

EFFECTS OF CAMBENDAZOLE AND MEBENDAZOLE ON THE
RESPIRATORY METABOLISM OF *MONIEZIA EXPANSA* (CESTODA).

by

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This thesis is my own work, except where specifically acknowledged.

A handwritten signature in cursive script, reading "M.S. Rahman". The signature is written in dark ink and is positioned above the printed name.

M.S. Rahman

"..... when you can measure what you are talking about and express it in numbers, you know something about it"

LORD KELVIN.

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ABSTRACT

Effects of cambendazole and mebendazole on respiratory metabolism in the anterior gram of *Moniezia expansa* were investigated under different experimental conditions. Under *in vitro* anaerobic conditions, in the presence of glucose, the drugs were found to inhibit glucose uptake by the worm. The inhibition of glucose uptake from the external source resulted in increased utilization of internal glycogen reserves by the worm, and thereby the carbon flux was largely maintained. The drugs also reduced the production of succinate by inhibiting the PEPCK reaction and the fumarate reductase system. The inhibition of carbon flow through the PEP-succinate pathway resulted in a shift of the flux from the succinate producing pathway to the lactate producing pathway. The increased lactate formed was accumulated in the worms. Decreased production of succinate resulted in a decrease in excretion. The drugs also diminished ATP generation and disturbed the synthesis and/or turnover of adenine nucleotides. This may be due to an inhibition of phosphorylation by the drugs. It was also considered that reduced energy levels in the worm might be responsible for decreased glucose uptake.

Under aerobic conditions, in the presence of glucose, the drugs exerted similar effects on glucose uptake, glycogen utilization and energy levels. However they had no effect on the formation of end-products and the carbon flux was largely maintained.

Under anaerobic and aerobic conditions, in the absence of glucose, some of the effects of the drugs were reproduced, but the overall metabolism was found to be adversely affected, irrespective of the presence of the drugs.

Investigations of the effects of CBZ and MBZ on selected glycolytic enzymes revealed that CBZ inhibited the activity of hexokinase

and phosphofructokinase whenever it was added to reaction mixtures containing cytosolic preparations of *M. expansa*. Both CBZ and MBZ inhibited the activity of HK and PFK when homogenates of *M. expansa* were incubated with the drugs. CBZ and MBZ did not affect the activities of these two enzymes in the supernatants prepared from the worms preincubated with the drugs; neither did they affect the activity of any other glycolytic enzymes, nor the commercial enzymes tested.

Further investigations with labelled substrates showed that both CBZ and MBZ inhibited the CO₂ fixing activity of PEPCK, and the fumarate reductase system.

In vivo experiments with infected lambs confirmed the effect on glucose uptake, glycogen utilization, succinate production and adenine nucleotide levels. The drugs exerted similar effects on energy metabolism in the gravid proglottids of *M. expansa*.

Therefore, it is concluded that a major effect of the drugs is the inhibition of phosphorylation at the substrate level and the electron transport level. They do not, however, uncouple phosphorylation from the electron transport chain.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CBZ	cambendazole
CoA	Coenzyme A
DHAP	dihydroxyacetone phosphate
DMA	dimethylacetamide
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDTA	ethylenediamine tetraacetate
EGTA	ethyleneglycol- <i>bis</i> (β -aminoethylether)- N,N'-tetraacetic acid.
F6P	fructose-6-phosphate
FDP	fructose-1,6-diphosphate
G6P	glucose-6-phosphate
G3P	glyceraldehyde-3-phosphate
GDP	guanosine 5'-diphosphate
GSH	reduced glutathion
GTP	guanosine 5'-triphosphate
IDP	inosine 5'-diphosphate
ITP	inosine 5'-triphosphate
K'	apparent equilibrium constant
KRP	Krebs-Ringer Phosphate solution
Lact	lactate
μ Ci	microCurie
mCi	milliCurie
MBZ	mebendazole

NAD+(H)	nicotinamide adenine dinucleotide (reduced)
NADP+(H)	nicotinamide adenine dinucleotide phosphate (reduced)
OAA	oxaloacetate
O.D.	optical density
PBZ	parbendazole
PEP	phosphoenolpyruvate
2PGA	2-phosphoglycerate
3PGA	3-phosphoglycerate
Pi	inorganic orthophosphate
PYR	pyruvate
sp. act.	specific activity
SD	standard deviation
Succi.	succinate
TBZ	thiabendazole
TCA	tricarboxylic acid
tris	tris-(hydroxymethyl)-aminoethane
x g	times gravity

ENZYMES

Abbreviation	Name Used	Enzyme Commission No.
Ald	aldolase	4.1.2.13
	enolase	4.2.1.11
	fumarase (fumarate hydratase)	4.2.1.2
FR	fumarate reductase	-
G6PDH	Glucose-6-phosphate dehydrogenase	1.1.1.49
G3PDH	glycerol -3-phosphate dehydrogenase	1.1.1.8
HK	hexokinase	2.7.1.1
LDH	lactate dehydrogenase	1.1.1.27
MDH	malate dehydrogenase	1.1.1.37
	malic enzyme	1.1.1.39-40
MK	myokinase	2.7.4.3
PEPCK	phosphoenolpyruvate carboxykinase	4.1.1.32
PFK	phosphofructokinase	2.7.1.11
PGI	phosphoglucose isomerase	5.3.1.9
PGM	phosphoglyceromutase	2.7.5.3
PK	pyruvate kinase	2.7.1.40
SDH	succinate dehydrogenase	1.3.99.1
STK	succinate thiokinase (Succinyl CoA synthetase)	6.2.1.4
TPI	triosephosphate isomerase	5.3.1.1

CHAPTER I: GENERAL INTRODUCTION

A. BIOLOGY OF *MONIEZIA EXPANSA*.

1.1 The Species.

Moniezia expansa Rudolphi, 1810, commonly known as the 'broad tapeworm' of sheep, inhabits the small intestine of sheep, goats, cattle and other ruminants in most parts of the world. This cestode may reach a length of six metres in about six weeks in the host's intestine (Lapage, 1965). The mature worms apparently do not normally live longer than three months in the host. Seddon (1931) reported that, in Australian lambs, *M. expansa* lives no longer than 65 to 70 days, but Gordon (1932) stated that the cestode produces eggs for at least 350 days in Australian sheep.

Lambs become infected very early in life and may pass gravid proglottids when they are as little as eight weeks old, which indicates that the worm grows very rapidly. The rapid growth rate and the prolific production of ripe segments suggest that much nourishment is required and obtained from the host, and that a large quantity of metabolic wastes is eliminated by the parasite in the host intestine.

M. expansa is an obligate parasite well adapted to its host. There is considerable controversy about its pathogenicity. Kates and Goldberg (1951) summarised the literature and concluded that light infections in older animals cause little harm, but that heavy infections in young animals cause serious digestive disturbances, stunting, emaciation, oedema, anaemia, and that considerable loss of wool may occur.

The scolex of *Moniezia* has no rostellum and its suckers are devoid of any hooks, so it cannot penetrate into nor hold itself firmly to the intestinal wall - rather, the scolex remains loosely

attached to the intestinal mucosa. Therefore, the main source of its nutrients is the host intestinal contents. Lacking an alimentary system, cestodes absorb nutrients from the ambient medium through their body surface. The external body surface of cestodes is quite different from the 'cuticle' of nematodes and trematodes, and it not only serves as a protective body covering but also as a metabolically active layer. Rothman (1959) suggested that it should more properly be termed a 'tegument'. Fine hairlike projections on the body surface of cestodes were recognised many years ago (Schiefferdecker, 1874; Steudener, 1877; Moniez, 1884; quoted from Read, 1966, p. 105). Electron microscopy has revealed the presence of these structures on the body surface of most of the cestodes belonging to several orders. Rothman (1959, 1960, 1963) termed these digitiform projections as 'microtriches' (singular microthrix), and Lumsden (1966) coined the term 'microvillus' for the tegumentary projection of *Hymenolepis diminuta*. Most of the authors have analogised these structures with the absorptive intestinal epithelial brush border of many other invertebrates and vertebrates. Lumsden (1966) described the tegumental zone of *H. diminuta* as 'an anucleate syncytium morphologically specialised for an absorptive function'.

With the help of scanning electron microscopy, Ubelaker, Allison and Specian (1973) found a density of 38 to 60 microtriches/ μm^2 on the mature proglottids of *H. diminuta*. Presence of projections on the body surface is significant because it results in amplification of the surface area, depending on the size of the projections. Lumsden (1966) reported that the microvilli of *H. diminuta* were an average of 750 nm long by 160 nm wide. It is not known whether the microtriches are capable of pinocytosis, but their absorptive function has certainly been demonstrated (Pappas and Read, 1975). Braten (1968) reported that an increase in the body

surface area favours greater absorption of nutrients. It is therefore also possible that this situation would make cestodes more susceptible than other helminths to drugs which act on the surface of the parasite.

During the entire life span in the host, *M. expansa* continues strobilation (production of segments) and destrobilation (casting off gravid segments), so that it requires a great deal of energy for growth and reproduction. The parasite is so closely adapted to its habitat that its life in the relatively crude laboratory imitations of this habitat is always brief. Disintegration of the animal is very rapid, whether kept in sterile or in oligoseptic media (Wardle, 1937). Accurate information regarding the longevity of *M. expansa in vitro* is scanty. Wardle (1937) reported that in saline media containing 2 mM glucose, whether sterile or non-sterile, at pH 6 to 9, the parasite cannot remain undulant longer than 12 hours.

1.2 The Habitat.

Cestodes differ markedly from nematodes and trematodes in that the adult stages generally occupy only one type of habitat i.e. the alimentary canal of chordate animals. The known exceptions also inhabit sites related to the digestive system, such as bile duct, gall bladder and pancreatic duct.

1.3 Physico-chemical Characteristics of the Small Intestine.

- (a) pH: The pH of the intestinal contents of mammals is rarely alkaline (Calloway, 1968; Levine, 1971; Mettrick and Podesta, 1974). It has been suggested (Smyth, 1969) that the pH of the intestinal contents of sheep is generally just on the acid side - about 6.7 to 6.8. Wilson (1962) measured a pH of 6.8 in the jejunum of the dog. Podesta and Mettrick (1974, 1975) reported that the pH of the bulk aqueous phase in the small intestine of rats infected with *H. diminuta* was significantly lower than that of the uninfected gut. The authors stated that a hydrogen ion

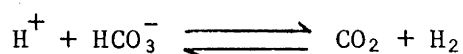
secretory mechanism in the worm tissue acidified the intestinal contents. They also reported that increased hydrogen ion concentration resulted in decreased absorption by the intestine, but stimulated absorption of salts, water and glucose by the parasite. This fact implies that, by lowering the pH of the environment, parasites can compete better with the host for absorption of nutrients.

