.5.1)

It was assayed according to the technique of Najjar (1948) as modified by Sutherland (1949). The reaction mixture contained: Tris-HCl buffer, pH 7.4, 50 μ moles; MgCl$_2$,
10 \mu{ moles}; cysteine, 10 \mu{ moles}; G-1-P, 10 \mu{ moles}; and enzyme protein 200-400 \mu{g}. After 30 min. incubation the reaction was stopped with 5N H_2SO_4 and labile phosphorus was estimated in the protein free filtrate.

**Glucosephosphate isomerase (D-glucose-6-phosphate ketol-isomerase; EC 5.3.1.9)**

It was assayed according to the method of Bodansky (1961). The reaction mixture contained: Tris-HCl buffer, pH 8.6, 50 \mu{ moles}; MgCl_2, 10 \mu{ moles}; G-6-P, 10 \mu{ moles}; and enzyme protein 80-100 \mu{g}. After 30 min. incubation, 1.0 ml of 10 \% TCA was added and fructose-6-phosphate was estimated in the protein free reaction mixture.

**Aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase; EC 4.1.2.13)**

It was assayed by the Sibley and Lehninger (1949) procedure adapted for microassay to suit the conditions of the present experiment. In a final volume of 1 ml, reaction mixture consisted of: Tris-HCl buffer, pH 8.6, 50 \mu{ moles}; hydrazine sulphate, 28 \mu{ moles}; FDP, 2.5 \mu{ moles}; and enzyme protein 40-60 \mu{g}. After 30 min. reaction was brought to end by an equal volume of 10 \% TCA and colour was developed in the aliquots of protein free supernate after hydrolysis of the triosephosphates. The method was standardized by the parallel determination of alkali-labile phosphate from the triosephosphates formed during aldolase action. Alkali-labile
phosphate formed from TCA filtrates obtained from reaction mixtures containing different concentrations of enzyme was plotted against Klett readings (540 nm) of the trapped trioses with dinitrophenyl hydrazine. A straight line was obtained from which a factor was calculated to facilitate the conversion of the Klett readings of the dinitrophenyl hydrazones into μ mole FDP transformed.

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9)

It was assayed according to the method of Swanson (1950) as modified by Shull et al. (1956). The reaction mixture contained: Tris-HCl buffer, pH 7.4 or citrate buffer, pH 6.5, 50 μ moles; MgCl₂, 10 μ moles; G-6-P, 10 μ moles; and enzyme protein 2-3 mg. The reaction was stopped with TCA after 60 min. and phosphorus estimated in the protein free supernatant fluid.

Hexosediphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase; EC 3.1.3.11)

It was assayed according to the method of Freedland and Harper (1959). The reaction mixture contained: Tris-HCl buffer, pH 8.6, 50 μ moles; MgCl₂, 10 μ moles; cysteine, 12 μ moles; FDP, 10 μ moles; and enzyme protein, 600-800 μg. Phosphorus was estimated in the protein free filtrates obtained by the addition of 1 ml of 10% TCA to the reaction mixture after 60 min. run and centrifugation.
\(\alpha\)-Ketoglutarate dehydrogenase (2-oxoglutarate: lipoate oxidoreductase (acceptor-acylating); EC 1.2.4.2)

It was assayed according to the procedure described by Srikantan and Krishna Murti (1955). The incubation mixture in a total volume of 3.0 ml consisted of phosphate buffer, pH 7.4, 100 \(\mu\) moles; \(\alpha\)-ketoglutaric acid, 100 \(\mu\) moles; 0.5 ml of 0.5% aqueous solution of triphenyl tetrazolium chloride salt, 4.0-8.0 mg (A. galli) or 2.0-4.0 mg (S. cervi) enzyme protein. Simultaneously a control was run which contained all the things except substrate which was added after the reaction was stopped. After incubation for 2-3 hours the colour was extracted by adding to the reaction mixture 6 ml each of glacial acetic acid and toluene. The colour intensities were read at 496 nm using Beckman Spectrophotometer, model DU. The O.D. values of the controls were subtracted from the experimental values for obtaining the net O.D. change.

Succinate dehydrogenase (succinate: (acceptor) oxidoreductase; EC 1.3.99.1)

It was assayed according to the method described by Slater and Bonner (1952) with slight modifications. The reaction mixture in a total volume of 3.0 ml contained: phosphate buffer, pH 7.2, 150 \(\mu\) moles; sodium succinate, 20 \(\mu\) moles; potassium cyanide, 30 \(\mu\) moles; potassium ferricyanide, 3 \(\mu\) moles; and 3-6 mg (A. galli) or 2-4 mg (S. cervi)
enzyme protein. After incubation for 30 min. the reaction was stopped by the addition of 2 ml of 10% TCA. In the control tube the substrate was added after the addition of TCA. The tubes were then centrifuged and the clear supernatant was read at 400 nm against water blank. The values of the controls were subtracted from the experimental values for obtaining net O.D. change.

Definition of unit and specific activity

One unit of the enzyme activity is the amount of enzyme required to catalyse the transformation of one n mole substrate or the formation of one n mole of the product per min. under the specified experimental conditions. Specific activity is units per mg of enzyme protein.

The units for the dehydrogenases were calculated using the extinction coefficients of the reduced NAD or NADP, $6.22 \times 10^6$/cm$^2$/mole (Horecker and Korenberg, 1948).

The molar extinction-coefficient used for aconitase is $3.3 \times 10^3$ cm$^{-1}$M$^{-1}$ for cis-aconitate, and for fumarase is $2.4 \times 10^3$ cm$^{-1}$M$^{-1}$ for fumarate, at 240 m$\mu$ (Mahler et al., 1958).

The activity of trypsin or chymotrypsin is expressed in $\mu$ moles of substrate converted per min. according to the proposal of King and Campbell (1960; 1961) and Well (1960; 1961).
The unit of trypsin is calculated according to the formula of Trautschold and Werle (1961):

\[ \frac{E_{283}/\text{min}}{0.359} = \mu \text{ moles substrate hydrolysed/min}. \]

The unit of chymotrypsin is calculated according to the formula of Schwert and Takenaka (1955):

\[ \frac{E_{283}/\text{min}}{0.210} = \mu \text{ moles substrate hydrolysed/min}. \]

One unit of the trypsin or chymotrypsin inhibitor was defined as the amount of inhibitor which inactivated one unit of the enzyme. Specific activity was defined as units per mg of inhibitor protein.

**ION EXCHANGE CHROMATOGRAPHY**

**Preparation of DEAE-cellulose column**

2.0 g of DEAE-cellulose (Mann Research) were suspended in glass-distilled water, thoroughly stirred and allowed to settle down. After 30 min. the supernatant was decanted off so as to remove the fine particles and the sediment was suspended in 1.0 N NaOH for 2 hours with occasional shaking. The supernatant was again removed and the residue was washed with glass-distilled water. A piece of glasswool was placed at the bottom of a column and the resin was then poured into it (1.5 x 15 cm or 1 x 15 cm as required) in such
a way so as to get a uniform packing and to avoid air
bubbles. A piece of sponge was kept at the top of the
column so as not to disturb the upper layers of the
column. The excess of alkali from the column was removed
by passing large amounts of water till the washings were
free from it. To convert the resin to the chloride form,
200 ml of 1.0 M HCl were slowly passed through the column
and the excess of the acid removed by passing several bed
volumes of glass-distilled water. The column was then
finally equilibrated with 0.02 M Tris-HCl buffer, pH 7.4
or 8.0 (as required) and kept in cold for further use.

Regeneration of the column

The DEAE-cellulose column could be employed for
repeated chromatography after regeneration of the resin.
Sodium chloride (1M) was passed through the column for
removing all the adhering proteins of the previous
experiment and the column was then washed with sodium
hydroxide followed by hydrochloric acid according to the
procedure described earlier.

Preparation of carboxymethyl-cellulose column

1.5 g of carboxymethyl cellulose powder (1213 Mannex-CM)
were suspended in glass-distilled water, stirred thoroughly
and allowed to settle down. After 30 min. the supernatant
was decanted off and sediment was again suspended in water
and poured in a column (1 x 11.5 cm). A piece of glasswool was previously packed at the bottom of the column for holding the cellulose ion-exchanger. The glasswool was kept for sometime in boiling water so as to remove air bubbles. The column was allowed to settle down under its own pressure while water continued to flow out of the column. A piece of sponage was placed on the top of the column to prevent any disturbance of the column during the addition of the eluting buffer. About 250 ml of glass-distilled water was passed through the column to wash and pack the resin.

100 ml of 0.01 M sodium chloride prepared in glass-distilled water were passed through the column which was then shifted to the cold room. A column height of 5-10 ml sodium chloride was always maintained at the top of the column.

The CM-cellulose column could be used repeatedly if the material loaded on it consisted of only soluble proteins. After use, 100 ml of molar sodium chloride were passed through the column for removing proteins adhering to the exchanger. The CM-cellulose was then poured into a beaker and repeatedly washed by decantation with distilled water until free from chloride, equilibrated and used as previously described.
PURIFICATION OF S. CERVI ALDOLASE

Step 1 - Ammonium sulphate fractionation

The ribosome-free 105,000 g supernatant was brought to 25% saturation with respect to ammonium sulphate by the addition of solid ammonium sulphate with constant stirring. The precipitate was allowed to settle for two hours and then removed by centrifugation at 4,000 g for 10 min. using Janetzki K24 refrigerated centrifuge. The supernatant thus obtained was taken to 45% saturation of ammonium sulphate, the mixture allowed to stand for three hours for complete precipitation and the resulting supernatant was then brought to 60% saturation with respect to ammonium sulphate and kept overnight (12-14 hours) at 4-6°C. The supernatant obtained was finally brought to 80% saturation of ammonium sulphate. All the precipitates were dissolved in small volumes of 10 mM Tris-HCl buffer, pH 7.4, dialysed against double distilled water with gentle agitation till free from ammonium sulphate and assayed for aldolase and a few other glycolytic enzymes for localization purposes.

All operations were carried out in cold room unless otherwise mentioned.

Step 2 - Chromatography on DEAE-cellulose column

The fraction obtained between 45-60% saturation of
ammonium sulphate having maximum aldolase activity was
applied in a quantity equivalent to 80-100 mg protein to
the top of a DEAE-cellulose column (1.5 x 16 cm) previously
equilibrated with 10 mM Tris-HCl buffer, pH 7.4. Elution
of the proteins was carried out stepwise with 60 ml aliquots
of 10 mM Tris-HCl buffer, pH 7.4 containing in sequence
0, 50, 100, 200, 300 and 500 mM NaCl. The buffer-NaCl
mixtures were allowed to flow through the column at a
rate of one ml per min. Five ml fractions were collected
and the protein content of each fraction was monitored
by determining the absorbancy at 260 and 280 nm. Fractions
possessing aldolase activity were pooled, dialysed against
distilled water for short period and stored frozen at
-20°C in small aliquots to avoid freezing and thawing.

Procedure for studying thermal inactivation of
S. cervi aldolase

0.5 ml aliquots of the enzyme fraction were taken
in a series of small test tubes and immersed in a water
bath at the desired temperature. The tubes were gently
shaken for half a minute and then corked so that no water
could evaporate. After heating for 30 min., each tube was
plunged into an ice bath, which quickly stopped any further
destruction of the enzyme. The corked tubes were tilted
and rotated horizontally to collect condensed moisture on
the walls and the residual aldolase activities of the
solutions heated to various temperatures as well as that of
an unheated control were recorded.
Procedure for studying action of inhibitors and activators on S. cervi aldolase

The compounds under study were preincubated with enzyme in a buffered medium for 15 min. at 37°C. Substrate was then added to the mixture and reaction allowed to proceed as described under enzyme assay procedures.

The effect of the protecting or reversing agent was studied by first incubating the enzyme with the activator for 15 min. followed by incubation with inhibitor for another 15 min. In certain instances the order of addition of activator and inhibitor was reversed, the substrate added and the enzyme reaction allowed to proceed for specified period.

Isolation and purification of proteolytic inhibitors of A. galli

Whole worms (A. galli) or the separate anatomical parts were cut into small segments, crushed in a pestle mortar and the pulp (10% w/v) was homogenized in 0.15 M potassium chloride using a Potter Elvehjem homogenizer fitted with a Teflon grinder. All the operations were performed at room temperature unless otherwise indicated. The homogenate was centrifuged at 800 g for 15 min. and the supernatant obtained was recentrifuged at 12,000 g for 45 min. The supernatant of the second centrifugation was further spun at 105,000 g for 60 min. The inhibitory activity was
assayed in all the fractions and the fraction exhibiting maximum activity was stored in deep-freeze until taken up for further processing.

Most of the inhibitory activity was found to be localized in the particulate-free supernatant of the cuticular layer. However, in view of the large quantities of starting material required for purification, the particulate-free supernatant of the whole worm was used. 105,000 g supernatant was treated with TCA (final concentration 5%) with constant stirring. It was now heated in a water bath at 70°C for 5 min., cooled, filtered and the clear filtrate was dialysed overnight against distilled water for removing TCA. The TCA free material was then lyophilized. This powder obtained was kept in cold till taken up for further purification by ion-exchange chromatography.

The lyophilized material was applied in a quantity equivalent to 10 mg protein to the top of a DEAE-cellulose (Mann Research) column (1 x 15 cm) previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0. Elution of the proteins was carried out stepwise with 50 ml aliquots of 0.02 M Tris-HCl buffer, pH 8.0 containing in sequence 0, 0.05, 0.1, 0.2, 0.3 and 0.5 M NaCl. The rate of flow of the buffer-NaCl mixture through the column was adjusted at 1 ml per min. Each fraction contained 5 ml volumes.
The two active peaks, one eluted with the equilibrating buffer, and the other with 0.05 M NaCl were pooled separately. The first peak (eluted with the equilibrating buffer) was dialysed against distilled water and then lyophilized. This lyophilized material was then passed through a carboxymethyl cellulose (1218 Mannex-CM) column (1 x 11.5 cm), and the proteins were eluted with 50 ml aliquots of 0.01, 0.05, 0.1, 0.2 and 0.5 M NaCl. Each fraction contained 5 ml. The flow rate was 1 ml per min. The active peaks eluting with 0.05 M and 0.1 M NaCl were pooled separately, dialysed against distilled water and then kept in frozen condition in small aliquots until taken out for further studies.

All the chromatographic runs were carried out at room temperature.

Effect of purified inhibitors on the enzyme activities

The effect of varying concentration of the inhibitors was studied by incubating 10 micrograms of trypsin or 100 micrograms of chymotrypsin with various concentrations of the inhibitors and then the remaining enzyme activities were assayed.

Complex formation between enzymes and the inhibitors

Enzyme and the inhibitor were mixed and incubated at different time intervals after which the remaining enzyme activity was assayed by the addition of substrate.
Effect of heat on the inhibitors

The inhibitor proteins in aqueous solution at a concentration of 50 μg/ml were incubated for 15 min. at 60°, 70°, 80°, 90° and 100°C. The samples were cooled to room temperature and the remaining inhibitory activities were assayed using 0.1 ml aliquots.

Effect of urea

Inhibitor solutions were mixed with urea solution (final concentration of urea being 1-8 M) and incubated for four hours at room temperature and residual inhibitory activities assayed.

pH stability

Aqueous solutions of the inhibitors were mixed with buffers of different pHs from 3-10 (the final concentration of buffer being 0.1 M). After 24 hours at 5°C, aliquots were taken and assayed for remaining inhibitory activities.

Effect of mercaptoethanol

Inhibitor solutions were added to β-mercaptoethanol (final concentration being 0.01-0.1 M) and left for 4 hours at room temperature, then aliquots were used for the assay of remaining inhibitory activities.
Effect of high salt concentration on the enzyme-inhibitor complex

A concentration range of the inhibitor was chosen which inactivated the enzyme to about 90-95%. At this particular concentration, the inhibitor was incubated with enzyme in presence of 1-4 M potassium chloride solution and the inhibitory activity was assayed. Simultaneously controls were also run where the enzyme was incubated with different concentrations of KCl alone and the enzyme activity was assayed.

Estimation of molecular weights of the trypsin and chymotrypsin inhibitors by Sephadex gel-filtration

The molecular weights were determined essentially according to Andrews (1964). Sephadex-gel filtration medium G-75 (Lot No. 70-5079; particle size (U.S. standard) 40-120 μ) was suspended in distilled water and allowed to swell for two days, and then the smaller particles were removed by decantation. The column was prepared by pouring a thin slurry of gel particles in buffer solution into a vertical tube (2 cm internal diameter) over a layer of acid-washed sea sand, already partly filled with buffer. The addition of gel was continued until a bed height of 52 cm was obtained and then a solvent reservoir was connected to the top of the column and the flow of buffer maintained at a rate of approximately 30 ml per hour for two days. By this time the bed had settled to a constant height, which
was then adjusted to 50 cm by addition or removal of gel. The void volume was determined by passing a solution of blue dextran (2 mg) through the column.