- (b) Gases: Distribution of gases in the gas phase of the small intestine of the sheep has been reported by Read (1950) as follows:

CO ₂	62 to 92%
O ₂	0%
CH ₄	0.04 to 6.6%
H ₂	0 to 37%
N ₂	1%

Most of the earlier workers, including Read, considered the intestinal lumen to be anoxic, except for the region adjacent to the mucosa where larger amounts of oxygen have been found (Rogers, 1949; Crompton, Shrimpton and Silver, 1965; Arme and Read, 1969). These workers, however, did not consider the fact that a considerable amount of oxygen might remain dissolved in the bulk aqueous phase of the intestine. More recent studies (Mettrick and Podesta, 1974; Podesta and Mettrick, 1974, 1975) have shown that the bulk aqueous phase of the rat intestine is aerobic with a partial oxygen tension (pO₂) of 40 to 50 mm Hg. The authors stated that free diffusion of oxygen between the mucosa and the luminal contents did occur, and that the pO₂ of the bulk aqueous phase is determined by that of the mucosa. Crompton *et al* (1965) reported that an oxygen tension of 5 mm Hg is required for the satisfactory functioning of the cytochrome

oxidase system. Therefore, it is apparent that the mammalian intestine is suitable for aerobic organisms. Carbon dioxide is the most common and abundant gas present in the intestinal lumen. Studies in rats revealed that the $p\text{CO}_2$ in the lumen of the parasitised intestine was significantly greater than that of the uninfected gut (Mettrick and Podesta, 1974; Podesta and Mettrick, 1974, 1975). It is suggested that one effect of the H^+ ions secreted by the helminth is to shift the equilibrium of the following reaction to the right:



The amount of O_2 and CO_2 in the intestinal lumen is of particular significance to the parasite because of the importance of these gases in metabolism. There is substantial evidence that CO_2 fixation in anaerobic carbohydrate metabolism is a most significant means of energy production in intestinal helminths (Saz, 1971a, 1972b; Bryant, 1972a; Behm and Bryant, 1975c).

Although some workers (Smith, 1969; Cheah, 1972a, b) considered intestinal helminths to be aerobic, most of the studies have shown that helminths are capable of both aerobic and anaerobic energy production (Agosin and Repetto, 1963; Prichard and Schofield, 1968; Davey and Bryant, 1969; Bryant, 1970, 1972a, b; Saz, 1971b, 1972a, b; Behm and Bryant, 1975a, b, c). The presence of both CO_2 and O_2 in the habitat is reflected in the adaptation of parasites to both aerobic and anaerobic energy production.

- (c) Redox Potential: The oxidation-reduction potential (E_h) of the intestinal contents is determined by the equilibrium between the rate of production of oxidised and reduced metabolites in the intestinal lumen. The quality and quantity of metabolites produced depend on the variety and number of microflora present. The intestinal lumen of rats exhibits strong reducing condition

with an Eh value of -100 mV (Bergeim, Kleinberg and Kirch, 1945). Mettrick (1975) reported that the bulk luminal phase of the uninfected rat intestine displayed a strong reducing condition (Eh - 46 to -158 mV), while the intestine harbouring *H. diminuta* showed an oxidised state (Eh + 23 to +75 mV). Other studies have revealed the same effect of the cestode on the redox potential of the intestine of rats (Podesta and Mettrick, 1974; Mettrick and Podesta, 1974). It has been reported (Mettrick, 1975) that the more positive Eh values reflected the greater worm biomass present *in situ*. Most of the authors concluded that helminths can alter the redox potential in two ways - directly, by secreting H^+ ions and indirectly, by reducing the microflora of the intestinal tract (Mettrick, 1971). As the distribution of bacteria is influenced by the intestinal hydrogen ion concentration (Percy-Robb and Collee, 1972), the regional difference in pH would result in a different Eh gradient along the intestine (Bergeim *et al.*, 1945; Hentges and Maier, 1972).

Since the dietary constituents have a significant effect on the intestinal redox potential (Mettrick, 1975), the Eh values for the intestinal content of sheep may be different. Moreover, the physico-chemical characteristics of the intestinal lumen of ruminants may be different from that of monogastric animals.

- (d) Osmotic Pressure: The osmotic pressure of the intestinal contents of sheep was measured by Schopfer (1932; quoted from Rogers, 1962) who found a freezing point depression of -0.825° compared with a value of -0.669° for the tissue extract of *M. expansa*. Therefore, the environment may be hypertonic to

the parasite. Read and Simmons (1963) observed that the change in wet weight of *Moniezia* was almost linear in response to a change in external osmotic pressure. Behm and Bryant (1975a) reported that there was a significant decrease in energy production in *M. expansa* when incubated in hypotonic (20% KRP) medium.

1.4 Nutritional Status of the Small Intestine.

Cestodes can absorb most of the low molecular weight nutrients, such as carbohydrates, amino acids, purine, pyrimidines and nucleosides, fatty acids, and vitamins through their body surface (Lumsden, 1966; Pappas and Read, 1975). There is no accurate information regarding the nutritional behaviour of *M. expansa*, but it is assumed to be same as other cestodes. As *M. expansa* cannot penetrate the mucosa, its main source of nutrients is the intestinal contents of the host. Therefore, an assessment of the nutritional status of the habitat is required for better understanding of the physiology and metabolism of the parasite.

Although the pre-ruminant stage of carbohydrate digestion and absorption in ruminants resembles that of monogastric animals (Jarrett and Potter, 1952; McCandless and Dye, 1959), the ruminant and post-ruminant phases differ markedly, since most of the ingested carbohydrate is converted to acetic, propionic and butyric acids in the rumen by microbial fermentation. Armstrong (1965) reported that, in ruminants fed conventional diets, no free glucose enters the small intestine and the glucose requirements of the animals are met by gluconeogenesis from propionate in the liver. In ruminants fed hay diets, little α -linked glucose polymer (starch) enters the small intestine, but when high grain diets are given the

amounts increase significantly (McRae and Armstrong, 1969). This starch (and other carbohydrates from the bodies of microbes) is hydrolysed to glucose in the jejunum (Wright, Grainger and Marco, 1966; Hembry, Bell and Hall, 1967). Another source of glucose in the jejunum may be leakage from intact mucosal cells (Behm, 1974). Therefore, a considerable amount of glucose may be available to *M. expansa* in its habitat.

The volatile fatty acids produced by microbial fermentation are absorbed through the rumen wall. Hence the concentrations of these nutrients cannot be high in the small intestine (Annison, 1954).

Amino acids in the small intestine may be derived from (i) digestion of protein in the abomasum and duodenum, (ii) bile, (iii) pancreatic secretions, and (iv) leakage from the mucosa. It has been reported (Nasset, 1957; Nasset and Ju, 1961) that the molar ratios of the pool of amino acids in the small intestine remain relatively constant, independent of the quantity ingested. It is recognised (Smyth, 1967) that mucosal cells of the small intestine are cast off and replaced by regeneration of new cells continuously and rapidly. Autolysis of these shed mucosal cells undoubtedly releases nutrients into the intestinal contents, which could therefore be available to the parasite.

1.5 Nutritional Requirements of *M. expansa*.

There is no experimental evidence as to the comparative importance of carbohydrate, protein and lipid in the energy metabolism of cestodes. Most of the parasitic tapeworms so far examined have been found to contain substantial quantities of the nutrients (von Brand, 1973). Cestodes have considerable carbohydrate requirement for their growth and reproduction. The levels of the host's dietary carbohydrate have a significant bearing on the well being of the

parasite (Smyth, 1969). Read and Phifer, (1959) observed that the size of *Hymenolepis citelli* and *H. diminuta* was significantly reduced in hamsters which received a restricted carbohydrate diet. Reid (1942) reported that 24 to 48 hours starvation of infested fowls resulted in the loss of strobila in *Raillietina cesticillus*, and that the scoleces were not affected and were able to regenerate new strobila upon resumption of normal feeding of the hosts. This author also noted that 20 to 24 hours starvation of the fowl hosts resulted in greatly reduced glycogen content of the worms. Levine (1938) found that complete starvation of infected fowls for 24 hours caused decreased production of proglottids in *Davainea proglottina* during the following week. The observation of Mettrick and Munro (1965) is much more spectacular. These authors reported that *H. diminuta* grew better in a host fed on protein-free carbohydrate diet than in one which received both carbohydrate and protein.

During short periods of maintenance *in vitro*, *M. expansa* can utilise exogenous glucose (this work, Ch. V). Wardle (1937) reported that incubation of *M. expansa in vitro* for six hours in media containing glucose resulted in a significant increase of glycogen level in the worm. He also found that absence of glucose from the media caused depletion of glycogen level in the worms, and that no other nutrients could substitute for glucose in this regard. In *M. expansa* the internal glucose pool size has been found to be high (Fairbairn, 1958; Behm and Bryant, 1975a; this work, Ch. III).

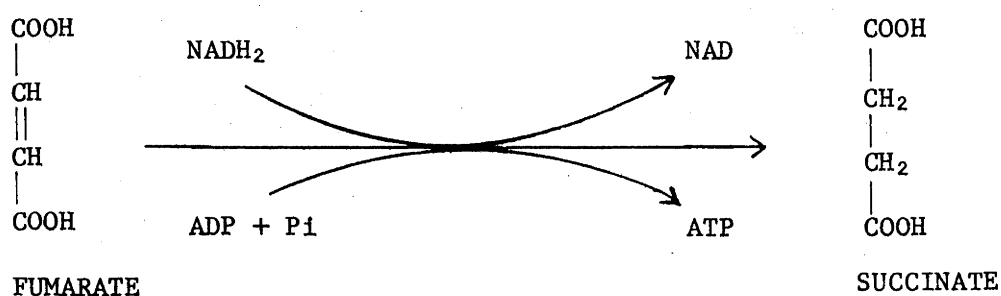
B. METABOLISM OF *M. EXPANSA*.

A considerable amount of information is available about the carbohydrate metabolism of *M. expansa*. There is no conspicuous experimental evidence regarding protein and lipid metabolism in this

cestode.

Behm and Bryant (1975a,b,c) carried out a thorough investigation of the intermediary metabolism of carbohydrate and metabolic regulation in *M. expansa*. A brief review of their work is considered worthwhile for the better understanding of metabolic pathways of energy production in this helminth.