**Procedure for column run**

The experiment was performed at room temperature with column equilibrated with 0.05M Tris-HCl buffer, pH 7.5, containing KCl (0.1M). Proteins were dissolved in the equilibration buffer and the solution was applied to the top of the column by layering under the buffer already present. The column effluents were collected in 3 ml fractions and assayed for various proteins using standard procedures. As molecular weight standards, the following proteins were used: bovine serum albumin, 70,000; chymotrypsin, 24,500; lysozyme, 14,400 and cytochrome C, 12,400.

**ANALYTICAL PROCEDURES**

Carbohydrate was determined as total hexoses by the anthrone reaction (Spiro, 1966) based on the earlier method of Roe (1955). The reaction employed is the formation in concentrated sulphuric acid of furfural derivatives which reacted with anthrone to form a blue green colour (Dreywood, 1946). Phosphorus was determined by the method of Fiske and SubbaRow (1925). Reducing sugars (glucose, mannose and galactose) were determined by the colorimetric
method of Nelson (1944) in aliquots of Ba(OH)₂-ZnSO₄ (Somogyi, 1945). Lactic and pyruvic acids in the protein free TCA supernates were estimated according to Barker and Summerson (1941) and Lardy (1964) respectively. Fructose content was analysed as described by Roe (1934). The protein content of the enzyme preparations was estimated spectrophotometrically according to Warburg and Christian (1941) or colorimetrically using a slight modification of the Folin phenol method of Lowry et al. (1951) using bovine plasma albumin as standard. Sialic acid was determined by thiobarbutaric acid method as described by James et al. (1966), which measures free N-acetyl, N-glycolyl and equine N-o-diacetylneuraminic acids. Glycogen from worms was isolated by digesting the fresh tissues with 30 % KOH at the temperature of boiling water bath for 15-20 min, and precipitating the polysaccharide with 2.5 volume of 95 % alcohol, and estimated according to the phenol-sulphuric acid method of Montogomery (1957) for total carbohydrates.
CHAPTER III

RESULTS AND DISCUSSIONS
SECTION I

METABOLIC STUDIES ON S. CERVI
Breakdown of endogenous carbohydrates

The filarial worms (*S. cervi*) excreted lactic and pyruvic acids when incubated in unsupplemented Krebs-Ringer bicarbonate medium; however, they did not liberate fructose or any reducing sugar. The breakdown of endogenous carbohydrates and formation of lactic and pyruvic acids are shown in Table 1. In the earlier periods of incubation (without any added sugar) a good amount of lactic acid was formed and it decreased along with the progress of time. During overnight (12-24 hours) incubation minimal amount of lactic acid was produced which reached zero level after 24 hours of incubation. In contrast, the production of pyruvic acid was more or less constant and did not change appreciably along with time. However, during overnight incubation the level was reduced to half and no pyruvic acid was produced on further incubation.

Utilization of sugars

When the worms were incubated aerobically in Krebs-Ringer bicarbonate medium fortified with glucose, mannose, galactose or fructose, they consumed the sugar and formed lactic and pyruvic acids. Tables 2-5 represent the average values for the quantitative utilization of glucose, mannose, fructose or galactose and lactic and pyruvic acids formed by four to five sets of adult motile *S. cervi*. Among the four
Table 1 - Breakdown of endogenous carbohydrates and formation of lactic and pyruvic acids by *S. cervi* during *in vitro* incubation (Mean with SE)

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>Lactic acid formed (μ moles/hr/g wet body weight at 37°C)</th>
<th>Pyruvic acid formed (μ moles/hr/g wet body weight at 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 2</td>
<td>16.95 ± 0.97</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>2 - 4</td>
<td>10.52 ± 0.59</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>4 - 6</td>
<td>9.77 ± 0.89</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>6 - 8</td>
<td>9.44 ± 0.41</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>8 - 10</td>
<td>7.44 ± 0.56</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>10 - 12</td>
<td>6.22 ± 0.77</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>12 - 24</td>
<td>0.93 ± 0.11</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>24 - 26</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Number of determinations were four in each case.
Table 2 - Glucose consumption and formation of lactic and pyruvic acids by *S. cervi* during in vitro incubation (Mean with SE)

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>μ moles/hr/g wet body weight at 37°C</th>
<th>Lactate/Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose consumed</td>
<td>Lactic acid formed</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>0 - 2</td>
<td>47.30 ± 6.54</td>
<td>26.57 ± 2.65</td>
</tr>
<tr>
<td>2 - 4</td>
<td>34.98 ± 3.06</td>
<td>29.90 ± 3.65</td>
</tr>
<tr>
<td>4 - 6</td>
<td>30.02 ± 2.33</td>
<td>30.83 ± 4.10</td>
</tr>
<tr>
<td>6 - 8</td>
<td>27.64 ± 2.99</td>
<td>30.40 ± 4.10</td>
</tr>
<tr>
<td>8 - 10</td>
<td>23.17 ± 3.12</td>
<td>32.20 ± 3.50</td>
</tr>
<tr>
<td>10 - 12</td>
<td>20.73 ± 2.77</td>
<td>33.13 ± 3.45</td>
</tr>
<tr>
<td>12 - 24</td>
<td>21.11 ± 2.08</td>
<td>21.90 ± 0.40</td>
</tr>
<tr>
<td>24 - 26</td>
<td>14.48 ± 3.21</td>
<td>26.87 ± 0.75</td>
</tr>
</tbody>
</table>

Number of determination were five in each case
Table 3 - Utilization of mannose and formation of lactic acid by *S. cervi* during *in vitro* incubation (Mean with SE)

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>µ moles/hr/g wet body weight at 37°C</th>
<th>Lactate/Mannose (µ mole/µ mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 2</td>
<td>42.2 ± 2.5</td>
<td>15.6 ± 1.0</td>
</tr>
<tr>
<td>2 - 4</td>
<td>29.3 ± 1.3</td>
<td>17.2 ± 1.3</td>
</tr>
<tr>
<td>4 - 6</td>
<td>19.5 ± 0.8</td>
<td>17.5 ± 1.2</td>
</tr>
<tr>
<td>6 - 8</td>
<td>12.2 ± 0.5</td>
<td>18.5 ± 1.1</td>
</tr>
<tr>
<td>8 - 10</td>
<td>11.9 ± 0.6</td>
<td>15.5 ± 1.0</td>
</tr>
<tr>
<td>10 - 12</td>
<td>7.6 ± 0.3</td>
<td>12.6 ± 0.9</td>
</tr>
</tbody>
</table>

Number of determinations were four in each case.
Table 4 - Utilization of fructose and formation of lactic acid by *S. cervi* during *in vitro* incubation (Mean ± with SE)

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>μ moles/hr/g wet body weight at 37°C</th>
<th>Lactate/Fructose (μ mole/μ mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fructose consumed</td>
<td>Lactic acid formed</td>
</tr>
<tr>
<td>0 - 2</td>
<td>9.4 ± 0.81</td>
<td>11.8 ± 1.0</td>
</tr>
<tr>
<td>2 - 4</td>
<td>6.4 ± 0.55</td>
<td>15.8 ± 1.2</td>
</tr>
<tr>
<td>4 - 6</td>
<td>6.1 ± 0.49</td>
<td>18.1 ± 1.3</td>
</tr>
<tr>
<td>6 - 8</td>
<td>5.3 ± 0.38</td>
<td>17.4 ± 1.4</td>
</tr>
<tr>
<td>8 - 10</td>
<td>5.0 ± 0.32</td>
<td>11.2 ± 0.9</td>
</tr>
<tr>
<td>10 - 12</td>
<td>2.2 ± 0.16</td>
<td>8.5 ± 0.7</td>
</tr>
</tbody>
</table>

Number of determinations were four in each case
Table 5 - Utilization of galactose and formation of lactic acid by *S. cervi* during *in vitro* incubation (Mean with SE)

<table>
<thead>
<tr>
<th>Period of incubation (hours)</th>
<th>Galactose consumed (µ moles/hr/g wet body weight at 37°C)</th>
<th>Lactic acid formed (µ moles/g wet weight)</th>
<th>Lactate/Galactose (µ mole/µ mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 2</td>
<td>8.3 ± 0.72</td>
<td>13.2 ± 1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>2 - 4</td>
<td>6.1 ± 0.54</td>
<td>13.3 ± 1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>4 - 6</td>
<td>3.3 ± 0.23</td>
<td>15.1 ± 1.6</td>
<td>4.5</td>
</tr>
<tr>
<td>6 - 8</td>
<td>2.8 ± 0.25</td>
<td>10.9 ± 0.9</td>
<td>3.9</td>
</tr>
<tr>
<td>8 - 10</td>
<td>2.2 ± 0.18</td>
<td>9.7 ± 0.8</td>
<td>4.4</td>
</tr>
<tr>
<td>10 - 12</td>
<td>0.5 ± 0.03</td>
<td>6.3 ± 0.6</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Number of determinations were four in each case
hexoses tried, glucose and mannose were consumed at a much faster rate than galactose or fructose. Quantitatively lactate accounted for 28, 20, 65 and 80% of glucose, mannose, fructose and galactose consumed respectively during the first two hours of incubation. Trace amounts (0.6%) of pyruvate were formed by glucose utilization.

The tables also include the mean values of the ratio between lactate formed and sugar utilized; the values were low during the initial stages and increased along with the time. It is apparent from these results that the rate of utilization of hexoses by the parasite was much faster during the initial stages of incubation and the rate decreased along with the time of incubation. At two hours incubation period the amount of glucose and mannose utilized was more or less the same but later on the utilization rate of mannose showed a steep fall. In case of glucose, the utilization rate continued to be 44% even during overnight incubation, while for mannose the rate decreased to 18% during 10-12 hours incubation and for fructose and galactose it was reduced to minimal values within this period. The worms kept in glucose were motile even after 24 hours, while in mannose they were motile up to 12 hours and in fructose and galactose the parasites became immobile within 8-10 hours. Usually 55.5 μ moles of various sugars were added to the medium for every two hours incubation period, but for overnight incubation, thrice
this amount of glucose was provided. The utilization rate of the sugars was found to be directly dependent on the motility of the worms or vice versa.

Fig. 4 illustrates the change in the rate of utilization of hexoses with the time of incubation. In case of fructose and galactose this fall was approximately linear for eight hours and then remained constant at 8-10 hours and reached zero level at about 13 hours. Mannose exhibited a similar pattern though its utilization was more and did not reach zero level even after 12 hours. However, the utilization rate of glucose showed a gradual fall though not linear up to 12 hours and remained stationary during overnight incubation.

**Incorporation of glucose-U-\(^{14}\)C and fructose-U-\(^{14}\)C into *S. cervi***

Adult female parasites were incubated aerobically in Krebs-Ringer bicarbonate buffer with uniformly labelled sugars along with carrier hexoses. The results obtained are summarised in Table 6. With glucose-U-\(^{14}\)C (1 \(\mu\) Ci per incubation medium) as the exogenous carbohydrate, the incorporation in whole worms was \(40.09 \times 10^4\) cpm/g wet weight of worms during two hours of incubation. The worms were processed for the isolation of glycogen, lipids, nucleic acids and proteins and the results obtained showed that 34.2% of the total recovered radioactivity was in the
Fig. 4  Effect of time on the utilization rate of different hexoses.

- Glucose
- Mannose
- Fructose
- Galactose
- Period not studied

Specific activity\(^+\) = \(\) mole sugar utilized/g wet worms/hr.
Table 6 - Incorporation of labelled sugars in *S. cervi* during *in vitro* incubation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Glucose-U-C&lt;sup&gt;14&lt;/sup&gt;</th>
<th>Fructose-U-C&lt;sup&gt;14&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm x 10&lt;sup&gt;4&lt;/sup&gt;/g</td>
<td>Per cent</td>
</tr>
<tr>
<td>Whole worms</td>
<td>40.09</td>
<td>100.0</td>
</tr>
<tr>
<td>Glycogen</td>
<td>13.69</td>
<td>34.2</td>
</tr>
<tr>
<td>PCA soluble fraction</td>
<td>13.46</td>
<td>33.5</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.19</td>
<td>0.5</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>0.16</td>
<td>0.4</td>
</tr>
<tr>
<td>Proteins</td>
<td>0.14</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The incorporation medium in 10 ml of KRB buffer, pH 7.4 contained: unlabelled (carrier) glucose or fructose, 55.5 μ moles; glucose-U-C<sup>14</sup> or fructose-U-C<sup>14</sup>, 1 μ Ci (Sp. activity, 17 and 20 m Ci per μ mole respectively for glucose and fructose); and 260 mg living *S. cervi*. The worms were incubated at 37°C for 2 hrs.
form of glycogen and 33.5% in the cold PCA soluble fraction (presumably, unidentified glycolytic intermediates, unpolymerized pyrimidine and purine nucleotides, and amino acids), while only traces, 0.5, 0.4 and 0.3% were incorporated in lipids, nucleic acids and proteins respectively. Under the same conditions employing fructose-U-\(^{14}\)C as the exogenous carbohydrate the incorporation in the whole body of the parasite was \(8.42 \times 10^4\) cpm/g wet worms out of which only 9% was incorporated into glycogen (Table 6).

Table 7 represents the data of another set of experiments where the worms were incubated first for 2 hours in Krebs-Ringer bicarbonate medium supplemented with glucose-U-\(^{14}\)C (along with carrier glucose) and then were starved in unsupplemented Krebs-Ringer bicarbonate medium for 8 hours at 37°C. In this case radioactivity in the excretory products was also recorded. The values for the excretory products, whole worms and glycogen were \(32.64 \times 10^4\), \(5.25 \times 10^4\) and \(2.28 \times 10^4\) cpm respectively.

**DISCUSSION**

**Utilization of monosaccharides**

The metabolism of carbohydrates and identification of end-products released as well as the different pathways of carbohydrate utilization in nematodes have received considerable attention. The results of these investigations
Table 7 - Incorporation of glucose-U-C\textsubscript{14} and excretion of labelled metabolic end-products by \textit{S. cervi} during \textit{in vitro} incubation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>cpm x 10\textsuperscript{4}/g</th>
<th>Per cent Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole worms</td>
<td>5.25</td>
<td>86.9</td>
</tr>
<tr>
<td>Glycogen</td>
<td>2.28</td>
<td>83.3</td>
</tr>
<tr>
<td>Excretory products</td>
<td>32.64</td>
<td>-</td>
</tr>
<tr>
<td>Whole worms + Excretory products</td>
<td>37.89</td>
<td>-</td>
</tr>
</tbody>
</table>

The incorporation medium in 10 ml of KRB buffer, pH 7.4 contained: unlabelled (carrier) glucose, 55.5 \( \mu \) moles; glucose-U-C\textsubscript{14}, 1 \( \mu \) Ci (Sp. activity, 17 m Ci per m mole); and 250 mg living \textit{S. cervi}. The worms were incubated at 37\textdegree C for 2 hours and then starved for 3 hours in unsupplemented KRB buffer.
can provide leads in devising suitable culture media for studying the utilization of metabolites as well as in the elucidation of the mechanism of action of antifilarial drugs. The helminth parasites are known to thrive in a culture medium fortified with sugars; however, their survival rates are usually dependent on the nature of the monosaccharides present in the medium. *Litomosoides carinii*, the cotton rat filarial worm, metabolizes glucose, mannose, galactose or fructose producing lactic and acetic acids both under aerobic and anaerobic conditions (Bueding, 1949). In contrast, *Dirofilaria insignis* (Bueding and Oliver-Gonzalez, 1950), *Dirofilaria uniformis* (von Brand et al., 1963) and Chandlerella hawkingi (Srivastava et al., 1968) metabolize considerable amounts of glucose. Cavier and Saval (1952) have reported the utilization of glucose and fructose by Ascaris lumbricoides. It can be inferred from the above that in spite of the tremendous need for energy, the spectrum of monosaccharides utilized by nematodes is rather narrow.

The results of the present study indicate that *S. cervi* utilizes glucose or mannose with equal efficiency during the initial stages of incubation. In this respect it resembles Himasthla quissetensis (Vernberg and Hunter, 1963), *C. hawkingi* (Srivastava et al., 1968) and *L. carinii* (Srivastava et al., 1970c). However, it differs from cestodes such as
Hymenolepis diminuta (Laurie, 1957), Hymenolepis nana (Read and Rothman, 1958) and Taenia taeniaeformis (von Brand et al., 1964) which can utilize both glucose and galactose.

Although the rate of glucose and mannose utilization by S. cervi was more or less same during the initial stages of incubation, the consumption rate of mannose decreased to half that of glucose even during and after 6-8 hours incubation. Glucose continued to be utilized at a rate of 31% even after 24 hours while the rate of mannose utilization decreased to 18% during 10-12 hours. Fructose or galactose did not show significant utilization and even the slow rate of metabolization of these sugars ceased completely after 13 hours of contact with the parasite. It appears, therefore, that the latter two sugars do not play any role in the survival of the worm which could live even in a simple mineral medium devoid of any sugar for an identical period. Glucose which can serve as energy supplier can, thus conveniently be used as the constituent of the medium employed for the maintenance of S. cervi under in vitro conditions. To some extent mannose can also be used for the purpose if the parasite is to be maintained for a short period.

The filarial worm C. hawkingi and L. carinii studied earlier in this laboratory did not release endogenous lactic and pyruvic acids when incubated in sugar free Krebs-Ringer
bicarbonate medium. However, *S. cervi* excreted these acids when incubated for longer periods in sugar free medium and thus in this respect it resembles intestinal nematodes especially *A. galli*.

Lactic acid accounts for nearly all the sugar consumed by *S. mansoni* (Bueding, 1949; 1950; Bueding and Oliver-Gonzalez, 1950), *D. insignis* (Bueding and Oliver-Gonzalez, 1950), *D. uniformis* (von Brand et al., 1963) and *C. hawkingsi* (Srivastava et al., 1968). But unlike these parasites, in *S. cervi* the amount of lactic acid formed and thus the ratio of the sugar utilized to the lactic acid formed was different at different periods of incubation. In case of glucose 28% of the sugar was converted to lactic acid as indicated by the lactate/sugar ratio to be 0.56 (Table 2), during the first 2 hours incubation period. This ratio increased with time and it reached maximum value during 24-26 hours incubation period when 90% of the sugar was converted to lactic acid. Similarly with mannose 20 and 80% of it was converted to lactic acid during initial and 10-12 hours incubation periods respectively. However, in case of fructose and galactose the ratio was higher even during the initial periods of incubation, being 1.3 and 1.6 which increased with the time and reached 3.8 and 12.6 respectively during 10-12 hours incubation. The higher ratio recorded in case of the latter two sugars
may be due to the fact that it also included the endogenous lactic acid produced. It also suggests that these two sugars did not serve as energy source since only small amount was utilized and simultaneously endogenous carbohydrate was also broken down even in their presence. The lower ratio (of lactate/sugar) in case of glucose and mannose during early periods of incubation may presumably be due to the fact that the sugar consumed may be utilized in synthesizing some body constituents like glycogen, and broken down to end-products other than lactic acid, as in the beginning the worms are very active and their metabolic processes must be at peaks. However, along the progress of time, metabolic activities of the worms seem to decrease as also indicated by their motility and lower rates of sugar utilization and hence the synthetic processes may stop and only degradation processes take place. The trace amounts of pyruvate detected in the incubates suggest that pyruvate, as soon as it is formed, is converted to lactate through the mediation of a fairly active lactate dehydrogenase activity present in *S. cervi*, or it may also enter the tricarboxylic acid cycle. The aerobic excretion of pyruvic acid as a metabolite has, however, been detected in the larvae of *Echinococcus granulosus* (Agosin, 1957) and larval as well as adult forms of *T. taeniaeformis* (von Brand and Bowman, 1961).
Incorporation of labelled sugars

Exploratory experiments indicated that when *S. cervi* was supplied with radioactive glucose, the label was incorporated into many constituents of the parasite, although the highest amount of incorporation was in glycogen. In case of fructose which was a poor energy source for the parasite, the incorporation was five times less than glucose and only a small fraction of it was incorporated into glycogen.

Polysaccharides stored in endoparasites seem to be derived in most cases from exogenous carbohydrates and a rapid rate of glycogen synthesis has been observed for several helminths. Using uniformly labelled glucose, Bueding (1949) observed rapid glycogen synthesis by *L. carinii*. Fernando and Wong (1964) also observed glycogen synthesis by Ancyclostoma caninum from glucose medium fortified with serum. Similarly *in vitro* synthesis of glycogen from glucose, fructose, sorbose, maltose and saccharose has been reported in *A. lumbricoides* (Cavier and Savel, 1952). Ether and Gonzalez (1959) using labelled glucose observed that half of the sugar consumed by *A. lumbricoides* was incorporated into glycogen.

Results of the present studies indicate that during the early periods of *in vitro* incubation *S. cervi* synthesizes
glycogen from the exogenous glucose and most of the glycogen is derived from glucose and not from fructose (9% of the total fructose-$U\cdot C^{14}$ incorporation goes to glycogen).

The results obtained with unlabelled glucose (Table 2) revealed a low ratio of lactate/glucose during the initial stages of incubation. This may be explained on the basis of the incorporation studies which indicate that whatever sugar was utilized was not converted 100% to lactate but was channelled towards glycogen synthesis giving a low lactate/glucose ratio.

In helminth parasites, glycogen is supposed to be the most important energy reserve and hence the parasites can live in a sugar free medium for a limited period at the expense of their body glycogen (von Brand, 1966). When _S. cervi_ was kept in a sugar free medium for eight hours after prior incubation for two hours with labelled glucose, there was a depletion of about 83% of the radioactivity incorporated into the glycogen. This was evident from the fact that only $2.28 \times 10^4$ cpm were recovered from the glycogen of the starved worms whereas the counts accounted for from the glycogen isolated from unstarved worms were $13.69 \times 10^4$ cpm. The depletion of radioactivity in the whole worms was of the order of 87%. The radioactivity recovered in the excretory products was as high as $32.64 \times 10^4$ cpm out of which about 35% could be
accounted due to the glycogen breakdown whereas rest might be coming from other body constituents, most probably from the pool. Although products other than lactic acid were not identified, excretion of other acidic and non-acidic end-products cannot be ruled out at this stage. When the radioactivity remaining in the starved worms and the radioactivity of the products excreted were added, the sum was $37.89 \times 10^4$ cpm which was more or less same as the radioactivity of unstarved whole worms ($40.09 \times 10^4$ cpm). Thus it may be inferred that the newly synthesized polysaccharide in S. cervi is being broken down into lactate and other metabolic end-products probably through the process of glycolysis.
SECTION II

ENZYMIC STUDIES ON S. CERVI AND A. GALLI
GLYCOLYTIC ENZYMES IN S. CERVI

Cell-free extracts of filarial parasite S. cervi showed the presence of glycogen phosphorylase, hexokinase, phosphoglucomutase, glucosephosphate isomerase, FDP-aldolase, phosphopyruvate hydratase, pyruvate kinase, lactate dehydrogenase and phosphoenol pyruvate-carboxykinase (Tables 8 - 11). The parasite also possessed glucose-6-phosphate dehydrogenase, G-6-Pase and hexosediphosphatase (FDPase) activities.

Distribution of glycolytic enzymes of S. cervi in various sub-cellular organelles

Cell-free homogenates of S. cervi were spun at different speeds of centrifugation for isolating mitochondria, ribosome like particles and particulate-free supernatants and assayed for the localization of various enzymes of glycolysis. The purity of mitochondria and ribosomes was tested by assaying the presence of marker enzymes viz. succinate dehydrogenase and G-6-Pase activities respectively.

In general, the specific activities of the enzymes were higher in the particulate-free (105,000 g) supernatant except for hexokinase where the specific activity was high in the cell-free extract. The presence of glycogen phosphorylase and G-6-Pase could be demonstrated in the
Table 8 - Distribution of glycolytic enzymes of *S. cervi* in the supernatates obtained after differential centrifugation

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300 g supernatant</td>
<td>12,000 g supernatant</td>
</tr>
<tr>
<td>Glycogen phosphorylase (5)</td>
<td>11.3 ± 1.1</td>
<td>-</td>
</tr>
<tr>
<td>Phosphoglucomutase (5)</td>
<td>244.7 ± 16.6</td>
<td>285.0 ± 9.1</td>
</tr>
<tr>
<td>Glucosephosphate isomerase (6)</td>
<td>523.3 ± 16.9</td>
<td>624.6 ± 13.6</td>
</tr>
<tr>
<td>G-6-P dehydrogenase (6)</td>
<td>16.7 ± 2.5</td>
<td>18.2 ± 3.6</td>
</tr>
<tr>
<td>Phosphopyruvate hydratase (7)</td>
<td>218.2 ± 10.2</td>
<td>290.3 ± 9.5</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (pH 6.5)(4)</td>
<td>3.8 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (pH 7.4)(4)</td>
<td>2.4 ± 0.4</td>
<td>-</td>
</tr>
<tr>
<td>Hexosediphosphatase (5)</td>
<td>13.2 ± 2.1</td>
<td>17.1 ± 2.2</td>
</tr>
</tbody>
</table>

Figures in brackets refer to the number of determinations.
supernatant from low speed (800 g x 15 min.) centrifugation as 105,000 g supernatant showed negligible activities. Aldolase and lactate dehydrogenase were the only two enzymes which were present in mitochondria besides other fractions, although specific activities in mitochondria were lower than in the supernatant fractions. An analysis of the particulate-free supernatant indicated high specific activities for phosphoglucomutase, aldolase, glucosephosphate isomerase, phosphopyruvate hydratase and lactate dehydrogenase, while pyruvate kinase and G-6-Pase were the least active.

The particulate-free supernatants did not oxidize reduced pyridine nucleotide coenzymes (NADH₂ or NADPH₂) without added substrate, indicating the absence of diaphorase activity in the system.

Hexokinases

Evidence for the presence of hexokinases in the extracts was sought by estimating the hexoses remaining in the reaction mixture after incubation. The rates at which different hexoses could be phosphorylated, were high for glucose and fructose and were low for mannose and galactose. The specific activities of hexokinases acting on glucose and fructose were more or less same in the 105,000 g supernate but the former was more active in the cell-free extract. The specific activity of hexokinase acting on fructose was 22% more in particulate-free as
compared to cell-free homogenate. In other cases, the specific activities were high in the cell-free homogenate. The results are summarized in Table 9.

### Phosphatases acting on G-6-P and FDP

$P_i$ liberated from G-6-P and FDP by the action of respective phosphatases were measured for the assay of these enzymes. G-6-Pase was assayed only in cell-free homogenate as the activity was very low in the particulate-free fraction. The assay of G-6-Pase carried out at pH 6.5 (as recommended by Harper, 1963) and at 7.4 (the pH in the usual hexokinase assay) indicated slightly higher activity at the former pH.

### G-6-P dehydrogenase

The extracts prepared from the worm were found to be active in mediating the dehydrogenation of G-6-P in the presence of NADP as shown in Fig. 5. The enzyme was NADP specific since there was no activity with NAD as cofactor. The enzyme was found to be mainly localized in the soluble fraction.

### Phosphopyruvate hydratase

The presence of phosphopyruvate hydratase in the different extracts of the worms is brought out in the data represented graphically in Fig. 6 which shows increase in
Table 9 - Hexokinase activities of *S. cervi* in the supernates obtained after differential centrifugation

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Specific activity (n moles min.(^{-1})(mg protein(^{-1}))</th>
<th>Mean with SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>800 g supernatant</td>
<td>12,000 g supernatant</td>
</tr>
<tr>
<td>Glucose</td>
<td>16.25 ± 2.20</td>
<td>13.05 ± 1.85</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.33 ± 0.25</td>
<td>1.70 ± 0.01</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.90 ± 0.05</td>
<td>1.20 ± 0.02</td>
</tr>
<tr>
<td>Fructose</td>
<td>9.60 ± 0.06</td>
<td>10.07 ± 0.04</td>
</tr>
</tbody>
</table>

Number of determinations were five in each case
absorbancy at 240 nm due to the formation of PEP. The specific activity of the enzyme was 41% more in the particulate-free fraction as compared to the cell-free extract (Table 8).

**Lactate dehydrogenase**

When extracts were incubated in the presence of pyruvate and NADH₂, there was a steady decrease in absorbancy at 340 nm indicating the oxidation of NADH₂ (Fig. 7) by the backward reaction of lactate dehydrogenase. The specific activity in particulate-free supernatant was 22% more in comparison to cell-free homogenate (Table 10).

**Pyruvate kinase**

In the presence of ADP, and MgCl₂, extracts of *S. cervi* converted PEP to pyruvate, which in turn, was transformed to lactate when the assay system was coupled to lactate dehydrogenase. Decrease in absorbancy at 340 nm due to the oxidation of NADH₂, as shown in Fig. 8, represents pyruvate kinase activity. The activity of this enzyme was very low as compared to the other enzymes of the glycolytic pathway (Table 11).

**PEP-carboxykinase**

Since PEP carboxykinase plays a very important role in metabolizing PEP formed during glycolysis in helminth
Table 10 - Distribution of aldolase and lactate dehydrogenase in particulate-free fractions and mitochondria of S. cervi

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity [n moles min⁻¹ (mg protein)⁻¹]</th>
<th>Mean with SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldolase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>800 g supernatant</td>
<td>309.1 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>12,000 g supernatant</td>
<td>637.4 ± 11.6</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>105,000 g supernatant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in brackets refer to the number of determinations.
Fig. 5  Glucose-6-phosphate dehydrogenase activity of *S. cervi*.  
(Enzyme protein 800 μg)

- ▲ ▲  800 g supernatant
- ■ ■  12,000 g supernatant
- ●●  105,000 g supernatant

Fig. 6  Phosphopyruvate hydratase activity of *S. cervi*.  
(Enzyme protein 200 μg)

- ▲ ▲  800 g supernatant
- ■ ■  12,000 g supernatant
- ●●  105,000 g supernatant

Fig. 7  Lactate dehydrogenase activity of *S. cervi*.  
(Enzyme protein 40 μg)

- ▲ ▲  800 g supernatant
- ■ ■  12,000 g supernatant
- ●●  105,000 g supernatant
- ■■  Mitochondria
### Table 11 - Pyruvate kinase and PEP-carboxykinase activities of *S. cervi* and *A. galli*

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzymes</th>
<th>Specific activity [n moles min.(^{-1})(mg protein)(^{-1})]</th>
<th>Mean with SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>800 g supernatant</td>
<td>12,000 g supernatant</td>
</tr>
<tr>
<td><em>S. cervi</em></td>
<td>Pyruvate kinase</td>
<td>(5)</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>PEP-carboxykinase</td>
<td>(4)</td>
<td>52.4 ± 1.3</td>
</tr>
<tr>
<td><em>A. galli</em></td>
<td>Pyruvate kinase</td>
<td>(5)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Body wall</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ovary</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Testis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PEP-carboxykinase</td>
<td>(4)</td>
<td>50.0 ± 1.1</td>
</tr>
</tbody>
</table>

Figures in brackets refer to the number of determinations.
* Values as reported by Srivastava et al. (1969).
Fig. 8  Pyruvate kinase activity of *S. cervi.*
(Enzyme protein 3.1 mg)

▲▲  800 g supernatant
■■  12,000 g supernatant
●●  105,000 g supernatant

Fig. 9  PEP-carboxykinase activity of *S. cervi* and *A. galli* in 105,000 g supernatant.

●●  *S. cervi*  (Enzyme protein 400 μg)
▲▲  *A. galli*  (Enzyme protein 350 μg)
parasites, an attempt was made to determine the comparative activity of this enzyme in *S. cervi* and *A. galli*.

The oxaloacetate required in the coupled assay system of PEP-carboxykinase was obtained by treating extracts of *S. cervi* or *A. galli* with PEP in the presence of GDP, MnCl₂ and NaHCO₃, and oxaloacetate in turn, was transformed to malate by coupling the system to malate dehydrogenase in presence of NADH₂.

Fig. 9 represents the comparative PEP-carboxykinase activities in *S. cervi* and *A. galli* and the results obtained with *S. cervi* and *A. galli* are summarized in Table II. A higher specific activity in the particulate-free system indicated that the enzyme was confined to the soluble fraction.

TRICARBOXYLIC ACID CYCLE IN *S. CERVI* AND *A. GALLI*

Dehydrogenation of TCA cycle intermediates and some amino acids by cell-free homogenates of *A. galli*

Homogenates of *A. galli* obtained after centrifugation for 15 min. at 800 g were employed for studying the dehydrogenation of TCA cycle intermediates and of a few representative amino acids under anaerobic conditions using Thunberg tubes. The results presented in Table 12
Table 12 - Dehydrogenation of TCA cycle intermediates and amino acids by A. galli extracts

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Q_{MB}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>0.87</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>1.04</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>1.32</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.73</td>
</tr>
<tr>
<td>Malate</td>
<td>0.33</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.12</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.76</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.27</td>
</tr>
<tr>
<td>Serine</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Q_{MB} = Microlitres of hydrogen transferred to methylene blue per hour per mg enzyme protein.

Number of determinations were three in each case.
indicate that all the substrates stimulated the reduction of the dye under the experimental conditions employed. Among the organic acids, succinate was most readily dehydrogenated followed by α-ketoglutarate, isocitrate, pyruvate and malate. Maximum dehydrogenation among amino acids was observed with alanine. Glutamate and serine were also actively dehydrogenated.

**TCA cycle enzymes in *S. cervi* and *A. galli***

The relevant data on the enzymes of TCA cycle present in the cell-free extracts of *S. cervi* and *A. galli* have been summarized in Tables 13 and 14. These assays were carried out using fresh homogenates at different periods. Table 13 records the TCA cycle enzymes present in *S. cervi* while Table 14 describes the distribution of TCA cycle enzymes in males and females of *A. galli* and in their different anatomical parts. Almost all the enzymes of the TCA cycle have been shown to be present in both these worms.