The above mentioned work revealed that in *M. expansa* glucose and/or glycogen is metabolised through the typical glycolytic sequences of the Embden-Meyerhof pathway down to the level of phosphoenolpyruvate (PEP). Thereafter the pathway is branched - one branch leads to the production of lactate via pyruvic kinase, and the other leads to the production of succinate. This latter branch includes the fixation of a molecule of CO_2 into PEP, mediated by the enzyme phosphoenolpyruvate carboxykinase (PEPCK). The product is oxaloacetate (OAA). OAA is reduced to malate by the cytosolic malic dehydrogenase (MDH). Malate produced in the cytosol then enters into the mitochondrion and is dehydrated to fumarate which in turn is reduced to succinate by an NADH linked particulate enzyme. This enzyme has been termed 'fumarate reductase' and there is evidence that the reaction is coupled to the formation of ATP (Bryant, 1970). The overall reaction can be represented as follows:



and is accomplished by an electron transport system analogous to that of an aerobic organism. In this reaction the oxidized molecule (fumarate)

is used to convert NADH to NAD with the coupled formation of ATP, and the production of a reduced molecule (succinate). It has been suggested that 'succinate dehydrogenase' in helminths acts in a manner opposite to that of mammalian tissues, i.e. as a fumarate reductase, and that its function is to reoxidize the NADH formed during glycolysis (Kmetec and Bueding, 1961; Saz, 1972a.) However, reoxidation of NADH from this source is achieved at the level of malate or lactate dehydrogenase, so that the reducing equivalents necessary to drive the fumarate reductase (FR) reaction must originate elsewhere.

Scheibel and Saz (1966) have listed some of the helminths which accumulate significant amounts of succinate or products presumably derived from succinate. The list includes nematodes [*Ascaris lumbricoides*, *Heterakis gallinae*, *Trichuris vulpis*, *Trichinella spiralis* (larvae), *Syphacia muris* and *Dictyocaulus viviparus*], cestodes [*H. diminuta*, *M. expansa*, *Taenia taeniaeformis* (adult and larvae) and *Echinococcus granulosus* cysts], the trematode *Fasciola hepatica*, and the acanthocephalan *Moniliiformis dubius*. It reveals that the fumarate reductase system is present in a wide variety of helminth parasites irrespective of their habitat in the host and that it must play an important role in the energy metabolism of the parasites.

In the lactate producing pathway, pyruvate kinase catalyses the production of pyruvate by transferring the phosphate bond from PEP to ADP with the generation of ATP. Lactate dehydrogenase (LDH) then catalyses the reduction of pyruvate to lactate by NADH. This reaction in *M. expansa* differs markedly from the mammalian system in that considerable activity occurs even in the presence of oxygen.

Lactate and succinate are the end-products of carbohydrate metabolism in *M. expansa* and are excreted.

Behm and Bryant (1975a) found that adenine nucleotides levels

were maintained whether or not the worms were incubated under aerobic conditions. They also observed that, under aerobic conditions, there was a small decrease in the flux through the pathway due to a disproportionate decrease in lactate production and accompanied by a large increase in the malate pool. The authors postulated that, in addition to the malate produced from glucose, reoxidation of succinate under aerobic conditions contributed to the increased malate pool, which then inhibited pyruvate kinase activity, resulting in decreased lactate production. No other significant difference was found between aerobiosis and anaerobiosis.

Addition of glucose to the incubation media significantly increased the flux through the pathway resulting in greatly increased production of end-products. Investigation of the activity of selected enzymes revealed that hexokinase, pyruvate kinase, phosphofructokinase and PEPCK have very low activities. Phosphofructokinase and pyruvate kinase have been found to play regulatory roles in *M. expansa*. The regulatory behaviour of hexokinase could not be ascertained because of the fact that G6P pool derives from both glucose and glycogen *in vivo*. However, the enzyme may control the flux by virtue of its very low catalytic activity. Because of the difficulty of assessing the equilibrium position of PEPCK it was not possible to demonstrate whether the enzyme plays some regulatory role in the branched pathway. However, as CO₂ fixation is an essential step for energy metabolism and as there is no other significantly active carboxylating enzyme in *M. expansa*, it has been predicted that PEPCK plays some regulatory role *in vivo*.

Malic enzyme activity was found to be so low that it is probably unimportant in *Moniezia* metabolism. Pyruvate carboxylase, phosphoenolpyruvate carboxylase and phosphoenolpyruvate transphosphorylase could not be detected in preparations from *M. expansa*. Malate dehydrogenase and lactate dehydrogenase were found to have very high

catalytic activities. It has been hypothesised that one of their functions is to maintain the redox balance of the cytosol by regenerating NAD.

Though the majority of the TCA cycle intermediates have been detected in the preparations from *M. expansa*, the existence of a functional cycle has been questioned (Davey and Bryant, 1969). Cornish and Bryant (1975) also failed to show a functional γ -aminobutyrate pathway in this cestode. Cheah and Bryant (1966) and Bryant (1970) reported that the major respiratory pathway in *M. expansa* utilizes fumarate as terminal electron acceptor. Mitochondrial preparations of this cestode were shown to contain functional cytochromes of types b,c and a (Cheah, 1968, 1972). Cheah (1967, 1968) suggested that *M. expansa* possesses a branched respiratory chain with two terminal oxidases. One, an O-type cytochrome commonly found in bacteria, uses fumarate as the terminal electron acceptor, and the other is a cytochrome similar to rat liver cytochrome oxidase, and uses oxygen.

In summary, therefore, energy metabolism in *M. expansa* involves the oxidation of a carbohydrate source to the level of PEP. PEP is then converted either to lactate or to succinate, with the former predominating during anaerobiosis. ATP is synthesised by substrate-linked phosphorylation at the 3-phosphoglycerate kinase, PK and (at one remove) PEPCK steps. It is synthesised by electron transfer linked phosphorylation at the fumarate reductase (anaerobic) or succinoxidase (aerobic) systems. The parasite is capable of adapting to a wide range of metabolic states, depending on the degree of aerobiosis. This capacity for compensation will prove important in subsequent discussions on the effects of benzimidazoles.

CHAPTER II

A. CHEMOTHERAPY OF HELMINTHIASIS

2.1 Introduction.

A large number of drugs belonging to different chemical groups are known to have cestocidal properties. The extracts of male fern, kamala, kousso, pumpkin seed, and areca nut are some which have been used for the treatment of cestode diseases of man and animals since the era of early Greek medicine. Copper sulphate, nicotine sulphate, arsenic compounds, and salts of metals are known to be highly effective, but have proved to be very toxic to mammals. As a result most of the latter compounds are not considered the anthelmintics of choice.

The synthetic organic compounds such as dichlorophen, bithionol, niclosamide, and bunamidine have proved to be satisfactory for both human and veterinary medication. None of the compounds for which cestocidal activity has been described appeared outstanding on the evidence of laboratory and field evaluations except niclosamide and bunamidine (Keeling, 1968).

Until recently, most of the anthelmintic formulations developed were effective against either a few or a number of species belonging to a particular helminth group and at least three different medications were required to eliminate a concurrent mixed infection of helminths from a host. In 1964 Merck and Co. Inc. reported that benzimidazole derivatives are effective against a wide variety of helminth parasites including cestodes. Since then, a number of benzimidazole compounds have been developed and marketed by pharmaceutical firms. They include thiabendazole, (TBZ), parabendazole (PBZ), cambendazole (CBZ), and mebendazole (MBZ).

Two more benzimidazole derivatives, namely, oxybendazole and fenbendazole have been developed but are not yet marketed.

2.2 The Benzimidazole Anthelmintics (Fig. 1).

2.2.1 Thiabendazole INN (Fig. 1-A)

2-(4-thiazolyl) benzimidazole INN.

MK 360.

Thiabendazole was the first benzimidazole compound reported to possess a broad spectrum of anthelmintic activity (Brown, Matzuk, Ilves, Peterson, Harris, Sarett, Egerton, Yakstis, Campbell, and Cuckler, 1961). Salunkhe (1966) reported that 1.25 g of TBZ, given twice daily after meals for three days to five human patients, effectively removed *Taenia saginata*. It has been observed that the anthelmintic had a destrobilating (reduction of the strobilar volume) effect on *M. expansa*, but had no effect on the scoleces (Kates, Colglazier, Enzie, Lindahl, and Samuelson, 1971).

Outstanding efficacy against most gastro-intestinal nematodes led to the wide use of TBZ in sheep parasite control until Theodorides, Scott and Laderman (1970) and Colglazier, Kates, and Enzie (1970) reported the development of strains of *Haemonchus contortus* that were resistant to the drug.

2.2.2 Parbendazole INN (Fig. 1-B)

Methyl 5-butyl-2-benzimidazolecarbamate INN.

SK&F 29044 (Helmatac).

The second benzimidazole for which broad anthelmintic spectrum has been described is PBZ (Actor, Anderson, Diccuollo, Ferlanto, Hoover, Pagano, Ravin, Scheidy, Stedman, and Theodorides, 1967). Subsequently its activity against a wide variety of nematodes of sheep and goats was confirmed (Bennett 1968; Danek, Pavlicek, and Zajicek, 1969;

Johns and Mendel, 1969; Lämmler, Sahai, and Zahner, 1969; Luque Forero, Boring, and Ehler, 1969; Ross, 1969; Theodorides et al, 1968, 1969). These authors found that PBZ had an anthelmintic spectrum similar to that of TBZ, but that it was effective at lower dose levels. Kates et al (1971) reported that the drug resulted in a decrease of strobilar volume of *M. expansa* in sheep, but had no effect on the scoleces.

Theodorides et al (1970) found that a number of TBZ-resistant strains of *H. contortus* were also resistant to PBZ.

2.2.3 Cambendazole INN (Fig. 1-C)

Isopropyl 2-(4-thiazolyl)-5-benzimidazolecarbamate INN.

5-isopropoxy-carbonylamino-2(4-thiazolyl) benzimidazole MK;
MK 905.

It has been reported that a number of derivatives of 5-aminothiabendazole have the same anthelmintic spectrum as TBZ but with an enhanced potency (Hoff, Fisher, Bichis, Lusi, Waksmunski, Egerton, Yakstis, Cuckler, and Campbell, 1970). From these compounds CBZ was selected, on the basis of overall safety and efficacy, for development. This compound is a modified form of TBZ with the addition of a carbamate function substituted at position 5.

CBZ is active against many gastrointestinal nematodes of sheep and cattle at a dose range of 15 to 40 mg/kg body weight (Egerton and Campbell, 1970; Egerton, Eary, Lanza, Wombolt, and Campbell, 1970; Gibbs and Gupta, 1971; Cvetkovic, Golosin, and Lepojev, 1971; Horak, Snijders, and Pienaar, 1972; Kates et al, 1973 and 1974). CBZ was also found to be highly effective against *Dictyocaulus viviparus* in cattle Hoff et al, 1970).

At a dose rate of 20 to 30 mg/kg body weight CBZ is highly effective against cestodes of sheep and cattle; *M. expansa* (Gibbs and Gupta, 1971; Cvetkovic et al, 1972; Horak et al, 1972; Campbell and Butler, 1973; Kates et al, 1973 and 1974), *Avitellina centripunctata* (Horak et al, 1972), *Thysaniezia giardi* (Cvetkovic et al, 1972), and *T. actinioides* (Allen, 1973).

Sibalic, Lepojevic and Miklijan (1971) reported that CBZ at 30 to 40 mg/kg body weight had about 87% efficacy against *Dicrocoelium dendriticum* in naturally infected sheep.

It has been reported that the TBZ resistant strains of *H. contortus* were also resistant to CBZ (Colglazier, Kates, and Enzie, 1972). Subsequent studies (Kates et al, 1973; Colglazier et al, 1974) confirmed the presence of CBZ resistant strains of *H. contortus* in a naturally occurring population. Kates et al (1974) reported that a CBZ-sensitive strain of *H. contortus* in lamb was rendered resistant after successive exposures of several parasite generations to the drug. These authors observed that, after the sixth drug exposure, CBZ at 20 mg/kg body weight was only 18% effective, whereas at the same dose level it was 90% effective against a sensitive strain.

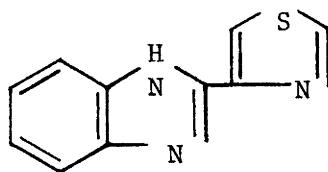
2.2.4 Mebendazole INN (Fig. 1-D)

Methyl 5-benzoyl-2-benzimidazolecarbamate INN.

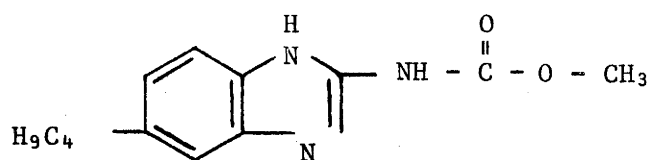
R 17635, Telmin (Janssen).

Mebendazole is a potent, orally active, anthelmintic effective against most nematodes and several species of cestodes in domesticated animals and man (Brugmans, Thienpont, van Wijngaarden, Vanparijs, Schuermans, and Lauwers, 1971).

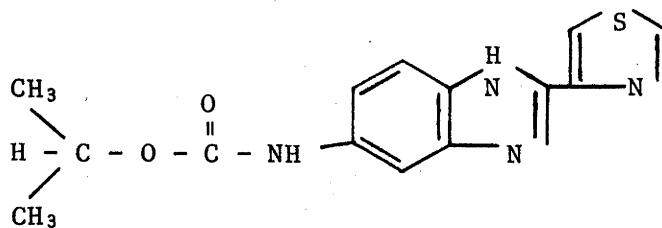
Subsequent studies have confirmed the broad spectrum anthelmintic activity of the drug (Callear and Neave, 1971; Walker and Knight,



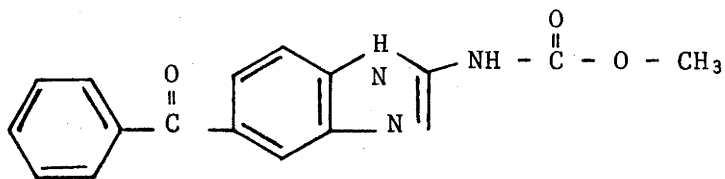
A. Thiabendazole



B. Parbendazole



C. Cambendazole



D. Mebendazole

Fig. 1 : Structure of benzimidazoles.

1972; Bradley and Radhakrishnan, 1973; Bennett, 1973; Varga, 1973; Guilhon, Couradeau and Barnabe, 1972; Grevel and Eckert, 1973; Drudge, Layons, and Tolliver, 1974).

Tabbaa (1972) reported that a dose of 10 to 15 mg/kg body weight was satisfactory against *M. expansa* in sheep and no adverse effects on pregnancy and lactation were observed. Goldsmid, Smith and Fleming (1974) reported that MBZ at 100 mg given twice daily for three days cleared *Moniliiformis* spp. two weeks after treatment from a 12 months old white girl in Rhodesia.

It has recently been demonstrated that the micronised MBZ is more effective than the coarse powder (Kelly, Chevis and Goodman, 1974 and 1975). Gemmell, Johnstone and Oudermans (1975) found that in order to remove 100 per cent *Taenia hydatigena* from dogs repeated doses of 8 x 20 or 4 x 80 mg/kg body weight of coarse-particle MBZ were required, whereas a single dose of micronised MBZ at 20 mg/kg removed all worms from all dogs. Micronised MBZ was also found effective against *Fasciola hepatica* in sheep (Kelly, Chevis, and Whitlock, 1975). Its activity against *T. pisiformis* in rabbits and multiplying tetrathyridia of *Mesocostoides corti* in mice has been reported by Heath, Christie and Chevis (1975). These authors also reported the efficacy of micronised MBZ against experimental secondary hydatid cysts in mice. Kelly and Bain (1975) reported that micronised MBZ at 15 mg/kg was 96% effective in removing *Anoplocephala perfoliata* from horses.

It has been reported that a few species of nematodes (such as *Haemonchus* and *Trichostrongylus* in sheep and *Strongylus* in horses) are resistant to MBZ (Chevis, 1975).

Oxybendazole and fenbendazole are being tested by

various workers for anthelmintic efficacy and nothing more is known about these compound.

Thiabendazole and parbendazole were not included in the present study due to lack of evidence as to their cesticidal properties. CBZ and MBZ were chosen because of

- (i) their proven efficacy against *M. expansa*,
- (ii) the microcrystalline MBZ could easily be suspended in the incubation media containing a very low concentration of 'Tween 80', and
- (iii) the water soluble salt of CBZ (Appendix I) made available by Merck Sharp and Dohme (Aust.) Pty. Ltd. was found satisfactory for *in vitro* biochemical studies.

2.3 Physical and Chemical Properties of CBZ and MBZ.

Cambendazole is a white crystalline powder, insoluble in water and any aqueous solution, including saline buffers. It is soluble to a certain extent in absolute ethanol (2.5%), dimethylsulfoxide (DMSO) and dimethylformamide (10%), and sparingly soluble in dimethylacetamide (DMA). When added to distilled water and saline buffers, solutions of CBZ in DMSO and ethanol turn milky and form insoluble clumps. Solutions in DMF and DMA were found to be miscible with aqueous solutions. CBZ can be suspended in 0.8% Tween 80, but the larger particles tend to sediment very quickly.

Mebendazole is an off-white to slightly yellow powder, insoluble or sparingly soluble in most common solvents such as water, alcohols, chloroform, acetone, ether, benzene (Janssen Pharmaceutica Report No. 2, 1969; Unpublished). It is soluble in formic acid (50%), benzaldehyde (10%), and 2.45% in DMSO (Marsboom, 1973). Solutions of MBZ in DMSO were found to be immiscible with any aqueous solution. However, the

microcrystalline powder could easily be suspended in 0.4% Tween 80 and the particles remained in suspension for about 4 hours depending on the concentration of the drug. At a concentration of 0.08% there was very little sedimentation. In the micronised form of MBZ, the majority of the particles are not greater than 5 μ and particles larger than 10 μ are virtually absent (Janssen Pharm. Rep No.520[701007], 1972, unpublished).

2.4 Toxicity.

Cambendazole has been described as a well tolerated drug devoid of any harmful effects on most domesticated animals at doses as high as 10 times the therapeutic dose (Hoff *et al*, 1970; Egerton and Campbell, 1970).

Mebendazole is also very well tolerated by mammals and birds (Chevis, 1975). Marsboom (1973) reported that a single oral dose ranging from 32 to 64 times the therapeutic dose did not produce any toxic effect in a variety of laboratory and domestic animals.

2.5 Pharmacology.

Studies in cattle show that between 20% and 80% of orally administered ^{14}C -CBZ is absorbed and that the peak plasma level, equivalent to 1.5 to 2.5 $\mu\text{g/ml}$, occurs approximately 24 hours after administration of the drug at 30 to 34 mg/kg body weight (MSD Report, 1974, unpublished).

Sixty percent of the radioactivity derived from orally administered ^{14}C -CBZ is excreted in faeces and 20% in urine. Excretion is rapid - 90% (72% of the total dose) of the radioactivity is excreted within three days. After six days the concentration of radioactivity in urine is less than 0.1 ppm CBZ equivalent, and in the faeces this level is reached in 10 to 14 days

(MSD Rep., 1974, unpublished).

Studies in rats with ^{14}C - MBZ administered orally revealed that most of the radioactivity was present in the gastrointestinal tract as unchanged drug. Low blood and tissue levels were found during a 32-hour period; levels in the liver never exceeded 4% of the dose. MBZ is mainly passed out with the faeces as unchanged compound; only a small percentage is excreted in the urine (Janssen Pharm. Rep. No. 182, 1972, unpublished).

2.6 Metabolism.

Cambendazole is rapidly metabolised in sheep, cattle, dogs and rats. In all species the molecule is attacked oxidatively at the thiazole moiety and degradation proceeds stepwise through a series of increasingly hydrophilic molecules which are all 2-substituted isopropyl 5-benzimidazolecarbamates. Six of these compounds have been isolated, structurally identified and quantitatively determined in urine, faeces and liver.

Further metabolic degradation of CBZ leads to small, water-soluble molecules which are ubiquitously and uniformly incorporated into all cell components, and are identified with the endogenous metabolic intermediates of the host, such as glutamate. The only relationship of such molecules with the drug is that the radiocarbon in them is derived from orally administered ^{14}C -CBZ (MSD report, 1974, unpublished).

Metabolic breakdown occurs within 24 hours after dosing, when only a small amount of the total dose was found to be unchanged compound. 5% of the radioactivity in the plasma, 5% (1% of the total dose) of the radioactivity excreted in the urine, and 2% of the radioactivity excreted in the faeces was found to be due to unchanged CBZ (MSD report, 1974, unpublished).

It has also been reported that CBZ undergoes metabolic transformation at both the thiazole-ring and isopropoxycarbonylamino moiety (Van den Heuvel, Buhs, Carlin, Jacob, Koniuszy, Smith, Trenner, Walker, Wolf, and Wolf, 1972). These authors determined the structures of three urinary metabolites of CBZ (one from calf and two from pig) using gas-liquid chromatography-mass spectrometry. From the calf they isolated the metabolite 5-isopropoxycarbonylamino-benzimidazole (Fig. 2-A). The structure suggests metabolic degradation of CBZ at the thiazole-ring.

The two metabolites from pig urine suggest transformation of CBZ at the isopropoxycarbonylamino moiety (Fig. 2-B and Fig. 2-C). The first one shows (Fig. 2-B) that the moiety has been transformed to 1-hydroxy-2-propoxycarbonylamine and the resultant compound is 5 (1-hydroxy-2-propoxycarbonylamino)-2(4-thiazolyl) benzimidazole. In the second urinary metabolite from pig it is observed that the isopropoxycarbonylamine moiety has been reduced to an N-acetylamine function and the resultant compound is 2-(4-thiazolyl)-5-N-acetylaminobenzimidazole (fig. 2-C).