Except for some minor differences in their specific activities, the distribution patterns of the TCA cycle enzymes in different anatomical parts of *A. galli* are similar. The level of aconitate hydratase activity is almost the same in various anatomical parts of *A. galli*. Aconitate hydratase, isocitrate dehydrogenase, fumarate hydratase and
Table 13 - Tricarboxylic acid cycle enzymes in cell-free homogenates of *S. cervi*

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Number of determinations</th>
<th>Specific activity [n moles min(^{-1}) (mg protein(^{-1})]&lt;sub&gt;1&lt;/sub&gt;]</th>
<th>Mean with SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aconitate hydratase</td>
<td>4</td>
<td>51.1 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>5</td>
<td>15.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate dehydrogenase</td>
<td>5</td>
<td>4.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>4</td>
<td>4.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Fumarate hydratase:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fumarate → malate</td>
<td>5</td>
<td>11.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>malate → fumarate</td>
<td>4</td>
<td>1749.7 ± 28.1</td>
<td></td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>5</td>
<td>15403.6 ± 157.4</td>
<td></td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>5</td>
<td>9.4 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>
Table 14 - Tricarboxylic acid cycle enzymes in cell-free homogenates of Agalli

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity n moles min.⁻¹(mg protein)⁻¹</th>
<th>Mean with SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Aconitate hydratase</td>
<td>39.6 ± 0.9</td>
<td>35.1 ± 1.3</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>30.3 ± 1.4</td>
<td>23.5 ± 0.7</td>
</tr>
<tr>
<td>α-Ketoglutarate dehydrogenase</td>
<td>7.2 ± 0.3</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>8.3 ± 0.7</td>
<td>8.6 ± 0.3</td>
</tr>
<tr>
<td>Fumarate hydratase:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumarate → malate</td>
<td>8.5 ± 0.3</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>Malate → fumarate</td>
<td>-</td>
<td>2018.6 ± 24.7</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>9666.2 ± 39.4</td>
<td>10221.3 ± 101.3</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>12.9 ± 1.3</td>
<td>10.1 ± 0.6</td>
</tr>
</tbody>
</table>

Values given are average of five determinations
malic enzyme are more active in the male worms than in the females, while the reverse is true for α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase. In general, the specific activities of the TCA cycle enzymes are higher in body walls and intestines than in the reproductive organs or in the whole worms. The levels of aconitate hydratase, α-ketoglutarate dehydrogenase, malate dehydrogenase and succinate dehydrogenase are higher in body walls while that of isocitrate dehydrogenase, fumarate hydratase and malic enzyme are more in intestines.

The specific activities of aconitate hydratase, fumarate hydratase (in forward direction) and malate dehydrogenase are higher in *S. cervi* than in *A. galli*. However, all the other enzymes studied, viz. isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, malic enzyme and fumarate hydratase (in backward direction) are more active in *A. galli* than in *S. cervi*.

Among the Krebs cycle enzymes of *S. cervi* and *A. galli* malate dehydrogenase and fumarate hydratase were most active, although comparatively high specific activity for aconitate hydratase was also observed. The least active was α-ketoglutarate dehydrogenase.

It should be pointed out that the enzyme assays were carried out in worms collected in batches on the same day.
or in different days. The standard deviations, therefore, if used for comparison of the activities of different tissues may underestimate the significance of the differences. Due to variations in the host physiology from day to day, there could have arisen related changes from batch to batch. The cell-free supernates were devoid of the ability to oxidise NADH₂ in the absence of added substrate. The absence of such reactions facilitated the assay of enzymes requiring these cofactors.

**Aconitase hydratase**

The assay of aconitase hydratase was carried out in both the forward and backward directions. It was found that the reaction: citric acid to cis-aconitic acid was more favourable. Figs. 10 and 11 represent the increase in absorbancy at 240 nm due to the formation of cis-aconitic acid by aconitase of *S. cervi* and *A. galli* respectively.

**Isocitrate dehydrogenase**

A NADP specific isocitrate dehydrogenase was detected in both *S. cervi* and *A. galli*. Figs. 12 and 13 represent the time activity relationship of isocitrate dehydrogenase of *S. cervi* and *A. galli* respectively by following the increase in absorbancy at 340 nm due to the reduction of NADP.
Fig. 10  Aconitate hydratase activity of *S. cervi*.

\[\text{△} \quad \text{△} \quad \text{Enzyme protein 400 µg} \]

\[\bullet \quad \bullet \quad \text{Enzyme protein 600 µg} \]

Fig. 11  Aconitate hydratase activity of *A. galli*.
(Enzyme protein 800 µg)

\[\text{△} \quad \text{△} \quad \text{Male} \]
\[\bigcirc \quad \bigcirc \quad \text{Female} \]
\[\square \quad \square \quad \text{Body wall} \]
\[\text{△} \quad \text{△} \quad \text{Intestine} \]
\[\bullet \quad \bullet \quad \text{Testis} \]
\[\square \quad \square \quad \text{Ovary} \]
Fig. 12 Isocitrate dehydrogenase activity of S. cervi.

- Enzyme protein 500 μg
- Enzyme protein 700 μg

Fig. 13 Isocitrate dehydrogenase activity of A. galli.
(Enzyme protein 650 μg)

- Male
- Female
- Body wall
- Intestine
- Testis
- Ovary
Fumarate hydratase

This enzyme was also assayed in both the forward and backward directions and the backward reaction i.e., malic acid to fumaric acid was more pronounced in both the worms. Transformation of fumaric acid to malic acid by fumarate hydratase of *S. cervi* and *A. galli* is shown in Figs. 14 and 15 respectively, while the backward reaction is illustrated in Fig. 16. Activity of the enzyme in backward direction was 150 and 250 times more than the activity in the forward direction in *S. cervi* and *A. galli* respectively (Tables 13 and 14).

Malate dehydrogenase

When cell-free extracts of *S. cervi* and *A. galli* were incubated in the presence of oxaloacetate and NADH$_2$ there was a steady decrease in absorbancy at 340 nm as shown in Figs. 17 and 18 indicating the oxidation of NADH$_2$ by the malate dehydrogenase enzymes of *S. cervi* and *A. galli* respectively.

Malate dehydrogenase (decarboxylating) (Malic enzyme)

In the presence of malic acid, the extracts prepared from *S. cervi* and whole worms of *A. galli* and its anatomical parts reduced added NADP indicating the presence of malic
Fig. 14  Fumarate hydratase (fumarate $\rightarrow$ malate) activity of *S. cervi*.

- Enzyme protein 600 µg
- Enzyme protein 900 µg

Fig. 15  Fumarate hydratase (fumarate $\rightarrow$ malate) activity of *A. galli*.
(Enzyme protein 730 µg)

- Male
- Female
- Body wall
- Intestine
- Testis
- Ovary

Fig. 16  Fumarate hydratase (malate $\rightarrow$ fumarate) activity of *S. cervi* and *A. galli*.
(Enzyme protein 21.5 µg)

- *S. cervi*
- *A. galli*
Fig. 17  Malate dehydrogenase activity of *S. cervi*.

- ▲▲  Enzyme protein 0.68 μg
- ●●  Enzyme protein 1.02 μg

Fig. 18  Malate dehydrogenase activity of *A. galli*.
(Enzyme protein 1.5 μg)

- ▲▲  Male
- ○○  Female
- □□  Body wall
- ▲▲  Intestine
- ●●  Testis
- ■■  Ovary
enzyme in these worms. The time activity curve of this enzyme has been shown graphically in Figs. 19 and 20 for \textit{S. cervi} and \textit{A. galli} respectively.

\textbf{DISCUSSION}

\textbf{Glycolysis in \textit{S. cervi}}

Extracts of \textit{S. cervi} have been found to be equipped with nearly all the enzymes required to operate the Embden-Meyerhof pathway of glycolysis. Except aldolase and lactate dehydrogenase which were present in mitochondria also, all the enzymes of the pathway were localized in the soluble fraction, showing a resemblance with the mammalian systems. The activity range of some of the enzymes in \textit{S. cervi} was similar to the range reported for the larvae of \textit{Haemonchus contortus} (Ward and Schofield, 1967a). Striking similarities were observed in glucosephosphate isomerase, G-6-Pase and G-6-P dehydrogenase where the corresponding values reported in \textit{H. contortus} were 616.0, 4.0 and 28.4 respectively. The specific activities for some of the enzymes of \textit{S. cervi} were higher than the corresponding enzymes of \textit{H. contortus} larvae, e.g. phosphoglucomutase, aldolase, phosphopyruvate hydratase and lactate dehydrogenase. Similarly G-6-Pase of \textit{S. cervi}
Fig. 19 Malic enzyme activity of *S. cervi*.

- ▲▲ Enzyme protein 500 µg
- ●● Enzyme protein 800 µg

Fig. 20 Malic enzyme activity of *A. galli*.
(Enzyme protein 850 µg)

- ▲▲ Male
- ○○ Female
- □□ Body wall and Testis
- ▲▲ Intestine
- ■■ Ovary
was comparable with the filarial parasite Chandlerella hawkingi (Srivastava et al., 1968), and glucosephosphate isomerase and FDPase compared with Litomosoides carinii (Srivastava et al., 1970b).

A hexokinase capable of phosphorylating all the four sugars (glucose, mannose, galactose or fructose) was detected in S. cervi. This suggests that the nematode probably contains a non-specific hexokinase of the type found in most animal tissues. The enzyme phosphorylated fructose at a rate similar to that of glucose; the fructose was also presumably phosphorylated at position 6, a further characteristic of a non-specific hexokinase (Walker, 1966). A hexokinase acting non-specifically on all the above four sugars has been reported in A. galli, L. carinii and C. hawkingi (Srivastava et al., 1970a,b; Srivastava and Ghatak, 1971). Ward and Schofield (1967a) have reported a similar type of hexokinase in H. contortus larvae and rat liver, acting on glucose and fructose.

On the other hand, Schistosoma mansoni (Bueding et al., 1954; Bueding and Mackinnon, 1955) and Echinococcus granulosus (Agosin and Aravena, 1959b) contained hexokinases specific for the phosphorylation of glucose, mannose, fructose and galactose; while a single enzyme or sometimes two designated as a non-specific glucokinase; EC 2.7.1.2 and a non-specific hexokinase; EC 2.7.1.1 (Walker, 1963), fulfill the same function in vertebrate tissues. In L. carinii, the non-specific
hexokinase perhaps accounts for the utilization of all the four sugars as observed by Bueding (1949). However, the slow utilization of fructose or galactose as compared to glucose or mannose by *S. cervi* could not be explained on the basis of a non-specific hexokinase. It appears that some permeability factor may be involved which restricted the entrance of the former two sugars inside the body of the parasite. Hexokinase activity was studied in the extracts of *S. cervi* where the enzyme was dispersed in the system through the process of homogenization, while the cuticular layer of the intact worm may act as a barrier for the entry of these sugars. It is also presumable that metabolites arising out of the early phase of utilization of galactose or fructose are toxic and also inhibit the further uptake and utilization of these sugars.

G-6-Pase activity is restricted in vertebrates to liver and kidney (Dixon and Webb, 1964), where it is principally of importance in the maintenance of blood glucose levels. No such physiological role can be visualized for this enzyme in a nematode; besides this enzyme as such may not be present. Thus the G-6-Pase activity recorded for *S. cervi* may be due to non-specific phosphatases as has been suggested for *Fasciola hepatica* (Hers and Dube, 1950).
Other pathways such as the pentose-phosphate pathway may be operating in *S. cervi* since the initial reaction, the oxidation of G-6-P by G-6-P dehydrogenase was shown to occur at a rate nearly similar to that in rat liver and *F. hepatica* (Ward and Schofield, 1967a) where the operation of this pathway has been suggested. On the contrary, the presence of this enzyme could not be detected in measurable amounts in two other filarial parasites *C. hawkingi* (Srivastava et al., 1968) and *L. carinii* studied in this laboratory. The presence of G-6-P dehydrogenase has been reported in *Parascaris equorum*, *Toxacara canis*, *Ascaridia galli*, *Ascaridia columbae*, *Heterakis gallinae*, *Strongylus edentus* (De Ley and Vercruysse, 1955), *Ascaris lumbricoides* muscle (De Ley and Vercruysse, 1955; Entner, 1957), *Trichinella spiralis* larvae (Agosin and Aravena, 1959a) and *Macracanthorhynchus hirudinaceus* (Dunagan and Scheifinger, 1966); and Srivastava et al. (1970a) have studied the properties of the partially purified G-6-P dehydrogenase of *A. galli*.

**TCA cycle in S. cervi and A. galli**

Although the tricarboxylic acid cycle is regarded as the main pathway of channelling carbon for energy in vertebrate tissues, very little is known about the routes of aerobic catabolism in nematode parasites. Slater (1925)
has shown that *A. lumbricoides* continuously stimulated
in *vitro*, lives longer if oxygen is available, and von Brand
(1937) using the same parasite observed resumption of
glycogen synthesis when oxygen becomes available after a
period of anaerobiosis. These results suggest that
oxidative mechanisms can be used by these parasites for
trapping energy.

Conflicting reports are available about the
presence or absence of TCA cycle in parasites. Based on
the results obtained with particulate preparations, several
workers have suggested the presence of a partial TCA
cycle in *A. lumbricoides*. Rathbone (1955) observed that,
in contrast to several other intermediates of the cycle,
citrate and cis-aconitate failed to stimulate oxygen
consumption by a particulate preparation from *A. lumbricoides*
muscle. However, this cannot be taken as conclusive
evidence for the absence of a full cycle in this parasite
since Plaut and Plaut (1952) also failed to demonstrate
oxygen consumption in the presence of citrate, cis-aconitate
and isocitrate using mitochondria from guinea-pig heart
muscle, a tissue known to contain very active TCA cycle.
Similar evidence for the presence of a partial cycle in
*A. lumbricoides* muscle was adduced by Seidman and Entner (1961)
who could not detect the presence of isocitrate dehydrogenase
or $\alpha$-ketoglutarate dehydrogenase in particulate preparations.
In contrast to the above results, Oya et al. (1965) succeeded in demonstrating the presence of all the enzymes of this cycle in a particulate fraction from *A. lumbricoides* muscle, although considerable difficulty was encountered in demonstrating the presence of aconitate hydratase. Bloom and Entner (1966) also failed to detect aconitate hydratase and isocitrate dehydrogenase in a particulate fraction from developing larvae of *A. lumbricoides*.

In the light of the above reports it would be relevant to mention that all the investigations that provided good evidence for the existence of all the TCA cycle enzymes in parasitic helminths have been carried out using whole homogenates and not confined to particulate preparations. Thus all the enzymes of this cycle have been demonstrated in *T. spiralis* larvae (Goldberg, 1957), infective larvae of *Strongyloides papillosus* (Costello and Grollman, 1959), unembryonated eggs of *A. lumbricoides* (Costello and Brown, 1962), scoleces of *Echinococcus granulosus* (Agosin and Repetto, 1963) and *H. contortus* larvae (Ward and Schofield, 1967b).

The results of the dehydrogenation of TCA cycle intermediates by *A. galli* coupled with the demonstration of most of the enzymes of TCA cycle in *A. galli* (whole worms and their different anatomical parts) and *S. cerevisiae* indicate
that a complete cycle can operate in these two parasites under aerobic conditions. However, the filarial parasite \textit{S. cervi} could not be differentiated from the ascarid (\textit{A. galli}) on the basis of TCA cycle, since no significant difference in the level of different enzymes mediating this pathway could be noticed. It is quite likely that the filarial parasite \textit{S. cervi}, living in an anaerobic habitat would behave more like intestinal nematode (\textit{A. galli}) than other filarial parasites like \textit{C. hawkingi} and \textit{L. carinii} which thrive in oxygen rich habitat and where enzymes of the cycle could not be detected in measurable amounts.

The assumption of a functional TCA cycle in some parasites does not imply correspondence in all details with the classical cycle in vertebrate tissues (von Brand, 1966). Since many parasites appear to be facultative anaerobes it is expected that in addition to possessing the mechanisms for aerobic metabolism, modifications to their basic metabolic pathways would occur to enable them to survive variable periods of anaerobiosis encountered in their normal habitat.

**PEP-oxaloacetate pathway in \textit{S. cervi} and \textit{A. galli}**

Among the different enzymes of the two parasites as measured in the present investigation the specific activity for pyruvate kinase was very low and this enzyme appears to
be least active in the whole sequence of glycolytic enzymes. Earlier, Ward and Schofield (1967a) failed to detect the presence of this enzyme in *H. contortus* larvae. Relatively low activity of pyruvate kinase in many parasitic helminths leads to the inference that this enzyme is perhaps the rate limiting factor in glycolysis.