Mebendazole is metabolised to its demethoxylated derivative which has no anthelmintic properties (Janssen Pharm. Report No. 182, unpublished).

In the absence of any other information it may tentatively be assumed that MBZ does not need to be metabolised by the host animal to exert its anthelmintic effect. It is also not known whether CBZ and MBZ are metabolised by the parasites.

2.7 Biochemical Mode of Action of Benzimidazoles.

There are a variety of ways in which chemotherapeutic agents exert their effects on helminths. Krotov (1970) classified most of the commonly used anthelmintics into the following categories on the basis on their mode of action.

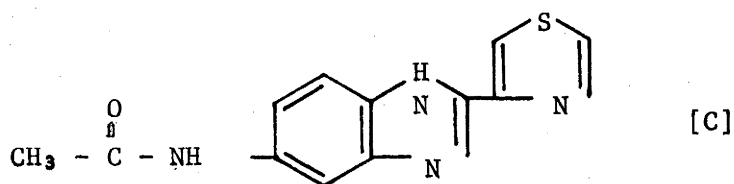
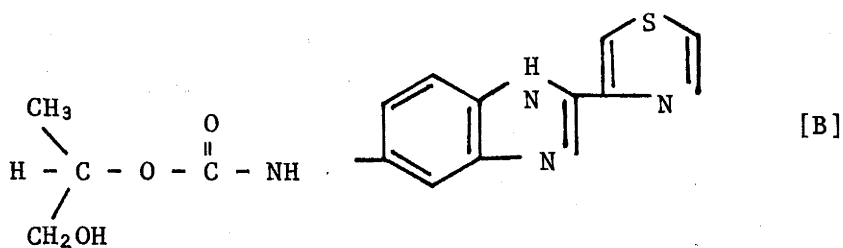
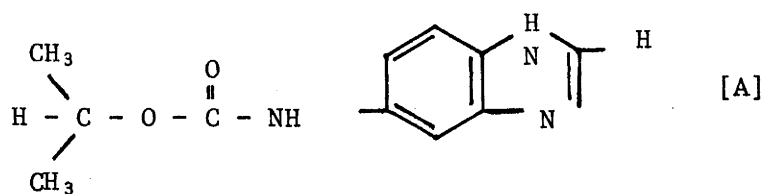


Fig. 2. Structure of urinary metabolites of cambendazole : A, 5-isopropoxycarbonylaminobenzimidazole, B, 5-(1-hydroxy-2-propoxycarbonylamino)-2-(4-thiazolyl) benzimidazole; C, 2-(4-thiazolyl)-5-N-acetylaminobenzimidazole.

- (i) Protoplasmic poisons e.g. carbon tetrachloride, tetrachloroethane, bromonaphthanol, iodothymol, heptyl- and hexyl- resorcinol, chenopodium oil.
- (ii) Neuromuscular blocking agents e.g. santonine, arecoline, nicotine, anticholinesterases like chlorophos, compounds with cholinolytic-like or adrenergic-like action (piperazine compounds).
- (iii) Paralysing the neuromuscular system and destroying the cuticle of flatworms e.g. dichlorophen, trichlorophen, hexachlorophen, phenesal, bithionol.
- (iv) Antimetabolic : (a) glycolysis inhibitors (ambilhar, tetramisole, copper sulphate), (b) enzyme inhibitors (chloroquine, phenothiazine, thiabendazole), and (c) inhibitions of carbohydrate absorption (cyanine dyes).
- (v) Substances with obscure mechanism of action e.g. cadmium compounds, methyridine, gentian violet.

This author, however, did not mention another important group of anthelmintics which are known to uncouple oxidative phosphorylation e.g. niclosamide, rafoxanide, dichlorophen, hexachlorophen.

There is not yet sufficient unequivocal evidence regarding the biochemical mechanism of action of benzimidazoles. Prichard (1970) reported that 1 mM TBZ completely inhibited the fumarate reductase (FR) system of *Haemonchus contortus* *in vitro*. Malkin and Camacho (1972) reported that 0.5 mM TBZ significantly inhibited the FR system of TBZ-sensitive strain of *H. contortus*, whereas the drug at 1.0 mM concentration had no effect on the system of TBZ-resistant strain. The authors also observed that CBZ was an effective and somewhat more potent inhibitor of the FR system from both the strains. Prichard (1973) observed that TBZ inhibited the

FR system of the susceptible strain of *H. contortus* only. CBZ inhibited the system in both strains, but the inhibition was much less in the resistant strain than in the susceptible one. The author also found that MBZ did not affect the FR system in either strain, and suggested that lack of effect of MBZ might be due to the experimental conditions, as the drug was used as a suspension.

Van den Bossche (1972a) reported that MBZ inhibited the uptake and/or transport of glucose by nematodes both *in vitro* and *in vivo*, and that this resulted in a significant decrease of glycogen levels in the worms. CBZ and PBZ exerted similar effects to MBZ, but TBZ had no significant effect on glucose uptake and glycogen levels. The author suggested that TBZ did not act in the same way as the other three benzimidazoles because MBZ, CBZ and PBZ are substituted in position 5 and have a carbamate function, whereas TBZ is not substituted in that position and has no carbamate function.

Van den Bossche and De Nollin (1973) stated that the MBZ-induced depletion of glycogen levels in *Ascaris* muscle was due to the inhibition of glucose uptake, and that the inhibitory effect on glucose uptake resulted in diminished uptake of 3-O-methylglucose, glycine, methionine and proline. MBZ also inhibited the uptake of fructose and palmitic acid. MBZ exerted similar effects on glucose uptake and glycogen levels in *Trichinella spiralis* larvae both *in vitro* and *in vivo* (De Nollin and Van den Bossche, 1973).

Borgers and De Nollin (1975) studied the cytochemical and ultrastructural changes in the intestinal cells of *Ascaris suum* after treating infected pigs with MBZ in the food. They found that after 6 to 9 hours, secretory granules were absent from the terminal web and accumulated in the Golgi region of the cells. There was also formation of autophagic vacuoles in the apical parts of the cells,

and loss of glycogen. After 15 to 24 hours of treatment the degenerative changes were very pronounced and involved the entire cytoplasm; the number of microvilli was decreased and most of them showed swelling, clustering or disruption. Some of the midgut cells were devoid of microvilli, the cytoplasm of most cells had a degenerative aspect, characterized by excessive formation of autophagic vacuoles, and accumulation of glycoproteins or polysaccharides was found in the Golgi apparatus. Acid phosphatase activity was present at the brush borders of less altered cells, whereas the activity diminished or was completely absent in the severely injured cells. No nuclear abnormalities were observed. These authors stated that such changes in the absorptive cells of the intestine might be the cause of diminished uptake and/or transport of nutrients.

Similar ultrastructural damage to the tegument of *Taenia taeniaeformis* and *Hymenolepis nana* has been reported (Verheyen, Borgers, Vanparijs and Thienpont, 1976) following the treatment of infected hosts with MBZ.

Van den Bossche (1972b) reported that benzimidazoles inhibit ^{32}P i incorporation in *Ascaris* mitochondria.

There is no report regarding the biochemical effects of benzimidazoles on *M. expansa*.

B. EXPERIMENTAL STRATEGY AND STUDY PLAN

Although few investigations on the biochemical mode of action of benzimidazoles have been carried out, such studies have put forward divided opinions on this subject. One group of workers (Van den Bossche, 1972a; Van den Bossche and De Nollin, 1973; De Nollin and Van den Bossche,

1973; Borgers and De Nollin, 1975) maintain the opinion that benzimidazoles act primarily by inhibiting glucose uptake and/or transport by helminth parasites. The other group (Prichard, 1970, 1973; Malkin and Camacho, 1972) holds the idea that the primary effect of these compounds is the inhibition of the fumarate reductase system in helminths. Neither of the conclusions is supported by thorough investigation, since possible effects on any other step(s) in the metabolic pathway have not been investigated.

Helminths have considerable ability to adjust their energy metabolism under adverse conditions (Cornish and Bryant, 1976; Bryant, Cornish and Rahman, 1976). Therefore, inhibition of a single reaction of the metabolic pathway may not be the primary cause of death of the parasite unless it is found to be the only effect (Mansour and Bueding, 1954). Saz and Bueding (1966) pointed out that many compounds are capable of altering a multitude of enzyme systems, but careful and critical analyses are necessary to determine which of the effects is responsible for chemotherapeutic action.

The various groups of helminths differ considerably from one another in their energy metabolism (Saz, 1970, 1972a; von Brand, 1973). It has been observed that the energy metabolism of *M. expansa* differs not only from that of nematodes and trematodes, but also to a certain extent from some cestodes (Behm and Bryant, 1975a,b,c). It is not yet known whether the anthelmintic effect of CBZ and MBZ against this cestode is due to the same biochemical effects that have been observed in nematodes.

On the basis of the above observations, the effects of the two benzimidazoles on the carbohydrate metabolism of *M. expansa* *in vitro* were investigated. Energy metabolism and metabolic regulation in the parasite *in vitro* have been thoroughly studied (Bryant, 1972a,b; Behm and Bryant, 1975a,b,c) and therefore it was considered advantageous to exploit

the known system with effective anthelmintics.

Since *Moniezia* does not live long outside the host, it is perhaps not a very good model for *in vitro* studies which require prolonged maintenance of the worm under laboratory conditions. However, it was chosen for the present work because it is abundant and easily obtained during most parts of the year in Canberra, and the incubation times used in this study were very short. The large body-surface of *M. expansa* was considered an additional advantage for this type of study.

In an attempt to correlate the *in vitro* biochemical effects with the anthelmintic action, *in vivo* studies were carried out.

CHAPTER III

EFFECTS OF CBZ AND MBZ ON THE METABOLISM OF *M. EXPANSA*.

3.1 Introduction.

The amount of information available regarding the biochemical effects of CBZ and MBZ is not adequate to ascertain the mode of action of the anthelmintics. Energy metabolism in parasitic helminths is a complex process. Inhibition of a particular function or a particular step in this process may not provide sufficient clues to ascertain the exact nature of effect on the whole process of metabolism. As there are interactions and interdependences amongst various components of the metabolic pathway, it is possible that inhibition of a particular step in the pathway would result in disturbances in the whole process. Therefore, an effect on a particular step may be a primary, a secondary or even a tertiary effect. To ascertain the exact nature of the effects thorough investigation of the whole process is required.

With these aims and objects in mind the intermediary metabolism of glucose in *M. expansa* was investigated. The experimental methods and the results are presented in this section.

3.2 Experimental Material.

The entire length of tapeworm represents a variety of developmental stages. Because of this, diversified metabolic activities relating to each stage may be taking place in different regions along the length of the strobila. A greater uniformity of metabolic activity may be found in the anterior scolex region of the worm, because the main function of this region is active strobilation. Following the examples of Bryant (1972b) and Behm

and Bryant (1975a,b,c) the anterior gram (scolex) of *M. expansa* was used in the present study.

3.3 Methods.

Worms were collected from the intestines of lambs, soon after slaughter at the Canberra abattoir, in ice-cold KRP (Umbreit, Burris and Stauffer, 1964). They were promptly transported to the laboratory. The scoleces were separated, washed several times in ice-cold KRP to remove detritus, and were preincubated in KRP containing 10 mM glucose for 30 mins at 37°C. The purpose of preincubation was to stabilise the metabolic activity of the worms, which was undoubtedly disturbed during collection and handling, and also to identify the healthy (undulating) ones.

Five preincubated scoleces were pooled for each incubation. Incubations were carried out in 50 ml conical flasks. The incubation media (appendix II) were pregassed for one hour with either oxygen-free nitrogen containing 5% CO₂ (anaerobic) or air containing 5% CO₂ (aerobic) depending on the experimental conditions desired. The incubation vials were then placed on a shaking water bath (Labmaster) inside a glove-bag. The appropriate gas mixture was passed through the bag continuously during the course of 30 mins incubation.

After incubation the worms were rinsed three times in distilled water, blotted on filter paper (Whatman No. 1), weighed and snap frozen in liquid nitrogen. The rest of the procedures for preparation of extracts and intermediate assays were performed according to Behm and Bryant (1975a).

Samples of incubation media were processed for lactate and succinate assays as described by Behm (1974). Media were

also assayed to determine the glucose remaining after incubations.

The following intermediates were assayed enzymatically using Gilford 2400 and Eppendorf 1101M recording spectrophotometers at 340 nm and 334 nm respectively with a light path of 1 cm ; glucose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, lactate, malate, and succinate.

Two of the metabolic intermediates could not be assayed - OAA, because its concentration is too small to measure and it is, in any case, labile; and fumarate because of the technical difficulties involved in its assay system (Behm and Bryant, 1975a).

To assess the suitability of using DMSO and Tween 80 in the metabolic studies, their effects on CO₂ fixation by *M. expansa* were determined. DMSO was found to inhibit this vital step of carbohydrate metabolism in the parasite (appendix III) and was considered not suitable for this study. Tween 80 (Sigma) did not interfere with CO₂ fixation by *M. expansa* (appendix IV) and was considered suitable for use as a suspending agent.

The sources of enzymes, nucleotides, co-factors, various substrates, and chemicals are listed in appendix V. Cambendazole was supplied by Merck Sharp and Dohme (Australia) Pty. Ltd., Ingleburn, N.S.W.; and MBZ was supplied by Ethnor Pty. Ltd., North Ryde, N.S.W., Australia. All other chemicals and reagents were of analytical grade and glass distilled water was used throughout.

3.4 Results and Discussions.

3.4.1 Effects of CBZ and MBZ on the Anaerobic Metabolism of *M. expansa* in the Presence of 10 mM Glucose.

The results of *in vitro* anaerobic experiments are presented in table 3.1. The concentrations of all the intermediates from glucose to lactate are shown here. G3P and DHAP have been added together and expressed as triosephosphates, and 2-PGA has been combined with 3-PGA to denote phosphoglycerates. This is desirable, because the pool sizes of G3P and 2-PGA are very small and variable. Two intermediates in the PEP-succinate pathway are not listed in the table for the reasons mentioned above. Lactate and succinate are the excretory end-products of *M. expansa*, so the total amount produced in 30 mins - i.e. the internal pool of the worm and the amount excreted in the media - are shown together. The total amount of metabolites is presented to compare the overall metabolic activity.

Table 3.1 shows that under anaerobic conditions both CBZ and MBZ significantly decrease the glucose pool in *M. expansa*. The F6P pool is also significantly decreased by the drugs. The production of succinate is significantly decreased, but that of lactate is significantly increased by both the drugs. Only CBZ significantly reduced the malate pool.

The changes in the pool sizes of various intermediates and in the formation of end-products, brought about on incubating *M. expansa* with CBZ and MBZ (table 3.1), may result from a single interference or several disturbances in the metabolic pathway.

The reduced glucose pool in the worm may either be due to diminished uptake or to increased utilization or both. Increased utilization should result in increased carbon flux through the pathway. This did not occur, as evidenced by the total formation of respiratory end-products. In the case of

Explanations for table 3.1 :-

- ^a The values are means $\pm$ SD. The units are nmoles/g wet weight.
- ^b Dihydroxyacetonephosphate + glyceraldehyde-3-phosphate.
- ^c 3-phosphoglycerate + 2-phosphoglycerate.
- ^d Total of substrates, intermediates and end-products.
Tests for significance by Student's t show e,f,g,h significantly different from control, $p < 0.0005$, < 0.0025 , < 0.005 , < 0.01 respectively.

Table 3.1 Effects of CBZ and MBZ on the Anaerobic Metabolism of *M. expansa* in the Presence of 10 mM Glucose (n = 5)^a.

Intermediate	Control	CBZ	MBZ
Glucose	10250 ± 1358	5194 ± 954 ^e	5475 ± 928 ^e
Glucose-6-phosphate	165 ± 64	121 ± 30	128 ± 29
Fructose-6-phosphate	58 ± 11	29 ± 11 ^f	32 ± 10 ^g
Fructose-1,6-diphosphate	55 ± 25	48 ± 14	62 ± 23
Triosephosphates ^b	66 ± 16	59 ± 10	64 ± 9
Phosphoglycerates ^c	252 ± 31	252 ± 42	254 ± 71
Phosphoenolpyruvate	71 ± 10	80 ± 18	81 ± 17
Pyruvate	204 ± 115	179 ± 69	119 ± 39
Lactate (total)	17292 ± 2489	21984 ± 3735 ^h	28828 ± 2004 ^e
Malate	948 ± 121	592 ± 90 ^e	780 ± 261
Succinate (total)	20881 ± 569	14764 ± 1272 ^e	13042 ± 814 ^e
Total end-product	38173 ± 3003	36749 ± 2833	41870 ± 2071
Total metabolites ^d	50980 ± 3005	44012 ± 3191 ^g	49449 ± 2961

For explanations see facing page.

the CBZ treated incubation there is instead a decrease of total metabolites. Therefore, it is most likely that the diminished glucose pool is due to an inhibitory effect of the drugs on glucose uptake by the worms. This effect of CBZ and MBZ on *M. expansa* is in conformity with the findings of other workers on nematodes (Van den Bossche, 1972a; Van den Bossche and De Nollin, 1973; De Nollin and Van den Bossche, 1973).

Cestodes need a carbohydrate source for their survival and reproduction. In the absence of external glucose they utilize the internal glycogen reserves for energy metabolism (Wardle, 1937; Reid, 1942; Read, 1959, 1966; von Brand, 1973). The results of the present study suggest, as a working hypothesis, that inhibition of glucose uptake from the medium resulted in increased utilization of glycogen and thereby the flux through the pathway was largely maintained. Therefore, no significant alterations of the over-all metabolic activity are observed.

The reduction of the F6P pool is statistically significant, yet may not be important, either because the pool size of this intermediate in *M. expansa* is small and variable or because it may be a consequence of the diminished glucose pool.

The second major effect of the drugs is the inhibition of the succinate production. It may be caused by inhibition of any of the reactions involved in the PEP-succinate pathway, where at least two steps of major importance to energy metabolism have been recognised - one is the CO₂ fixation by PEPCK and the other is the electron transport reaction of the FR system. These reactions are known to be energy yielding---

(Bryant, 1972b, 1975; Behm and Bryant, 1975c), and any interference with either of them would alter succinate production. Inhibition of CO_2 fixation by PEPCK would lead to the decreased flow of carbon through the pathway and thereby the production of succinate would be reduced. Inhibition of the FR system will also result in reduced production of succinate, but with an accumulation of carbon along the pathway. It is possible that in the case of the CBZ treated incubation either PEPCK or PEPCK and FR have been inhibited, as evidenced by the significantly reduced malate pool. In case of the MBZ treated incubation, inhibition of the FR system might be greater than that of the PEPCK reaction as there is no significant decrease in the malate pool. However, due to lack of quantitative data on all the intermediates involved in the PEP-succinate pathway, it is not possible to conclude whether one or both of the reactions have been inhibited by the drugs.

There are various possibilities which might result in increased formation of lactate. Allosteric activation of the PEP-lactate pathway and/or inhibition of the PEP-succinate pathway may lead to increased formation of lactate. In *M. expansa*, PK is a regulatory enzyme and is activated by FDP and inhibited by high levels of malate and ATP (Behm and Bryant, 1975a,b). An inspection of the results in table 3.1 reveals that there is no increase in the FDP pool and in the case of MBZ treated incubation, no significant decrease in the malate pool, although greater lactate production has occurred. Therefore, it appears probable that these allosteric effectors did not play a role in the increased production of lactate. However, the concentration of ATP is significantly reduced by

CBZ and MBZ treatment (Ch. IV). The reduction of ATP level may result in increased activity of pyruvate kinase.

In anaerobic organisms which have no PEP-succinate pathway, one molecule of G6P provides two molecules of lactate. In *M. expansa*, where both the pathways function simultaneously, and where PK competes with PEPCK for PEP, inhibition of one branch of the pathway is likely to increase the flux to the other branch of the pathway. So, in the present situation, inhibition of the flow in the succinate pathway might have resulted in a shifting of the flux from the succinate pathway to the lactate pathway. Inhibition, by the drugs, of the PEPCK reaction is thus a possibility.

Davey and Bryant (1969) reported that incubation of homogenates of *M. expansa* with succinate resulted in the production of significant quantities of lactate via fumarate, malate and PEP. The authors also reported the production of lactate from malate. Behm and Bryant (1975c) observed that PEPCK in *M. expansa* is equally active in catalyzing both carboxylation and decarboxylation reactions. Therefore, it is evident that all the reactions in the PEP-succinate pathway are freely reversible. As *M. expansa* scoleces in the presence of CBZ and MBZ produce large amounts of lactate from $\text{NaH}^{14}\text{CO}_3$ and ^{14}C -glucose (this study, Ch. VII), it seems probable that the drugs do not inhibit the decarboxylating activity of PEPCK. The decarboxylation of malate by malic enzyme to yield pyruvate, and hence lactate, can be ruled out because the activity of this enzyme in both the cytosol and mitochondria is very low (Behm and Bryant, 1975c).

Therefore, it is concluded that the increased production of lactate is a consequence of at least one

obstruction in the PEP-succinate pathway.