Simpson and Awapara (1966) found that in several species of invertebrates although the reactions of the Embden-Meyerhof scheme of glycolysis were operative, a major modification of the pathway was noted at the level of PEP which permits these organisms to operate an alternative scheme for the production of succinate as the end product in preference to lactate. According to their proposed pathway, glucose was degraded to PEP most of which was readily carboxylated to oxaloacetate by the action of a very active PEP-carboxykinase, the oxaloacetate so formed being further metabolized to succinic acid. Succinate rather than pyruvate or lactate has been detected as the end-product of anaerobic glucose degradation in several parasitic helminths (Fairbairn, 1954; Bueding and Farrow, 1956; Saz and Vidrine, 1959; Fairbairn et al., 1961; Graff, 1964; Kmetec and Bueding, 1965; Ward and Schofield, 1967b), but no definite evidence was available at that time to show whether CO₂ fixation was mediated by PEP carboxykinase or malic enzyme.
According to Agosin (1957) and von Brand and Bowman (1961) much greater amounts of succinate are produced anaerobically than under aerobic conditions, a situation analogous to lactate accumulation in vertebrate tissues. Bueding (1963) suggested the production of succinate in many helminths possessing low lactate dehydrogenase activities as a means of reoxidizing NADH\_2 formed during glycolysis. Succinate formation has a distinct advantage for the parasite over the lactate dehydrogenase reaction in the anaerobic habitat of intestinal helminths (Saz and Bueding, 1966). Although both the reactions provide a means for reoxidising NADH\_2 making NAD available for glycolysis, in contrast to the lactate dehydrogenase reaction, the reduction of fumarate to succinate by NADH\_2 catalyzed by a mitochondrial electron transport system, generates energy in the form of ATP (Chin and Bueding, 1954; Seidman and Entner, 1961; Kmetec and Bueding, 1961; Scheibel and Saz, 1966). In *A. lumbricoides*, according to Saz and Vidrine (1959), pyruvate by CO\_2 fixation produces malate which is subsequently metabolized to succinate by fumarate hydratase and succinate dehydrogenase. The operation of such a scheme proposed in *A. lumbricoides* by Saz and Vidrine (1959) is doubted in *S. cervi* and *A. galli*, since in both these parasites glycolysis proceeds normally up to PEP formation stage due to the very low activity of pyruvate kinase, thus indicating that the involvement of malic enzyme is unlikely.
The present investigations agree with the results obtained for glycolysis in several invertebrates by Simpson and Awapara (1966) who further demonstrated that the carboxylation of PEP rather than of pyruvate was involved in succinate production by these organisms - a possibility not excluded by the results of Saz and Vidrine (1959) in *A. lumbricoides*. Similar mechanism may operate in other helminths since an active PEP carboxykinase has been demonstrated in *E. granulosus* (Agosin and Repetto, 1963; 1965), *H. diminuta* (Prescott and Campbell, 1965), *H. contortus* larvae (Ward and Schofield, 1967b) and *T. spiralis* larvae (Ward et al., 1969).

The following results observed in the present studies may support conclusively the operation of the alternate PEP-oxaloacetate pathway in *S. cervi* and *A. galli* identical with that proposed by Simpson and Awapara in invertebrates. The low levels of pyruvate kinase in these two parasites result in high levels of PEP which is the substrate for PEP-carboxykinase. Further, very high activities of malate dehydrogenase and fumarate hydratase will rapidly convert any oxaloacetate formed in the system by PEP-carboxykinase (which is 28 times and 57 times higher than pyruvate kinase activity in *S. cervi* and *A. galli* respectively) to an equilibrium mixture of malate and fumarate and finally fumarate may be reduced to succinate by succinate
dehydrogenase. No attempts were made in the present study to identify end-products of anaerobic glycolysis other than lactic and pyruvic acids. It is probable, however, that there are end-products of anaerobic glycolysis, other than these two acids as has been repeatedly observed in other parasitic worms. A similar mechanisms of succinate production has been suggested in *H. contortus* larvae by Ward and Schofield (1967b) who also reported that succinate dehydrogenase of *H. contortus* resembles that of facultative anaerobes (Singer, 1969; Hammen and Lum, 1966) and can reduce fumarate to succinate.

Thus parasites which possess all the enzymes of the TCA cycle are biochemically adapted to the environmental conditions encountered in their habitat. Aerobically a full TCA cycle can operate, succinate can be oxidised and electrons from NADH$_2$ can be transferred to oxygen as demonstrated by Kmetec and Bueding (1961) and Cheah and Bryant (1966). Anaerobically the cycle cannot function as such but the parasites may satisfy some of the enzyme requirements by the mechanism of succinate production as discussed above.

It can be concluded that parasites having low activities for pyruvate kinase and lactate dehydrogenase have significantly high activities for P$_5$-carboxykinase and malate dehydrogenase and those having low activities for
the latter enzymes have good activities for the former ones. Thus, the activities of terminal enzymes are in good agreement with the PEP-oxaloacetate pathway and supports its operation in helminth parasites explaining the formation of various end-products. On this basis helminths can be classified into two major groups:

1. Those excreting lactic acid as the only or predominant end-product, viz. S. mansoni (Bueding and Saz, 1968), L. carinii (Srivastava et al., 1970b) and C. hawkingi (Srivastava and Ghatak, 1971) etc. having an usual glycolytic pathway with high activities of pyruvate kinase and lactate dehydrogenase.

2. Those excreting succinic and volatile fatty acids other than lactic acid as the predominant end-product, such as A. lumbricoides (Bueding and Yale, 1951; Bueding and Saz, 1968), T. spiralis (Agosin and Aravena, 1959a; Ward et al., 1969), H. contortus (Ward and Schofield, 1967a,b) and H. diminuta (Fairbairn et al., 1961; Bueding and Saz, 1963) etc. These parasites possess modified glycolysis branching off at PEP level due to low activities for pyruvate kinase and lactate dehydrogenase.

The relevant data on these parasites along with the present observations are summarized in Table 15.

In S. cervi having 24 times higher MDH activity when compared to LDH where only 30 % of the carbohydrate (glucose)
<table>
<thead>
<tr>
<th>Species</th>
<th>% carbohydrate converted into lactic acid</th>
<th>Pyruvate kinase/PEP</th>
<th>LDH/MDH</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trematodes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. hepatica</td>
<td>4 - 9 (Mansour, 1959)</td>
<td>0.00</td>
<td>-</td>
<td>Prichard and Schofield, 1968a,b,c.</td>
</tr>
<tr>
<td>S. mansoni</td>
<td>81 - 91 (Bueding, 1950)</td>
<td>5.0 - 9.7</td>
<td>4.00</td>
<td>Bueding and Saz, 1968.</td>
</tr>
<tr>
<td><strong>Cestode:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. diminuta</td>
<td>6 (Fairbairn et al., 1961)</td>
<td>0.18</td>
<td>0.15</td>
<td>Bueding and Saz, 1968.</td>
</tr>
<tr>
<td><strong>Nematodes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. lumbricoides</td>
<td>Negligible (Bueding and Yale, 1951)</td>
<td>0.04</td>
<td>0.03</td>
<td>Bueding and Saz, 1968.</td>
</tr>
<tr>
<td>T. spiralis larvae</td>
<td>Appreciable (Agosin and Aravena, 1959a)</td>
<td>0.31</td>
<td>0.12</td>
<td>Ward et al., 1969.</td>
</tr>
<tr>
<td>L. carinii</td>
<td>50 - 55 (Bueding, 1949)</td>
<td>2.5 - 3.0</td>
<td>1.00</td>
<td>Srivastava et al., 1970 b.</td>
</tr>
<tr>
<td>C. hawkingii</td>
<td>85 - 95 (Srivastava et al., 1968)</td>
<td>8.50</td>
<td>123.00</td>
<td>Srivastava and Ghatak, 1971.</td>
</tr>
<tr>
<td>H. contortus larvae</td>
<td>-</td>
<td>0.22</td>
<td>0.26</td>
<td>Ward et al. (1967a,b; 1968a,b)</td>
</tr>
<tr>
<td>A. galli</td>
<td>-</td>
<td>0.02</td>
<td>0.0006</td>
<td>Present studies.</td>
</tr>
<tr>
<td>S. cervi</td>
<td>28 (present studies)</td>
<td>0.04</td>
<td>0.04</td>
<td>Present studies.</td>
</tr>
</tbody>
</table>
utilized was converted to lactic acid during the early periods of incubation, the ratio of pyruvate kinase/PEP-carboxykinase was around 0.04. Similarly in _A. galli_ having 1572 times higher MDH activity than LDH the ratio of pyruvate kinase/PEP-carboxykinase was 0.02. It would thus appear that the filarial parasite _S. cervi_ does not resemble the other filarial parasites _L. carinii_ and _C. hawkingi_ having higher pyruvate kinase/PEP-carboxykinase ratio and producing lactic acid as the major end-product but behaves more closely with the intestinal parasite _A. galli_ and other nematodes e.g. _A. lumbricoides, T. spiralis, H. contortus_ and the cestode _H. diminuta_ which have been reported to produce succinate and other volatile fatty acids.
SECTION III

PURIFICATION AND CHARACTERIZATION OF FDP-ALDOASE OF S. CERVI
Distribution pattern of FDP-aldolase and certain other glycolytic enzymes of *S. cervi* in various ammonium sulphate precipitated fractions

The ribosome free 105,000 g supernatant of the filarial parasite *S. cervi* was brought to various saturations with respect to ammonium sulphate and the levels of FDP-aldolase, phosphoglucomutase, phosphopyruvate hydratase and hexokinase were assayed in all the fractions. It is apparent from Table 16 that fraction III had the highest activities of phosphoglucomutase, aldolase and phosphopyruvate hydratase, while maximum hexokinase activity was recovered in fraction IV. The distribution pattern of phosphopyruvate hydratase was diffused, as 44% of the enzyme activity was brought down in I, II and IV fractions. The proteins sedimenting in fraction III possessed 80 and 85% phosphoglucomutase and aldolase activities respectively, while the proteins sedimenting beyond 60% saturation of ammonium sulphate (fraction III) were devoid of these two enzymes. Although 55% of hexokinase activity was recovered in fraction IV, the residual amount was distributed in the first three fractions sedimenting up to 60% saturation. Aldolase was subjected to further purification employing DEAE-cellulose chromatography and its properties are described. At this step (45-60%) of ammonium sulphate fractionation the recovery and purification of aldolase was 85% and 4.5 fold respectively.
Table 16 - Distribution on pattern of certain glycolytic enzymes of *S. cervi* in various ammonium sulphate precipitated fractions

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>% Ammonium sulphate saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>(I)</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>0</td>
</tr>
<tr>
<td>FDP- Aldolase</td>
<td>6</td>
</tr>
<tr>
<td>Phosphopyruvate hydratase</td>
<td>5</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>8</td>
</tr>
</tbody>
</table>

The values reported are per cent activities of various enzymes.
Purification of *S. cervi* aldolase employing DEAE-cellulose chromatography

Fractions separating between 45-60% saturation of ammonium sulphate and possessing maximum aldolase activity were further purified by chromatography on DEAE-cellulose column. Fig. 21 illustrates a typical elution profile of the soluble proteins and aldolase activity of *S. cervi*. Five main protein fractions were eluted with buffer containing 0, 50, 100, 200 and 300 mM sodium chloride respectively. The two fractions eluting with 50 and 100 mM sodium chloride exhibited aldolase activity indicating the presence of two isomeric forms of this enzyme. Nearly 85% of the aldolase activity recovered was present in the fraction eluting with 50 mM sodium chloride. The above two fractions were pooled, freed from buffer and salts and then recyclyzed through a fresh column. Aldolase activity was recovered in eluates emerging in buffer fronts containing concentrations of sodium chloride identical with the ones used in the preparatory column. The recovery and purification of the enzyme by DEAE-cellulose chromatography (for 50 mM eluate) was 37.1% and 11.5 fold respectively. Table 17 summarises the purification and recovery data at different stages of purification.

Properties of the partially purified aldolase

Since most of the enzyme activity was recovered in the
<table>
<thead>
<tr>
<th>Description</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Recovery %</th>
<th>Recovery %</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude (800 g) supernatant</td>
<td>47.5</td>
<td>356.3</td>
<td>100.0</td>
<td>100.0</td>
<td>350.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Particulate-free (105,000 g)</td>
<td>45.7</td>
<td>287.9</td>
<td>80.8</td>
<td>96.2</td>
<td>422.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Dialysed solution of (NH₄)₂SO₄ paste</td>
<td>6.4</td>
<td>96.0</td>
<td>27.0</td>
<td>85.6</td>
<td>1570.9</td>
<td>4.5</td>
</tr>
<tr>
<td>DEAE-cellulose (50 mM NaCl eluate)</td>
<td>30.0</td>
<td>15.0</td>
<td>4.4</td>
<td>37.1</td>
<td>4047.0</td>
<td>11.5</td>
</tr>
<tr>
<td>DEAE-cellulose (100 mM NaCl eluate)</td>
<td>25.0</td>
<td>10.4</td>
<td>2.9</td>
<td>6.4</td>
<td>1271.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>
Fig. 21  DEAE-cellulose chromatography of the soluble proteins of *S. cervi* sedimenting between 45-60 % saturation of 

\[(NH_4)_2SO_4\] and possessing maximum aldolase activity.

- - - Protein

▲▲▲ Aldolase

Size of the column  1.5 x 16 cm

Flow rate  1 ml/min.

Volume of each fraction  5 ml
protein band eluting with buffer containing 50 mM sodium chloride, investigations for studying the properties were confined to this fraction.

**Stability during storage**

An analysis of aliquots of the purified enzyme preparations kept at +5° and -10°C for different time intervals indicated that the enzyme was fairly stable. 100% activity remained even when preserved at -10°C for 10 days, while 48% and 95% loss in activity was observed on keeping the enzyme at +5°C for 12 and 30 days respectively (Table 18).

**Thermal stability**

Studies on sensitivity to temperature indicated that the purified preparation was thermolabile and lost 9% activity after keeping at 40°C for 30 min. Nearly 50% inactivation took place when the enzyme was incubated at 45°C for identical period (Table 19).

**Time-activity relationship**

A linearity in the reaction rate was observed when buffered enzyme and substrate were incubated at 37°C up to 40 min. reaching a maximum after 80 min. of incubation (Fig. 22).

**Enzyme concentration**

A direct relationship between FDP cleaved and the amount of enzyme used was observed up to 5 micrograms of enzyme protein (Fig. 23).
Table 18 - Stability of *S. cervi* aldolase during storage at +5 and -10°C

<table>
<thead>
<tr>
<th>No. of days</th>
<th>% Remaining activity (+5°C)</th>
<th>% Remaining activity (-10°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>3</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>6</td>
<td>96.0</td>
<td>100.0</td>
</tr>
<tr>
<td>9</td>
<td>90.3</td>
<td>100.0</td>
</tr>
<tr>
<td>12</td>
<td>52.4</td>
<td>90.1</td>
</tr>
<tr>
<td>15</td>
<td>30.2</td>
<td>85.2</td>
</tr>
<tr>
<td>18</td>
<td>20.0</td>
<td>82.3</td>
</tr>
<tr>
<td>21</td>
<td>18.3</td>
<td>81.4</td>
</tr>
<tr>
<td>24</td>
<td>14.3</td>
<td>78.5</td>
</tr>
<tr>
<td>27</td>
<td>10.3</td>
<td>76.4</td>
</tr>
<tr>
<td>30</td>
<td>5.1</td>
<td>75.4</td>
</tr>
<tr>
<td>Temperature of incubation (°C)</td>
<td>% Activity lost in 30 min.</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>37.8</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>47.8</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>68.9</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>77.8</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>86.7</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>87.8</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 22  Time-velocity relationship of \textit{S. cervi} aldolase.

Fig. 23  Effect of enzyme protein concentration on \textit{S. cervi} aldolase activity.
Effect of pH

Using veronal and Tris-HCl buffers, the optimum pH for *S. cervi* aldolase was observed in the pH range of 8.5 - 8.7. Although no significant change in the enzyme activity was observed in these two buffers at the optimum pH, veronal buffer recorded much lower activity at pH 8.2 as compared to Tris-HCl buffer (Fig. 24).

Effect of substrate concentration

Fig. 25 illustrates the effect of fructose-1,6-diphosphate concentration. The Michaelis constant (K_m) as computed from the Lineweaver-Burk plot was found to be of the order of 7.14 x 10^{-4}M of FDP (Fig. 25 insert).

Effect of inhibitors and activators

The results of the inhibition and activation studies with -SH reactants, carbonyl agents, divalent cations and metal chelators are summarized in Tables 20-22. p-Chloromercuribenzoate at 1 x 10^{-3}M concentration inhibited the enzyme by 83% while β-mercaptoethanol at identical concentration activated the enzyme by 11% and at 1 x 10^{-2}M concentration by 73%. When β-mercaptoethanol was added after the enzyme was preincubated with pCMB complete reversal of the activity was not achieved. However, when pCMB was added after preincubation with β-mercaptoethanol
Fig. 24 Effect of pH and buffers on *S. cervi* aldolase activity.

- Tris-HCl buffer
- Veronal buffer

Fig. 25 Effect of FDP concentration on *S. cervi* aldolase activity.

Insert shows the reciprocal of substrate concentration against reciprocal of activity (Lineweaver Burk plot), for the verification of $K_m$ value.
(i.e., in the reverse way) 92% enzyme activity was retained, thereby indicating that the inhibition by pCMB could be reversed by β-mercaptoethanol. Cysteine and reduced glutathione both at 1 x 10^{-2} M concentration activated the enzyme by 51 and 80% respectively, whereas N-ethylmaleimide and iodoacetate at the same concentration proved to be ineffective in blocking the essential -SH groups of the enzyme. Hydroxylamine and semicarbazide were inhibitory to the enzyme (Table 20).

Among the divalent cations, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+} and Co^{2+} were inhibitory while Mg^{2+} had no effect even at a high concentration (1 x 10^{-2} M). The inhibition caused by Zn^{2+} was maximum while Ca^{2+} was least effective. Of the anions tried, borate was inhibitory while phosphate and fluoride had no effect. Thymol at 1 x 10^{-3} M concentration inhibited the enzyme by 27% while veronal at 1 x 10^{-2} M concentration did not inhibit the enzyme (Table 21).

Among the metal chelators, α-α-dipyridyl and EDTA activated the purified enzyme by 20 and 35% respectively at 1 x 10^{-2} M concentration while 8-hydroxyquinoline and o-phenanthroline at 1 x 10^{-3} M concentration inactivated the enzyme by 34% and 9% respectively. Pyrophosphate at 1 x 10^{-2} M concentration had no effect whereas salicylic acid at identical concentration activated the enzyme by 15%. The pronounced inhibition observed with Cu^{2+} was
Table 20 - Effect of -SH reactants and carbonyl reagents on aldolase of S. cervi

<table>
<thead>
<tr>
<th>Addition</th>
<th>Final Concentration (M)</th>
<th>% of activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100.0</td>
</tr>
<tr>
<td>pCMB</td>
<td>$1 \times 10^{-3}$</td>
<td>12.3</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>$1 \times 10^{-3}$</td>
<td>110.8</td>
</tr>
<tr>
<td>8-Mercaptoethanol</td>
<td>$1 \times 10^{-2}$</td>
<td>173.0</td>
</tr>
<tr>
<td>pCMB + β-Mercaptoethanol</td>
<td>$1 \times 10^{-3}$ and $1 \times 10^{-3}$</td>
<td>52.3</td>
</tr>
<tr>
<td>β-Mercaptoethanol + pCMB</td>
<td>$1 \times 10^{-3}$ and $1 \times 10^{-3}$</td>
<td>92.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>$1 \times 10^{-2}$</td>
<td>151.0</td>
</tr>
<tr>
<td>Gluathione (reduced)</td>
<td>$1 \times 10^{-2}$</td>
<td>180.0</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>$1 \times 10^{-2}$</td>
<td>100.0</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>$1 \times 10^{-2}$</td>
<td>100.0</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>$1 \times 10^{-3}$</td>
<td>52.0</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>$1 \times 10^{-3}$</td>
<td>77.0</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>$1 \times 10^{-2}$</td>
<td>37.0</td>
</tr>
</tbody>
</table>
Table 2.1 - Effect of divalent cations, some anions, veronal and thymol on aldolase of *S. cervi*

<table>
<thead>
<tr>
<th>Addition</th>
<th>Final concentration (M)</th>
<th>% of activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>$1 \times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>$5 \times 10^{-3}$</td>
<td>70</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>$1 \times 10^{-2}$</td>
<td>46</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>$1 \times 10^{-4}$</td>
<td>26</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>$1 \times 10^{-4}$</td>
<td>40</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>$1 \times 10^{-3}$</td>
<td>90</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>$1 \times 10^{-3}$</td>
<td>87</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>$1 \times 10^{-3}$</td>
<td>49</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$1 \times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>Borate</td>
<td>$1 \times 10^{-2}$</td>
<td>72</td>
</tr>
<tr>
<td>Fluoride</td>
<td>$1 \times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>Veronal</td>
<td>$1 \times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>Thymol</td>
<td>$1 \times 10^{-3}$</td>
<td>73</td>
</tr>
</tbody>
</table>
completely reversed by salicylic acid when the enzyme was preincubated with salicylic acid before the addition of Cu$^{2+}$. On the other hand when salicylic acid was added after inhibiting the enzyme with Cu$^{2+}$ complete reversal was not observed (Table 22).

**Effect of certain antifilarial compounds and metabolic inhibitors**

Among the antifilarial compounds and metabolic inhibitors tried, hetrazan (diethylcarbamazine citrate; Cyanamid Co., U.S.A.), 67/255 (1-ethyl-6-methyl-2-oxo-perhydro(1,2-c) piperazine pyrimidine; Saxena, 1968 - a compound prepared in Central Drug Research Institute, Lucknow, and shown to be three times more active than hetrazan in its antifilarial activity in cotton rats), neoarsphenamine, sodium arsenate, sodium fluoride, sodium azide and dinitrophenol had no inhibitory effect on the purified enzyme. Cyanine-863, however, strongly inhibited the enzyme activity at a concentration of $1 \times 10^{-4}$M (Table 23).

**DISCUSSION**

Relatively little information is available on the properties of the glycolytic enzymes of nematodes and virtually none concerning filarial parasites. On account of the key role played by aldolase in carbohydrate metabolism,
<table>
<thead>
<tr>
<th>Addition</th>
<th>Final concentration (M)</th>
<th>% of activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>L-α-Dipyridyl</td>
<td>$1 \times 10^{-2}$</td>
<td>120</td>
</tr>
<tr>
<td>EDTA</td>
<td>$1 \times 10^{-2}$</td>
<td>135</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>$1 \times 10^{-3}$</td>
<td>66</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>$1 \times 10^{-3}$</td>
<td>91</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>$1 \times 10^{-2}$</td>
<td>62</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>$1 \times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>$1 \times 10^{-2}$</td>
<td>115</td>
</tr>
<tr>
<td>Cu$^{2+}$ + Salicylic acid</td>
<td>$1 \times 10^{-2}$ and $1 \times 10^{-4}$</td>
<td>88</td>
</tr>
<tr>
<td>Salicylic acid + Cu$^{2+}$</td>
<td>$1 \times 10^{-2}$ and $1 \times 10^{-4}$</td>
<td>100</td>
</tr>
</tbody>
</table>
### Table 23 - Effect of certain antifilarial compounds and metabolic inhibitors on aldolase of *S. cervi*

<table>
<thead>
<tr>
<th>Addition</th>
<th>Final concentration (M)</th>
<th>% of activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Sodium arsenate</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>Hetrazan</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>67/255</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>Neoarsphenamine</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>Cyanine-363</td>
<td>$1 \times 10^{-4}$</td>
<td>60</td>
</tr>
<tr>
<td>Sodium cyanide</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
</tbody>
</table>
the present study on the purified aldolase of *S. cervi* has a relevance to the problem of chemotherapy particularly against the background of the observations of Srivastava et al. (1971) who reported cyanine-863 blocked glycolysis in the filarial worms *Chandlerella hawkingi* and *Litomosoides carinii* specifically at the aldolase step. It may be mentioned, however, that Srivastava et al. (1971) had used only crude extracts of the worms due to the unavailability of these parasites in quantities adequate for purification. The present report would appear to be the first on a highly purified preparation of an aldolase from a filarial parasite.

Ammonium sulphate treatment of the particulate-free supernatant enriched besides aldolase a major fraction of phosphoglucomutase, phosphopyruvate hydratase and 25% of hexokinase could be recovered in the fraction sedimenting between 45-60% saturation of ammonium sulphate. The purified enzyme of *S. cervi* could be separated by DEAE-cellulose chromatography into two isomeric forms unlike partially purified aldolase from *A. galli* (Srivastava et al., 1970a) where no isoenzymes could be detected. The finding of the isomeric forms of aldolase in *S. cervi* is not unusual for worms since the presence of malate dehydrogenase (Rhodes and Marsh, 1964; Zee and Zinkham, 1968) and lactate dehydrogenase (Nagase, 1968) isoenzymes have earlier been reported in *Ascaris* species employing starch gel and cellulose acetate electrophoresis.
The partially purified aldolase of *A. galli* (Srivastava et al., 1970a) was stable at 56°C, however, the *S. cervi* enzyme was thermolabile and lost 50% activity even at 46°C.

While studying the stability of bacterial aldolase in *Clostridium perfringens*, Bard and Gunsalus (1950) reported a rapid decline on ageing. Baernstein and Rees (1952) and Baernstein (1955) observed similar results in *Trypanosoma cruzi* and *Trichomonas vaginalis* respectively. On the other hand *Taenia crassiceps* (Phifer, 1958) and *A. galli* (Srivastava et al., 1970a) aldolase were stable and could be stored for 3 and 6 months respectively under frozen condition. The observations on the stability of aldolase of *S. cervi* indicated that the enzyme was fairly stable and did not loose any activity up to 10 days when stored at -10°C and was found to retain 75% activity when stored for a month under frozen condition.

Using Tris buffer, Sibley and Lehninger (1949) reported optimum pH 8.5 - 9.0 for rabbit muscle aldolase while Bard and Gunsalus (1950) and Baernstein (1955) working with no specific buffer system reported pH optimum of 7.5 and 7.0 for *C. perfringens* and *T. cruzi* respectively. Baernstein and Rees (1952), using both acetate-veronal and collidine buffers, reported an optimum of 7.3 - 7.6 for *T. vaginalis.*
Similar broad pH optimum was also observed in *T. crassiceps* (8.5 - 9.0, Phifer, 1958), *T. spiralis* larvae (6.0 - 8.5, Agosin and Aravena, 1959a) and *A. galli* (7.3 - 7.8, Srivastava et al., 1970a). Thus the pH optimum of the aldolase of filarial parasite *S. cervi* (8.5 - 8.7) is much closer to that of mammalian and *T. crassiceps* aldolase than that of protozoa.

The $K_m$ values for the aldolase of *T. spiralis* larvae (Agosin and Aravena, 1959a), *T. vaginalis* (Baernstein, 1955), *T. cruzi* culture (Baernstein and Rees, 1952) and *A. galli* (Srivastava et al., 1970a) have been reported to be $3.0 \times 10^{-4} M$, $1.0 \times 10^{-3} M$, $1.2 \times 10^{-3} M$ and $4.5 \times 10^{-3} M$ FDP respectively, while Dounce and Beyer (1948) reported a constant of $9.0 \times 10^{-3} M$ for rabbit muscle aldolase. Hence the observed $K_m$ of *S. cervi* ($7.1 \times 10^{-4} M$) is in close agreement with the reported values in other parasites.

Inhibition and activation studies with purified aldolase of *S. cervi* revealed some interesting properties. The enzyme was not metal activated and thus resembled the aldolase of *Trypanosoma lewisi* (Ryley, 1953), *T. spiralis* (Agosin and Aravena, 1959a) and *A. galli* (Srivastava et al., 1970a) where pronounced inhibition was observed with certain divalent cations. The aldolase of *T. vaginalis*, on the other hand, differed sharply from the above aldolases by its metal...
relationship and strong inhibition by \( \kappa-\alpha \)-dipyridyl and EDTA (Baernstein, 1955). However, Phifer (1958) working with \textit{T. crassiceps} aldolase reported that divalent cations tested apparently did not inhibit aldolase activity nor were they necessary for optimal functioning.

According to Rutter (1961) aldolase derived from various sources may be grouped into two types on the basis of their behaviour towards chelating agents. Aldolase type I is not sensitive to chelating agents and hence is not activated by metal ions. To this category belong mammalian aldolase like the enzyme prepared from skeletal muscle. Aldolase type II is highly sensitive to metal chelators. The inhibition by metal chelators is reversed by certain divalent metal ions. This group included enzymes derived from microbial sources. \textit{S. cervi} aldolase may, therefore, be classified as aldolase I in the light of differentiation discussed by Rutter (1964) and Kobes \textit{et al.} (1969).

The activation by \( \kappa-\alpha \)-dipyridyl and EDTA observed in \textit{S. cervi} was presumably due to the removal of traces of heavy metallic inhibitors carried by the enzyme in spite of its passage through the anion exchange column. Since \( \text{Fe}^{2+} \) and \text{o-phenanthroline} were individually inhibitory to the enzyme, the effect of the latter reagent was not due to the removal of essential \( \text{Fe}^{2+} \) ions. According to Kobashi and Horecker (1967), \text{o-phenanthroline} brings out conformational
changes on crystalline muscle aldolase. Activation by salicylic acid observed in the present study may presumably be due to the removal of traces of Cu$^{2+}$ ions present by the process of chelation since Cu$^{2+}$ has been shown to strongly inhibit the enzyme. Borate and thymol were also significantly inhibitory to aldolase, the mechanism of this inhibition remains unelucidated.

pCMB effectively blocked the enzyme activity whereas NEM and iodoacetate failed to exert any significant inhibitory action. Presumably the active -SH groups of the S. cervi aldolase were not accessible to iodoacetate and NEM but only to pCMB. The reversal of aldolase inhibition due to pCMB, by a reducing agent like $\beta$-mercaptoethanol (when added prior to the preincubation of the enzyme with the inhibitor) was indicative of the presence of functional -SH groups in the enzyme molecule. In this respect S. cervi aldolase resembles the enzyme isolated from T. vaginalis (Baernstein, 1955) and A. galli (Srivastava et al., 1970a), but differs from T. crassiceps (Phifer, 1958) and T. spiralis larvae (Agosin and Aravena, 1959a) where the presence of functional -SH groups could not be detected.

Cyanine inhibition is not limited to S. cervi as similar inhibitory action of this dye has been observed by Srivastava et al. (1971) on the cell-free aldolase and glycolytic rate of C. hawkingi and L. carinii. The dye also
inhibited partially purified aldolase of *A. galli* (Srivastava et al., 1971). The present observations on the inhibition of *S. cervi* aldolase may be attributed to the same general phenomenon as in other filarial parasites.
SECTION IV

ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF TRYP SIN AND CHYMOTRYPSIN INHIBITORS FROM A. GALLI
Subcellular localization of the inhibitors

The whole worm (*A. galli*, male or female), cuticular layers, intestines, ovaries or testes were used for the localization of the inhibitors. Various subcellular fractions obtained by centrifuging the cell-free homogenate at 800 g, 12,000 g and 105,000 g were examined for antitryptic and chymotryptic activities. The results indicated that most of the inhibitory activity was localized in the particulate-free supernatant of the cuticular layers irrespective of sex.

Isolation of the inhibitors

All the subsequent work concerning the purification of these inhibitors was carried out using whole worms.

In preliminary experiments the proteolytic inhibitors were found to withstand heat up to 80°C, were unaffected by 5% (w/v) TCA in which they were soluble. These two properties were utilized for the preparation of these inhibitors from the 105,000 g supernatant of whole worms. About 90% inactive protein was removed by TCA and the heat treatment and the inhibitor complex was purified to 36.0 and 12.59 fold respectively when assayed for trypsin and chymotrypsin inhibiting activities with a recovery of 100% and 64.5% respectively (Table 24) as compared to the starting material.
Purification of the inhibitors employing ion-exchange chromatography

The inhibitor complex obtained after lyophilization was subjected to ion-exchange chromatography. Fig. 26 shows a typical elution profile of the proteins present in the lyophilized inhibitor preparation using DEAE-cellulose chromatography. The figure also describes activities with respect to trypsin and chymotrypsin inhibitors. Out of the five protein bands, the first two showed proteinase inhibitory activities.

The first band eluting with equilibrating buffer exhibited inhibitory activity against both trypsin and chymotrypsin; while the second band eluting with 0.05 M sodium chloride inhibited only chymotrypsin activity. Thus chymotrypsin inhibitor was found to be resolved into two bands suggesting the possible occurrence of isomeric forms of this inhibitor.

On recycling through freshly prepared DEAE-cellulose column after the removal of buffer and salts, the two inhibitory activities were recovered in eluates emerging in the equilibrating buffer and sodium chloride concentration identical with the ones used in the preparatory columns. The purification and recovery of these inhibitor proteins at different stages of purification are summarized in Table 24. The recovery and purification of the trypsin
Table 24 - Recovery and purification of trypsin and chymotrypsin inhibitors of A. galli

<table>
<thead>
<tr>
<th>Description</th>
<th>Volume</th>
<th>Protein (mg)</th>
<th>Trypsin inhibitor</th>
<th>Chymotrypsin inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Recovery</td>
<td>Total activity (units)</td>
<td>Specific</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% Recovery</td>
<td>Total activity (units)</td>
<td>Specific</td>
</tr>
<tr>
<td>800 g supernatant</td>
<td>169.3</td>
<td>914.2</td>
<td>22.0x10^3</td>
<td>100</td>
</tr>
<tr>
<td>105,000 g supernatant</td>
<td>164.0</td>
<td>754.4</td>
<td>24.6x10^3</td>
<td>100</td>
</tr>
<tr>
<td>105,000 g supernatant after TCA heat treatment</td>
<td>240.0</td>
<td>46.8</td>
<td>40.8x10^3</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography (peak I)</td>
<td>-</td>
<td>3.5</td>
<td>43.6x10^3</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography (peak II)</td>
<td>-</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

131
Fig. 26 Elution profile of 105,000 g supernatant of A. galli obtained after TCA and heat treatment, employing stepwise elution from DEAE-cellulose column.

- Protein
- --- --- Trypsin inhibitor
- ▲▲~▲ Chymotrypsin inhibitor

Size of the column: 1 x 15 cm
Flow rate: 1 ml/min.
Volume of each fraction: 10 ml
FIG. 26

[Sodium Chloride Concentration (mM) vs. Inhibitor Units/ml]

- Protein
- Trypsin Inhibitor
- Chymotrypsin Inhibitor

SODIUM CHLORIDE CONCENTRATION (mM)

Fraction Numbers

Inhibitor Units/ml

Protein mg/ml

Concentrations: 0.05 → 0.1 → 0.2 → 0.3 → 0.4
inhibitor by DEAE-cellulose chromatography was 100% and 252 fold respectively while for chymotrypsin inhibitor the corresponding values were 67.3% and 37 fold and 46.1 fold for peak I and peak II respectively (Table 24).