3.4.2 Effects of CBZ and MBZ on the Aerobic Metabolism of *M. expansa* in the Presence of 10 mM Glucose.

The results are presented in table 3.2. It appears from the table that there are fewer effects of the drugs under aerobic conditions. Both CBZ and MBZ produce significant decreases in the pool size of glucose. The other significant effect is the decrease in total metabolites by MBZ. No other effect is significant at the level $p = 0.01$.

The decreased glucose pool in the worm may be due to inhibition of uptake from the media, as mentioned in section 3.4.1. The notable feature in this case is that the effects of the drugs on the production of succinate and lactate, observed under anaerobic conditions, are removed when the experiment is repeated under aerobic conditions. In the case of the anaerobic incubations it was found that the drugs diminished the production of succinate which in turn resulted in the increased production of lactate.

A possible explanation may be related to the availability of molecular oxygen. At least two electron transport pathways operate in *M. expansa*, one of which utilizes oxygen while the other utilizes fumarate as the terminal electron acceptors (Bryant, 1970). As oxygen is a more efficient acceptor of electrons than fumarate, it is probable that under aerobic conditions the role of the FR system is diminished. Benzimidazole compounds are reported to have a specific effect on this system (Prichard, 1970, 1973; Malkin and Camacho, 1972). Therefore, under aerobic conditions any effect on the production of succinate by this pathway will be minimised.

Explanations for table 3.2 :-

^a The values are means $\pm$ SD. The units are nmolles/g wet weight.

^b Total of substrates, intermediates and end-products.

Tests for significance by Student's *t* show c,d,e significantly different from control, $p < 0.0005$, < 0.0025 , < 0.01 respectively.

Figures in parentheses refer to the number of experiments in each category.

Table 3.2 Effects of CBZ and MBZ on the Aerobic Metabolism of *M. expansa* in the Presence of 10 mM Glucose^a.

Intermediate	Control (5)	CBZ (4)	MBZ (4)
Glucose	10995 ± 1844	6511 ± 675 ^d	4994 ± 500 ^c
Glucose-6-phosphate	183 ± 83	189 ± 88	151 ± 55
Fructose-6-phosphate	48 ± 24	70 ± 58	49 ± 25
Fructose-1,6-diphosphate	59 ± 28	49 ± 10	62 ± 10
Triosephosphates	89 ± 26	79 ± 32	55 ± 31
Phosphoglycerates	331 ± 63	280 ± 54	296 ± 106
Phosphoenolpyruvate	102 ± 32	119 ± 31	95 ± 47
Pyruvate	146 ± 47	159 ± 33	112 ± 41
Lactate (total)	20533 ± 5262	15659 ± 4654	16596 ± 3598
Malate	1036 ± 187	887 ± 268	808 ± 252
Succinate (total)	18928 ± 4143	18932 ± 5244	14502 ± 3374
Total end-product	39462 ± 6601	32631 ± 9736	30542 ± 7611
Total metabolites ^b	52047 ± 6754	40157 ± 11581	37687 ± 6728 ^e

^aFor explanations see facing page.

3.4.3 Effects of CBZ and MBZ on the Metabolism of *M. expansa* in the Absence of Glucose.

In the foregoing sections it has been observed that, despite the presence of glucose in the media, reduction in glucose pools in the worms occurs due to CBZ and MBZ treatment. It is assumed that an inhibition of uptake from the medium may be responsible for the diminished glucose pool in the worms. In this section the results of experiments without added glucose in the incubation media are presented.

The procedures for incubation, sample preparation and assays of intermediates were the same as has been described above.

The results of anaerobic incubation are presented in table 3.3 and those of the aerobic incubation are shown in table 3.4. The results are means of three separate observations in each category.

Table 3.3 shows that, under anaerobic conditions, CBZ significantly increases lactate and decreases succinate production. MBZ significantly reduces the concentrations of PGAs and PEP, and diminishes the production of both the end-products. Another effect of the drug is the increased pool of malate.

Table 3.4 shows that, under aerobic conditions, both the drugs result in increased PGAs and PEP pools and increase the production of lactate. Only CBZ increases the production of succinate to a significant level. Both the drugs increase the total flux through the pathway.

The data in tables 3.3 and 3.4 reveal that the pool size of glucose in the worms depends on external glucose supply

Explanations for table 3.3 :-

^a The values are means $\pm$ SD. The units are nmoles/g wet weight.

^b Total of substrates, intermediates and end-products.

Tests for significance by Student's t show c,d,e,f significantly different from control, $p < 0.0005$, < 0.0025 , < 0.0125 , < 0.025 respectively.

Table 3.3 Effects of CBZ and MBZ on the Anaerobic Metabolism of *M. expansa* in Absence of Glucose (n = 3)^a.

Intermediate	Control	CBZ	MBZ
Glucose	1264 ± 125	1194 ± 30	1279 ± 100
Glucose-6-phosphate	37 ± 3	25 ± 5	30 ± 8
Fructose-6-phosphate	11 ± 2	6 ± 2	4 ± 3
Fructose-1,6-diphosphate	39 ± 8	37 ± 1	45 ± 3
Triosephosphates	50 ± 7	46 ± 4	45 ± 1
Phosphoglycerates	261 ± 11	214 ± 3	153 ± 11 ^c
Phosphoenolpyruvate	79 ± 4	73 ± 6	58 ± 11 ^f
Pyruvate	89 ± 3	120 ± 11	75 ± 4
Lactate (total)	14262 ± 863	16617 ± 513 ^e	11010 ± 277 ^d
Malate	714 ± 68	732 ± 34	1079 ± 163 ^e
Succinate (total)	11168 ± 665	7154 ± 108 ^c	9216 ± 425 ^e
Total end-product	25258 ± 1319	23771 ± 612	20226 ± 652 ^d
Total metabolites ^b	28820 ± 1201	26847 ± 1626	23627 ± 1796 ^f

For explanations see facing page.

and that the drugs have no effect on the internal glucose pool of the worms when glucose is excluded from the medium. Behm and Bryant (1975a) also reported that the glucose pool in *M. expansa* decreased significantly when there was no glucose in the incubation medium, that the absence of glucose in the medium resulted in significant decreases in the flow through the pathway and decreased production of end-products. A comparison of the data in table 3.3 and 3.4 with those in tables 3.1 and 3.2 respectively, would reveal that, both anaerobically and aerobically, the flux and the overall metabolic activity of *M. expansa* is influenced by the external glucose supply.

As Behm (1974) rightly pointed out, the incubation of the worms in glucose free medium is actually the imposition of an adverse condition which is detrimental to their metabolic integrity. Therefore, the results of *in vitro* incubation without glucose may not approximate to what would happen *in vivo*. However, the changes observed due to treatment with the drugs are consistent, so hypotheses can be constructed to interpret them.

PGAs are linked to PEP by equilibrium enzymes, so the changes in these pools probably reflect changes in the PEP pool. Interpretation of the changes in the PEP concentration is complicated by the fact that PEP is the substrate for two enzymes and the product of one of them, OAA, could not be measured. Since there is no significant change in the pool size of pyruvate, it cannot be said whether PK activity has been interfered with.

In case of the MBZ treated anaerobic incubation (table 3.3) there are significant decreases in the production

Explanations for table 3.4 :-

^a The values are means $\pm$ SD. The units are nmoles/g wet weight.

^b Total of substrates, intermediates and end-products.

Tests for significance by Student's t show c,d,e,f significantly different from control, $p < 0.0005$, < 0.0025 , < 0.005 , < 0.01 respectively.

Table 3.4 Effects of CBZ and MBZ on the Aerobic Metabolism of *M. expansa* in Absence of Glucose ($n = 3$)^a.

Intermediate	Control	CBZ	MBZ
Glucose	990 ± 118	740 ± 140	970 ± 118
Glucose-6-phosphate	76 ± 14	49 ± 6	66 ± 21
Fructose-6-phosphate	26 ± 4	24 ± 6	25 ± 5
Fructose-1,6-diphosphate	37 ± 11	42 ± 10	62 ± 24
Triosephosphates	62 ± 10	59 ± 3	67 ± 10
Phosphoglycerates	182 ± 29	305 ± 29 ^e	353 ± 21 ^d
Phosphoenolpyruvate	58 ± 11	100 ± 13 ^f	117 ± 18 ^e
Pyruvate	57 ± 12	85 ± 22	113 ± 47
Lactate (total)	3612 ± 581	5304 ± 182 ^e	7843 ± 585 ^c
Malate	584 ± 145	844 ± 220	858 ± 179
Succinate (total)	10113 ± 652	13247 ± 828 ^e	11753 ± 1242
Total end-product	13724 ± 730	18550 ± 851 ^c	19596 ± 1160 ^c
Total metabolites ^b	15796 ± 272	20799 ± 953 ^d	22247 ± 897 ^d

For explanations see facing page.

of end-products and an increase in the malate pool. The diminished succinate production may be due to inhibition of either PEPCK or the FR system or both. As has been mentioned earlier (section 3.1), any inhibition in the PEP-succinate pathway would result in a shift of flux to lactate. The statement possibly will not apply in this case, as there is a significant decrease in the throughput, due to lack of exogenous substrate (glucose). The increased malate concentration may be due to an inhibition in the pathway between malate and succinate. This high concentration of malate may partly be responsible for decreased lactate production by inhibiting PK.

From the results in table 3.3 it appears probable that CBZ exerts the same effects on the anaerobic metabolism of *M. expansa* irrespective of the presence or absence of glucose in the medium. The explanations for the decreased succinate and increased lactate production, without change in either the flux or the overall metabolic activity, caused by CBZ treatment, have been given earlier in this chapter (section 3.1).

Under aerobic conditions and in the absence of external glucose (table 3.4) it seems that the parasite responds to the drugs in a different manner from that observed on incubation with CBZ and MBZ under anaerobic and aerobic conditions in presence of glucose. Increased flux through the pathway has occurred. The most likely explanation for this situation is interference with the adenylate system. The components of the adenylate system i.e. ATP, ADP and AMP are responsible for the driving mechanism of metabolic regulation. Therefore, disturbance of the generation and/or turnover of adenine nucleotides, especially ATP, may result in increased

or decreased flow through the pathway. However, it may also arise from a degenerated metabolism as the worms were under stress due to lack of glucose. This possibility is considered in more detail in chapter IV.

It should be noted that the number of samples available for statistical analysis was often only three. The small number of observations may not provide sufficient information regarding the level of significance. The Student's t analysis of small numbers of samples may be used instead of simple arithmetic means for comparison of results, but the significance level shown by such analysis may not be reliable (T.G. Marples, personal communication). Therefore, the results presented in this section are not beyond questioning.

CHAPTER IV

EFFECTS OF CBZ AND MBZ ON METABOLIC REGULATION AND ATP SYNTHESIS IN *M. EXPANSA*.