Since the fractions under peak I possessing both trypsin and chymotrypsin inhibitory activities were not held by the DEAE-cellulose, they were pooled, dialysed, lyophilized and then passed through a freshly prepared cation exchanger (CM-cellulose column). The elution profile of the proteins separated by CM-cellulose chromatography and their activities with respect to trypsin and chymotrypsin inhibitors are given in Fig. 27.

The protein obtained from DEAE-cellulose chromatography, when applied to CM-cellulose chromatography was further resolved into five bands. Out of these five protein bands separated, the second and the third showed inhibitory activities against both trypsin and chymotrypsin. The band eluting with 0.05 M sodium chloride carried most of the trypsin inhibitory activity and only 20% was eluted by 0.1 M sodium chloride. Chymotrypsin inhibitory activity was present in 0.05 M and 0.1 M sodium chloride eluates, the former being more active than the latter.

Properties of the purified inhibitors

Since most of the trypsin inhibitory activity was recovered in the protein band eluting with buffer containing
Fig. 27  CM-cellulose chromatography of the protein band containing trypsin and chymotrypsin inhibitory activities, obtained after DEAE-cellulose chromatography (peak I).

- Protein
- Trypsin inhibitor
- Chymotrypsin inhibitor

Size of the column  1 x 11.5 cm
Flow rate  1 ml/min.
Volume of each fraction  5 ml
FIG. 27

SODIUM CHLORIDE CONCENTRATION (MOL/L)

INHIBITOR UNITS/mL (y-axis)

FRACTION NUMBER
0.05 M sodium chloride (CM-cellulose peak II), properties were studied with this fraction. Likewise properties of the protein band eluting with buffer containing 0.05 M sodium chloride (DEAE-cellulose peak II) and possessing only chymotrypsin inhibitory activity were studied in detail.

Molecular weights of the inhibitors

The molecular weights of the purified trypsin and chymotrypsin inhibitors as determined by gel-filtration method of Andrews (1964) were found to be approximately 15,500 and 15,000 respectively.

Complex formation between enzymes and the inhibitors

The data on the complex formation between trypsin and its inhibitor and chymotrypsin and its inhibitor have been presented in Table 25. Complex formation was complete within 60 and 30 seconds for trypsin and chymotrypsin respectively.

Effect of the purified inhibitors on the enzyme activity

Fig. 27 represents the effect of varying concentrations of the inhibitor on the enzyme activity employing 10 micrograms crystalline trypsin. A plot of the enzyme activity against inhibitor concentration gave a sigmoidal curve suggesting that more than one molecule of the inhibitor was bound to
Table 25 - Complex formation between enzymes and the inhibitors

<table>
<thead>
<tr>
<th>Time in min.</th>
<th>% Remaining enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibitor + Trypsin</td>
</tr>
<tr>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>0.5</td>
<td>50.6</td>
</tr>
<tr>
<td>1.0</td>
<td>37.4</td>
</tr>
<tr>
<td>2.0</td>
<td>37.4</td>
</tr>
<tr>
<td>3.0</td>
<td>37.4</td>
</tr>
<tr>
<td>4.0</td>
<td>37.3</td>
</tr>
<tr>
<td>5.0</td>
<td>37.1</td>
</tr>
<tr>
<td>10.0</td>
<td>37.4</td>
</tr>
</tbody>
</table>
the enzyme and these sites were interacting. According to Hill's equation:

\[ \log \left( \frac{v}{V_{\text{max}} - v} \right) = n \log (s) - \log k \]

is plotted against the log of inhibitor concentration, a straight line is obtained. The insert in Fig. 27 shows that when such a plot for the data was used, a straight line with slope of 2.4 was obtained.

More or less similar results were obtained in case of chymotrypsin inhibitor (Fig. 28) where the Hill plot was found to give a straight line having a slope of 2.3 (Fig. 28 insert).

**Effect of heat on the inhibitors**

It is evident from Table 26 that both trypsin and chymotrypsin inhibitors are active up to 80°C. Chymotrypsin inhibitor could withstand heating at 100°C up to 15 min. without any loss of inhibitory activity. However, 5% loss of activity was observed in case of trypsin inhibitor under identical conditions.

**Effect of urea, β-mercaptoethanol and stability at different pHs**

Table 27 presents the results of a study on the effect of urea, β-mercaptoethanol treatment and pH stability of the two purified inhibitors. Treatment with 8 M urea or
Fig. 28  Effect of increasing concentrations of the purified trypsin inhibitor on the activity of 10 μg crystalline trypsin.

Insert shows Hill plot.

\[ [i] \quad \text{Concentration of the trypsin inhibitor} \]
\[ v \quad \text{Activity of trypsin in presence of the inhibitor} \]
\[ V_{\text{max}} \quad \text{Activity of trypsin in absence of the inhibitor} \]

Fig. 29  Effect of increasing concentrations of the purified chymotrypsin inhibitor on the activity of 100 μg crystalline chymotrypsin.

Insert shows Hill plot.

\[ [i] \quad \text{Concentration of the chymotrypsin inhibitor} \]
\[ v \quad \text{Activity of chymotrypsin in presence of the inhibitor} \]
\[ V_{\text{max}} \quad \text{Activity of chymotrypsin in absence of the inhibitor} \]
FIG. 28

Log \( \frac{V}{V_{\text{max}}} \)

% Trypsin Activity

μg Trypsin Inhibitor

FIG. 29

Log \( \frac{V}{V_{\text{max}}} \)

% Chymotrypsin Activity

μg Chymotrypsin Inhibitor
Table 26 - Effect of temperature on the proteolytic inhibitors

<table>
<thead>
<tr>
<th>Temp. °C</th>
<th>% Remaining inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypsin inhibitor</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>90</td>
<td>98</td>
</tr>
<tr>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Treatment</td>
<td>% Remaining inhibitory activity</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td></td>
<td>Trpserin inhibitor</td>
</tr>
<tr>
<td>Urea (1 - 8 M)</td>
<td>100</td>
</tr>
<tr>
<td>pH (3 - 10)</td>
<td>100</td>
</tr>
<tr>
<td>β-Mercaptoethanol (0.01 - 0.1 M)</td>
<td>100</td>
</tr>
<tr>
<td>Potassium chloride (1 - 4 M)</td>
<td>-</td>
</tr>
</tbody>
</table>
with 0.1 M mercaptoethanol had no effect on these inhibitors. The inhibitors were active over a wide pH range of 3-10.

**Dissociation of the enzyme-inhibitor complex**

A study of the dissociation of the enzyme-inhibitor complex using high salt concentrations indicated that the complex was not dissociated even in the presence of 4.0 M KCl (Table 27).

**DISCUSSION**

The proteinase inhibitors of *Ascaris* have received wide attention in recent years and several groups of workers have isolated, purified and characterized the trypsin and chymotrypsin inhibitors of *Ascaris lumbricoides* (see review by Kassell, 1970). The data reported in the present studies suggest the possible existence of two trypsin and three chymotrypsin inhibitors in *Ascaridia galli*. The functions of these isomeric forms of the inhibitors is not clear. As far as the inhibitory action is concerned, probably the same general mechanism is involved. Earlier, Rhodes *et al.* (1963) and Rola and Pudles (1966) have reported the presence of at least two different chymotrypsin inhibitors in *A. lumbricoides* suum, although electrophoresis on cellulose acetate indicated the existence of three components (Rola and Pudles, 1966). Likewise, Kucich and
Peanasky (1970) showed that the body walls of *A. lumbricoides suis* contained two trypsin inhibiting fractions differing with respect to $K_I$. Multiple forms of trypsin inhibitors have previously been noted in lima beans (Jones and Stein, 1963; Haynes and Feeney, 1967), soybeans (Frattali et al., 1968), dog submandibular glands (Fritz et al., 1968), porcine pancreatic juice (Green et al., 1963; Tschesche et al., 1969) and porcine clostrum (Lawrence et al., 1971).

The localization studies of these inhibitors in the various subcellular fractions and also in the different anatomical parts of *A. galli* revealed the existence of most of the inhibitory activity in the particulate-free fraction of the cuticular layers from both male and female parasites. Rhodes et al. (1963), however, reported the presence of these inhibitors not only in the cuticular layers but also in the perienteric fluids, intestines, ovaries and uteri of *A. lumbricoides suum*. Both trypsin and chymotrypsin inhibitors of *A. galli* were soluble and stable in TCA (final concentration 5%), a property which was effectively utilized in the isolation of these inhibitors. Out of the three chymotrypsin inhibitors, the one eluting with 0.05 M sodium chloride (DEAE-cellulose chromatography peak II) was specific for inhibiting only chymotrypsin activity while the other two inhibitors separated by CM-cellulose chromatography inhibited both trypsin and chymotrypsin activities.
The inhibitors for proteolytic enzymes studied so far are polypeptides. Like ovomucoid a proteinase inhibitor, the TCA treated crude inhibitor preparation of *A. galli* also contained protein and carbohydrates suggesting that they are protein carbohydrate complex. The fraction separated by ion-exchange chromatography possessing trypsin and chymotrypsin inhibitory activity contained carbohydrates along with protein. However, the fractions possessing the maximum inhibitory activities were devoid of carbohydrates. None of these fractions contained sialic acid. This rules out the possibility of a protein–carbohydrate complex nature of the *A. galli* inhibitors.

The inhibitors were unaffected by urea and β-mercaptoethanol treatment, showed a broad pH stability and were resistant to heat. Similar stabilities towards heat, urea and pH were observed for chymotrypsin and trypsin inhibitors of *A. lumbricoides* suum (Rola and Pudles, 1966; Pudles et al., 1967). However, β-mercaptoethanol inactivated the chymotrypsin and trypsin inhibitors of *A. lumbricoides* suum and s-s bridges were assumed to be present in these inhibitors on the basis of reduction, reoxidation and alkylation studies (Rola and Pudles, 1966; Pudles et al., 1967). The results of the present study suggest the absence of non-covalent linkages and disulphide bonds in the inhibitors of *A. galli*. The complex formation between the enzyme and the inhibitor is not a time dependent reaction and the complex is not dissociated by high salt concentrations.
The sigmoidal nature of the curves obtained by studying the effect of various inhibitor concentrations on trypsin and chymotrypsin activities suggest that there are at least two binding sites if not more for the inhibitors.

The $K_i$ for trypsin inhibitor of *A. lumbricoides* suum as determined by Green (1957) was $3 \times 10^{-9}$ M, while Kucich and Peanasky (1970) reported $9 \times 10^{-8}$ M and $1.3 \times 10^{-8}$ M for peak I and II respectively for *A. lumbricoides* suis trypsin inhibitor. According to Peanasky and Szucs (1964) $K_i$ against chymotrypsin A and B were $6.9 \times 10^{-9}$ and $3.2 \times 10^{-8}$ M respectively, while according to Rola and Pudles (1966), $K_i$ against chymotrypsin was of the order of $4.7 \times 10^{-8}$ M. Thus the observed values in *A. galli* (which are $2.6 \times 10^{-8}$ and $1.05 \times 10^{-7}$ M for trypsin and chymotrypsin inhibitors respectively) are in close agreement with the previous investigations in other *Ascaris* species.

The proteinase inhibitors of *A. galli* appear to differ from the corresponding inhibitors of other *Ascaris* species in the molecular size. The molecular weights of trypsin and chymotrypsin inhibitors of *A. galli* are of the order of 15,500 and 15,000 respectively, while the corresponding values reported for *Ascaris* suum are 8200 and 8600 respectively. According to Kucich (1970) the trypsin inhibitor of *Ascaris* suis comprising of 50 amino acid residues has a molecular weight of 5520.
The presence of tryptic or chymotryptic inhibitors could not be detected in the filarial parasites \textit{S. cervi}, \textit{L. carinii} and \textit{C. hawkingi} which thrive in the peritoneal cavity, pleural cavity and heart and lung of the corresponding hosts. It can be generalized, therefore, that proteinase inhibitors may be playing important role in protecting the intestinal parasites from destruction by the digestive juices of the hosts and are not of much significance to the tissue parasites.
CHAPTER IV

SUMMARY AND CONCLUSIONS
The results obtained on certain aspects of the carbohydrate metabolism in *Setaria cervi*, the filarial parasite of buffaloes and *Ascaridia galli*, the intestinal nematode of fowls, and proteinase inhibitors present in *A. galli* are described in the present dissertation.

The filarial parasite *S. cervi* released lactic and pyruvic acids during *in vitro* incubation in KRB buffer, pH 7.4, using air as the gas phase indicating breakdown of endogenous polysaccharides.

The worm could utilize exogenously added glucose and mannose efficiently, while fructose and galactose showed poor utilization rates.

Quantitatively lactate accounted for 28, 20, 65 or 80% of glucose, mannose, fructose or galactose consumed respectively during first 2 hours of incubation period. Trace amounts of pyruvate were also formed by glucose utilization.

The rate of utilization of hexoses by the parasite was much faster during the initial periods of incubation which decreased along with the time of incubation.

During the first 2 hours of incubation, the amount of glucose and mannose utilized was more or less the same but afterwards the utilization rate of mannose showed a steep fall.
The worms remained motile in KRB medium fortified with glucose even after 24 hour incubation period whereas they lost their motility in media supplemented with fructose or galactose within 6-12 hours.

Glucose, which could serve as the best energy source can, therefore, conveniently be employed as the constituent of a mineral medium for the maintenance of *S. cervi* under in vitro conditions. This medium could be employed in the testing of antifilarial drugs using motility as one of the parameters.

When the parasite was incubated aerobically in KRB buffer, pH 7.4 with glucose-\(^{14}C\) or fructose-\(^{14}C\), the label was incorporated into different macromolecular constituents of the worm. About 34% of the total radioactivity of glucose-\(^{14}C\) incorporated in the body was recovered in glycogen, while 0.5, 0.4 and 0.3% were distributed in lipids, nucleic acids and proteins respectively. With fructose-\(^{14}C\), the incorporation of radioactivity was one fifth that of glucose, out of which 9% was recovered in glycogen.

The worms from the above incorporation experiments when incubated in KRB medium devoid of any sugar released radioactivity into the medium indicating that during starvation the reserve food material stored as glycogen was broken down.
Homogenates of *S. cervi* were assayed for enzymatic activities associated with the glycolytic pathway of glucose assimilation. The extracts contained significant activities of glycogen phosphorylase, a non-specific hexokinase (phosphorylating glucose, mannose, galactose and fructose), phosphatases acting on G-6-P and FDP, phosphoglucomutase, glucosephosphate isomerase, FDP-aldolase, phosphopyruvate hydratase, pyruvate kinase and lactate dehydrogenase.

Sub-cellular localization of these enzymes indicated that specific activities of glycogen phosphorylase, G-6-Pase and hexokinase were higher in cell-free (800 g) supernatant than in the particulate-free (105,000 g) fraction. Except for aldolase and lactate dehydrogenase which were also present in mitochondria, the remaining enzymes of the pathway were localized in the particulate-free fraction. The particulate-free supernatant did not show by themselves any oxidation of NADH₂ or NADPH₂ without added substrates.

The evidence adduced, indicated that the parasite was equipped with the enzymatic machinery required to mediate the anaerobic breakdown of glucose and to derive energy by this mechanism.

Among all the enzymes of the glycolytic pathway in *S. cervi*, pyruvate kinase was least active. Presence of this enzyme in the worm could account for the detection of trace amounts of pyruvate among the metabolites.
Extracts of *S. cervi* and *A. galli* possessed the enzyme PEP-carboxykinase which catalyzed the transformation of PEP to oxaloacetate. The specific activity of this enzyme was 28 times and 57 times higher than pyruvate kinase activity in *S. cervi* and *A. galli* respectively.

Cell-free extracts of *A. galli* were shown to dehydrogenate almost all the tricarboxylic acid cycle intermediates. The presence of aconitate hydratase, isocitrate dehydrogenase, succinate dehydrogenase, fumarate hydratase, malate dehydrogenase and malic enzyme was detected in *S. cervi* and *A. galli* providing conclusive evidence for the functioning of TCA cycle in these parasites.

All the enzymes of the TCA cycle in *A. galli* were assayed in whole worm (male or female) and its different anatomical parts. The levels of aconitate hydratase, isocitrate dehydrogenase, fumarate hydratase and malic enzyme were higher in male worms than in the females, while the reverse was true for α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase. In general, the specific activities of the TCA cycle enzymes in *A. galli* were higher in body walls and intestines than in the reproductive organs or in the whole worms.

The specific activities of aconitate hydratase, fumarate hydratase (in forward direction) and malate
dehydrogenase were higher in *S. cervi* than in *A. galli*, while isocitrate dehydrogenase, α-keto-glutarate dehydrogenase, succinate dehydrogenase, malic enzyme and fumarate hydratase (in backward direction) were more active in *A. galli* than in *S. cervi*.

Among the TCA cycle enzymes of *S. cervi* and *A. galli* malate dehydrogenase and fumarate hydratase were most active.

Aconitate hydratase was more active in catalyzing the forward (citrate to cis-aconitate) reaction, while in case of fumarate hydratase the backward (malate to fumarate) reaction was 150 and 250 times more pronounced than the forward (fumarate to malate) reaction in *S. cervi* and *A. galli* respectively.

*S. cervi* could not be differentiated from *A. galli* since no significant difference in the levels of different enzymes mediating this pathway could be noticed. It is quite likely that the filarial parasite *S. cervi* living in an anaerobic habitat would behave more like the intestinal nematode *A. galli* than *G. hawkingi* or *L. carinii* which thrive in an oxygen rich habitat.

The presence of PEP-carboxykinase, low level of pyruvate kinase and high activities of malate dehydrogenase and fumarate hydratase suggested the operation of PEP-
oxaloacetate pathway in *S. cervi* and *A. galli*. Further, production of certain acids e.g. succinate by the parasites could be explained on the basis of the operation of this alternate pathway. Succinate production has a distinct advantage over the LDH reaction in the anaerobic habitat of helminth parasites. Both the above reactions provide means for reoxidizing NADH$_2$ thereby making NAD available for glycolysis. In contrast to the LDH reaction, the reduction of fumarate to succinate by NADH$_2$ catalyzed by a mitochondrial electron transport system in the parasites, generates energy in the form of ATP.

The particulate-free supernatant of *S. cervi* was fractionated with various concentrations of ammonium sulphate and the levels of aldolase, phosphoglucomutase, phosphopyruvate hydratase and hexokinase were assayed in all the fractions. The fraction sedimenting between 45-60% saturation of ammonium sulphate was the richest source of phosphoglucomutase, aldolase and phosphopyruvate hydratase activities, while maximum hexokinase activity was recovered in 60-80 % saturated fraction.

The fraction separating between 45-60% saturation of ammonium sulphate and possessing maximum aldolase activity was further purified by chromatography on DEAE-cellulose column following a stepwise elution scheme using increasing molarity of sodium chloride in Tris-Cl buffer, pH 7.4.
The purified enzyme of *S. cervi* could be separated by DEAE-cellulose chromatography into two isomeric forms.

Properties of the partially purified aldolase were studied with respect to stability during storage, thermal stability, time-activity relationship, enzyme concentration, effect of pH, buffer and substrate concentration and action of divalent cations, some anions, metal chelating agents, $-\text{SH}$ as well as carbonyl agents and effect of certain antifilarial compounds and metabolic inhibitors.

The purified enzyme was thermolabile, but fairly stable when stored frozen. Optimum pH using Tris-HCl and veronal buffers was 8.5 - 8.7 and the $K_m$ value was $7.1 \times 10^{-4} \text{M}$.

The $-\text{SH}$ groups of the aldolase were readily blocked by pCMB and presumably also by $\alpha$-phenanthroline. Iodoacetate and NEM failed to exert any inhibitory action, the active $-\text{SH}$ groups of the enzyme were presumably not accessible to these reactants.

No requirement of any divalent cation was shown by this enzyme, instead $\text{Zn}^{2+}$ and $\text{Cu}^{2+}$ were highly inhibitory. On the other hand, it was activated by cysteine, $\beta$-mercaptoethanol, salicylic acid, $\alpha$-$\alpha$-dipyridyl and EDTA. On the basis of different properties studied the enzyme was classified as aldolase I.
Among the metabolic inhibitors and antifilarial compounds tested, sodium arsenate, sodium fluoride, sodium azide, dinitrophenol, hetrazan, 67/255 and neoarsphenamine had no inhibitory effect on the purified enzyme. Cyanine-863, however, strongly inhibited the enzyme activity.

A. galli was found to contain inhibitors for trypsin and chymotrypsin activities. The presence of similar proteinase inhibitors could not be detected in the filarial parasites, S. cervi, C. hawkingi and L. carinii.

The proteinase inhibitors were found to be localized mostly in the 105,000 g particulate-free supernatant of the cuticular layers from either sexes.

The inhibitors were found to withstand heat up to 80°C and were active and soluble in 5% TCA.

A method for the isolation and purification of these inhibitors employing ion-exchange chromatography on DEAE-cellulose and CM-cellulose was developed. Two trypsin and three chymotrypsin inhibitors were separated by ion-exchange chromatography. Out of the three chymotrypsin inhibitors, the one eluting with 0.05 M NaCl (DEAE-cellulose chromatography peak II) was specific for inhibiting only chymotrypsin activity, while the other two inhibitors separated by CM-cellulose chromatography inhibited both trypsin and chymotrypsin activities.
The molecular weights of the trypsin and chymotrypsin inhibitors as determined by gel-filtration technique were 15,500 and 15,000 respectively.

The inhibitors were unaffected by heat, urea and β-mercaptoethanol suggesting the absence of non-covalent linkages and disulphide bonds in these molecules. Two binding sites for the inhibitors were found from kinetic analysis. The $K_i$ values were $2.6 \times 10^{-8}$ and $1.05 \times 10^{-7}$M for trypsin and chymotrypsin inhibitors respectively. The complex formation between enzymes and the inhibitors was not a time dependent reaction and was not dissociated by high salt concentrations.

These inhibitors are assumed to protect the intestinal parasite *A. galli* from destruction by the digestive enzymes of the host.
Agosin, M. (1957)  

Agosin, M. and Aravena, L. (1959a)  

Agosin, M. and Aravena, L. (1959b)  

Agosin, M. and Aravena, L. (1960)  

Agosin, M. and Repetto, Y. (1963)  

Agosin, M. and Repetto, Y. (1965)  

Andrews, P. (1964)  

Baernstein, H. D. (1955)  

Baernstein, H. D. and Rees, C. W. (1952)  

Bard, R. C. and Gunsalus, I. C. (1950)  

Barker, B. S. and Summerson, W. H. (1941)  

Battelli, F. and Stern, L. (1910)  

Bernard, Ch. (1859)  

Bloom, S. and Entner, N. (1965)  

Bodansky, O. (1961)  


Exp. Parasitol., 6, 586.  

Exp. Parasitol., 8, 10.  


Exp. Parasitol., 10, 28.  

Comp. Biochem. Physiol., 8, 245.  

Comp. Biochem. Physiol., 14, 299.  

Biochem. J., 21, 222.  

Exp. Parasitol., 4, 323.  

Exp. Parasitol., 1, 215.  


J. Biol. Chem., 138, 635.  

Biochem. Z., 30, 172.  


Arch. Biochem., 13, 291.
Bossche, H.V.D. and Janssen; P.A.J. (1967)

Bowman, D.E. (1944)

Bryant, C. and Nicholas, W.L. (1965)

Bryant, C. and Smith, M.J.H. (1963)

Bryant, C. and Williams, J.P.G. (1962)

Bueding, E. (1949)

Bueding, E. (1950)

Bueding, E. (1951)

Bueding, E. (1953)

Bueding, E. (1959)

Bueding, E. (1962a)

Bueding, E. (1962b)

Bueding, E. (1963)

Bueding, E. (1969)

Bueding, E. and Charms, B. (1952)

Bueding, E. and Farrow, G.W. (1956)

Bueding, E. and Mackinnon, J.A. (1955)

Life Sciences, 2, 1781.


Comp. Biochem. Physiol., 15, 103.

Comp. Biochem. Physiol., 9, 189.

Exp. Parasitol., 12, 372.


J. Gen. Physiol., 33, 475.


J. Biol. Chem., 202, 205.


Biochem. Pharmacol., 11, 17.

Federation Proc., 21, 1039.


Biochem. Pharmacol., 18, 1541.

J. Biol. Chem., 196, 615.

Exp. Parasitol., 5, 345.

J. Biol. Chem., 215, 495.
Bueding, E. and Mansour, T. E. (1957)

Bueding, E. and Most, H. (1953)

Bueding, E. and Oliver-Gonzalez, J. (1950)

Bueding, E. and Saz, H. J. (1968)

Bueding, E. and Yale, H. W. (1951)


Bueding, E., Saz, H. J. and Farrow, G. W. (1959)

Burge, W. E. and Burge, G. L. (1915)

Bushnell, L. D. and Erwin, L. E. (1949)

Cavier, R. and Savel, J. (1952)

Cheah, K. S. and Bryant, C. (1966)


Collier, H. B. (1941)


Microbiol., 7, 295.


Comp. Biochem. Physiol., 24, 511.


Biochem. Pharmacol., 5, 311.


J. Parasit., 1, 179.


Comp. Rend., 234, 2562.

Comp. Biochem. Physiol., 12, 491.

Biochim. Biophys. Acta, 12, 331.


Goldberg, E. (1958)

Good, C.A., Kramer, H. and Somogyi, M. (1933)

Graff, D.J. (1964)

Green, N.M. (1957)


Ham, W.E. and Sandstedt, R.M. (1944)

Hamill, J.M. (1966)


Harned, B.K. and Nash, T.P. (1932)


Hers, H.G. and de Duve, C. (1950)

Horecker, B.L. and Kornberg, A.C. (1948)

James, T.C., George, W.J. and Saul, R. (1966)

J. Parasitol., 44, 363.

J. Biol. Chem., 100, 485.


J. Biol. Chem., 242, 1804.

J. Biol. Chem., 154, 305.

J. Physiol. (London), 32, 479.

Comp. Biochem. Physiol., 19, 775.

J. Biol. Chem., 97, 443.


J. Biol. Chem., 242, 5378.


J. Biol. Chem., 175, 385.


Kassel, B. (1970)

Kmetec, E. and Bueding, E. (1961)

Kmetec, E. and Bueding, E. (1965)

Kobashi, K. and Horecker, B.L. (1967)

Kobes, R.D., Simpson, R.T., Vallee, B.L. and Rutter, W.J. (1969)

Kornberg, A. (1955)


Kunitz, M. (1938)

Kunitz, M. and Northrop, J.H. (1933)


King, E.J. and Campbell, D.M. (1960, 61)

Biochemistry,
2, 66.

J. Parasitol.,
41, 48.


J. Biol. Chem.,
233, 584.

Comp. Biochem. Physiol.,
15, 271.

Arch. Biochem. Biophys.,
121, 178.

Biochemistry,
8, 585.


Biochim. Biophys. Acta,
202, 47.

J. Gen. Physiol.,
22, 207.

Science,
78, 558.

J. Gen. Physiol.,
18, 433.

J. Gen. Physiol.,
19, 991.

Lardy, H.A. (1964)  


Laurie, J.S. (1957)

Exp. Parasitol., 6, 245.

Laurie, J.S. (1959)

Exp. Parasitol., 8, 188.

Lawrence, F.K., Susan, R.M. and Laskowski, M. Sr. (1971)


Mahler, H.R., Wittenberg, M.H. and Brand Ludwig (1953)


Mansour, T.E. (1962)

J. Pharmacol. Exp. Ther., 135, 94.

Mansour, T.E. and Bueding, E. (1954)


Massey, V. and Rogers, W.P. (1949)

Nature (London), 163, 909.

Massey, V. and Rogers, W.P. (1950)


J. Exp. Med., 84, 569.

Mendel, L.B. and Blood, A.F. (1910)

J. Biol. Chem., 8, 177.

Montgomery, R. (1957)

Nagase, K. (1968)  

Kiseichugaku Zasshi, 17, 86.

Najjar, V.A. (1948)  

J. Biol. Chem., 175, 281.

Nelson, N. (1944)  


Northrop, J.W. (1926)  

J. Gen. Physiol., 2, 497.

Northrop, J.H. and Kunitz, M. (1931)  

Science, 73, 262.

Northrop, J.H. and Kunitz, M. (1932)  


Northrop, J.H. and Kunitz, M. (1934)  

Science, 80, 505.

Norton, S. and De Beer, E.J. (1957)  


Ochoa, S. (1948a)  


Ochoa, S. (1948b)  


Ochoa, S. (1955)  


Oya, H., Costello, L.G. and Smith, W. (1963)  

Exp. Parasitol., 14, 186.

Oya, H., Kikuchi, G., Bando, T. and Hayashi, H. (1965)  

Exp. Parasitol., 17, 229.

Pandaya, G.T. (1961)  

Z. Parasitenk., 20, 466.
Peanasky, R.J. (1963)

Peanasky, R.J. and Laskowski, M. (1960)

Peanasky, R.J. and Szucs, M.M. (1964)

Phifer, K. (1958)

Plaut, G.W.E. and Plaut, K.A. (1952)

Prescott, L. and Campbell, J.M. (1965)

Prichard, R.K. and Schofield, F.J. (1968a)

Prichard, R.K. and Schofield, F.J. (1968b)

Prichard, R.K. and Schofield, F.J. (1968c)

Pudles, J. (1962)

Pudles, J. and Rola, F. (1964)


Pushkarev, I.A. (1966)

Racker, E. (1950)


Rathbone, L. (1955)


J. Biol. Chem., 239, 2525.

Exp. Parasitol., 7, 269.

J. Biol. Chem.; 199, 141.

Comp. Biochem. Physiol., 14, 491.

Comp. Biochem. Physiol., 24, 697.


Ciencia e Cultura, 14, 216.


Arch. Biochem. Biophys., 120, 594.


Indian J. Malar. iol., 14, 457.

Biochem. J., 61, 574.
Rathbone, L. and Rees, K.H. (1954)
Read, C.P. (1951)
Read, C.P. and Rothman, A.H. (1958)
Reid, W.M. (1945)
Rhodes, M.B. and Marsh, C.L. (1964)
Roe, J.H. (1934)
Roe, J.H. (1955)
Rogers, W.P. and Lazarus, M. (1949)
Rola, F.H. and Pudles, J. (1966)
Rothman, A.H. (1959)
Rutter, W.J. (1961)
Rutter, W.J. (1964)
Ryley, J.F. (1953)
Ryley, J.F. (1955)

Exp. Parasitol., 1, 1.
Exp. Parasitol., 7, 217.
Exp. Parasitol., 15, 403.
Exp. Parasitol., 13, 266.
J. Parasitolog., 49, Suppl. 51.
J. Parasitolog., 51, 129.
J. Biol. Chem., 107, 15.
J. Biol. Chem., 212, 335.
Parasitology, 39, 302.
Arch. Biochem. Biophys., 113, 134.
J. Parasitolog., 45, 379.

Ryley, J.F. (1956)

Biochem.J.,

Sang, J.H. (1938)

Parasitology,
30, 141.

Saxena, R. (1968)

Thesis entitled "Studies on Synthetic Drugs", University of Lucknow.

Saz, H.J. and Bueding, E. (1966)

Pharmacol.Rev.,
18, 871.


Exp.Parasitol.,
12, 204.

Saz, H.J. and Hubbard, J.A. (1957)

J.Biol.Chem.,
225, 921.

Saz, H.J. and Vidrine, A. (1959)

J.Biol.Chem.,

Saz, H.J. and Weil, A. (1960)

J.Biol.Chem.,
235, 914.

Saz, H.J. and Weil, A. (1962)

J.Biol.Chem.,
237, 2053.


Exp.Parasitol.,
7, 477.

Scheibel, L.W. and Saz, H.J. (1966)

Comp.Biochem.Physiol.,
13, 151.

Schwabe, C.W. (1957)

Am.J.Hyg.,
65, 325.

Schwert, G.W. and Takenaka, Y. (1955)

Biochim.Biophys.Acta,
16, 370.

Seidman, I. and Entner, N. (1961)

J.Biol.Chem.,
236, 915.

Shonk, C.E. and Boxer, G.E. (1964)

Cancer Research,
24, 709.


Arch.Biochem.Biophys.,


Singer, T. P. (1959)

Slater, W. K. (1925)

Slater, E. C. and Bonner, W. D. (1952)

Smith, W., Costello, L. C. and Oya, H. (1963)

Somogyi, M. (1945)

Spiro, R. G. (1966)


J. Biol. Chem., 177, 859.

Comp. Biochem. Physiol., 18, 537.


Biochem. J., 52, 185.

J. Parasitol., 49, 51.


Indian J. Biochem. Biophys., 8, 108.

Indian J. Biochem. Biophys., 8, 97.

Exp. Parasitol., 28, 176.

Exp. Parasitol., 23, 339.

Parasitology, 60, 157.
Stewart, J. (1933)
Sutherland, E.W. (1949)
Swanson, M.A. (1950)
Szent-Gyorgyi, A. (1924)
Tallqvist, T.W. (1907)
Thorsell, W. (1963)
Ueno, Y., Oya, H. and Bando, T. (1960)
Utter, M.F. and Kurahashi, K. (1954)
Vernberg, W.B. and Hunter, M.S. (1963)
Vogel, R., Trautschold, I. and Werle, E. (1968)
von Bonsdorff, B. (1939)


Ward, C.W., Schofield, P.J. and Johnstone, I.L. (1963b)


Warrern, L.G. and Guevarra, A. (1962)

Warren, L.G. and Guevarra, A. (1963)

Webb, E.C. (1960, 61)

Weinland, E. (1901)

Weinland, E. (1903)

Wendel, W.B. (1946)

Werle, E. and Boetsch, K.B. (1959)

Whitlock, B. and Strong, F.M. (1963)

Wilson, T. (1968)

Yonesawa, T. (1953)

Zee, D.S. and Zinkham, W.H. (1968)

Comp. Biochem. Physiol., 26, 537.

J. Parasitol., 55, 67.


Naturwissenschaften, 46, 559.

Arch. Biochem. Biophys., 103, 459.


Arch. Biochem. Biophys., 126, 574.