4.1 Regulation of Metabolism and the Effects of the Drugs.

The rate and direction of metabolism in a living tissue is controlled by effectively irreversible enzymatic reactions which are out of thermodynamic equilibrium (Krebs, 1946). The flux through a metabolic pathway is controlled by the slowest reaction in the sequence and the enzyme involved must be a nonequilibrium one (Krebs, 1969). Atkinson (1969) stated that equilibrium enzymes are required to have catalytic activities much greater than the regulatory enzymes to avoid uncontrolled rises in the concentration of intermediates. Therefore, according to definition, regulatory enzymes are non-equilibrium enzymes having very low catalytic activities.

An enzymatic reaction can be identified as non-equilibrium *in vivo* by comparing the mass-action ratio (Γ), with the apparent equilibrium constant (K') of the reaction determined *in vitro*. According to published guidelines (Rolleston, 1972), if Γ/K' is <0.05 the reaction is not at equilibrium *in vivo*; if it is >0.20 the reaction is at equilibrium. Behm and Bryant (1975a) reported that in *M. expansa* HK, PFK and PK are non-equilibrium enzymes with very low catalytic activities and, therefore, may play regulatory roles in glycolysis. The authors, however, questioned the regulatory property of HK considering the fact that glycogen also contributes to the G6P pool in the worm. In some other tissues (for example, mouse brain) the glycolytic flux is absolutely controlled by hexokinase and phosphofructokinase (Lowry

and Passonneau, 1964).

The mass-action ratios for the glycolytic enzymes of *M. expansa* have been calculated from the results in table 3.1. In *M. expansa* no significant differences in the mass-action ratios of the enzymes were observed during the change from aerobiosis to anaerobiosis (Behm and Bryant, 1975a). In the normal, *in vivo*, situation the worms are not expected to experience a glucose-free environment. Considering these points, the mass-action ratios were calculated for the anaerobic incubation in the presence of 10 mM glucose. The mass-action ratio for LDH could not be determined because lactate is an excretory product. Lack of quantitative values for OAA and fumarate prevented the calculation of mass-action ratios for the reactions in the PEP-succinate pathway.

Results and Discussion.

The apparent equilibrium constants published in the literature are variable, so in calculating the ratios of Γ/K' the highest values available were arbitrarily chosen. The results are presented in tables 4.1 and 4.2.

Table 4.1 shows that both CBZ and MBZ increase the mass-action ratios of HK and PFK, and that MBZ significantly reduces that of PK.

The apparently decreased mass-action ratio of enolase due to CBZ treatment (table 4.1) may not be important, because it is an equilibrium enzyme (Behm and Bryant, 1975a) and its equilibrium status has not been changed significantly to affect the flux through the pathway (table 4.2). The pool size of 2-PGA in *M. expansa* is very small (which prevents accurate assay); extremely variable and may be the cause of this alteration, as there is no change in the pool size of PEP (table 3.1). The

Table 4.1 Effects of CBZ and MBZ on the Mass-Action Ratios of Glycolytic Enzymes of *M. expansa* ($n = 5$)^a.

	Control	CBZ	MBZ
Hexokinase	0.007 ± 0.003	0.023 ± 0.008 ^c	0.020 ± 0.003 ^b
Phosphoglucose isomerase	0.386 ± 0.155	0.246 ± 0.088	0.237 ± 0.093
Phosphofructokinase	1.427 ± 0.159	2.057 ± 0.173 ^b	2.103 ± 0.551 ^e
Aldolase ($\times 10^{-6}$ M)	9.928 ± 2.615	7.694 ± 1.235	8.298 ± 1.483
Triosephosphate isomerase	0.195 ± 0.019	0.225 ± 0.052	0.201 ± 0.051
Phosphoglyceromutase	0.114 ± 0.014	0.138 ± 0.023	0.122 ± 0.010
Enolase	3.139 ± 0.319	2.439 ± 0.272 ^d	2.890 ± 0.242
Pyruvate kinase	2.568 ± 0.368	2.203 ± 0.567	1.163 ± 0.146 ^b

^a Values are means ± SD, calculated from data of individual experiment as in table 3.1.

Tests for significance by Student's *t* show b,c,d,e significantly different from control, $p < 0.0005$, < 0.0025 , < 0.005 , < 0.01 respectively.

ratios Γ/K' for the other equilibrium reactions are also unaffected by the drugs (table 4.2).

The mass-action ratio depends on the concentration of reactants and products of an enzymatic reaction, as represented by the formula:

$$\text{Mass-action Ratio } (\Gamma) = \frac{\text{Product(s)}}{\text{Reactant(s)}}$$

The diminished levels of glucose and F6P in the worms incubated with the drugs (table 3.1) are responsible for the increased mass-action ratios of HK and PFK respectively. The other reactant in each case is ATP, which is also diminished by CBZ and MBZ treatment (table 4.3). As there is no change in the level of ADP in the worms due to drug treatments, then clearly the decreased level of ATP has also contributed to the increased mass-action ratios of HK and PFK. In addition, decreases in the pool sizes of pyruvate (table 3.1) and of ATP (table 4.3) in the worms due to MBZ treatment have resulted in the decreased mass-action ratio of pyruvate kinase.

Table 4.2 shows that though the drugs significantly alter the Γ/K' values for HK, PFK, and PK, as compared with the control, yet the enzymes maintain their non-equilibrium position. Therefore, it may be assumed that the interference caused by the drugs is not of such magnitude to change the regulatory capacity of the enzymes, so far as this criterion is concerned.

4.2 Effects of CBZ and MBZ on the Energy Metabolism of *M. expansa*.

Introduction.

Glycolysis is linked to the adenylate system for energy-yielding and energy-requiring functions of the cell. In fact the

Table 4.2 Comparison of Mass-Action Ratios and Apparent Equilibrium Constants, (Γ/K') ($n = 5$)^a.

	Control	CBZ	MBZ	K'
Hexokinase	1.30×10^{-6}	4.25×10^{-6} b	3.60×10^{-6} b	5500 (i)
Phosphoglucose isomerase	0.82	0.53	0.51	0.47 (i)
Phosphofructokinase	1.19×10^{-3}	1.71×10^{-3} b	1.75×10^{-3} b	1200 (i)
Aldolase ($\times 10^{-6}$ M)	0.11	0.09	0.09	90(ii)
Triosephosphate isomerase	5.41	6.24	5.58	0.036(ii)
Phosphoglyceromutase	0.67	0.81	0.71	0.17 (i)
Enolase	0.68	0.53	0.63	4.6 (ii)
Pyruvate kinase	1.71×10^{-4}	1.47×10^{-4}	0.78×10^{-4}	15000(ii)

i Hess, 1963; ii Lowry and Passonneau

a The values are means $\pm$ SD.

b Student's t tests show significantly different from control, $p < 0.0005$.

main purpose of glycolysis is the generation and turn-over of high-energy components of the adenylate system. In the glycolytic sequence all the intermediates, except pyruvate, are esters of phosphoric acid. The phosphate group has special significance in glycolysis, because it is the chemical vehicle required for regeneration of ATP from ADP. The other physiological significance of the phosphate ester intermediates is that, due to their inability to diffuse through the cell membrane, they are in effect locked inside the cell and thereby the rate of glycolysis is maintained. It is to be expected that any disturbance of glucose metabolism would be reflected in the concentrations of adenine nucleotides in the worms. The effects of CBZ and MBZ on the relative concentrations of ATP, ADP and AMP in *M. expansa* have therefore been investigated.

Materials and Methods.

Collection and incubation of worms, and preparation of samples, have been described in chapter III. The spectrophotometric assays for ATP, ADP and AMP were performed following the method of Behm and Bryant (1975a) with modifications as required.

- (i) ATP was determined with the help of hexokinase in the presence of glucose (2.5 mM) and $MgCl_2$ (5 mM). The reaction was linked to G6P dehydrogenase reaction and the change in optical density due to reduction of NADP was recorded. The hexokinase reaction is specific for ATP and does not measure ITP or GTP. Sample volume was 100 μ l in 2 ml final volume.
- (ii) ADP and AMP were assayed in the same cuvette using lactate dehydrogenase, pyruvate kinase and myokinase. This assay also measures IDP and GDP. Sample volume

was 0.5 ml in 3 ml assay mixture.

Results.

The results of anaerobic and aerobic incubations in the presence of 10 mM glucose are presented in table 4.3. Incubations without glucose were not considered suitable for the investigation of energy metabolism.

Table 4.3 shows that CBZ and MBZ significantly reduce the level of ATP in *M. expansa* both anaerobically and aerobically. The levels of ADP and AMP are not affected, but the total adenine nucleotides are markedly decreased by the drugs.

Discussion.

Reduced level of ATP indicates a reduced rate of phosphorylation, which implies inhibition of the energy-yielding reactions in the metabolic pathway. Increased utilization may also result in a decreased level of ATP, but in that situation there would be an increase in the ADP level. In *M. expansa*, ATP is generated in the cytosol by substrate linked phosphorylation and, in the mitochondria, by the electron transport mediated phosphorylation. In the PEP-succinate pathway GTP is produced from GDP by the PEPCK reaction and the fumarate reductase reaction is coupled to phosphorylation of ADP. Inhibition of any of these reactions would result in a reduced energy level in the worms. It has been observed (chapter III) that both CBZ and MBZ diminish the production of succinate, probably by inhibition of some or all of the reactions between PEP and succinate. Scheibel, Saz and Bueding (1968) reported that omission of CO₂ from the incubation media resulted in marked decrease in ATP and ³²P incorporation in *H. diminuta*. The authors stated that the CO₂ deprivation reduced the rate of PEPCK activity and the availability of fumarate, and

Table 4.3 Effects of CBZ and MBZ on the Adenine Nucleotides in *M. expansa* in the Presence of 10 mM Glucose^a.

	Control	CBZ	MBZ
ANAEROBIC (n = 5)			
ATP	940 ± 218	509 ± 83 ^b	633 ± 154 ^e
ADP	418 ± 143	442 ± 91	379 ± 97
AMP	71 ± 35	111 ± 37	74 ± 9
Total nucleotides	1429 ± 214	1061 ± 144 ^d	1087 ± 73 ^c
AEROBIC			
ATP	1001 ± 177 (5)	640 ± 91 ^c (4)	541 ± 58 ^b (4)
ADP	642 ± 231 (5)	586 ± 170 (4)	553 ± 177 (4)
AMP	40 ± 16 (5)	23 ± 4 (4)	22 ± 10 (4)
Total nucleotides	1682 ± 246 (5)	1248 ± 87 ^d (4)	1166 ± 155 ^c (4)

^a Values are means ± SD. The units are nmoles/g wet weight.

Tests for significance by Student's *t* show b,c,d,e significantly different from control, p<0.0025, <0.005, <0.01, <0.025 respectively.

Figures in parentheses refer to the number of experiments in each category.

thereby both the substrate level phosphorylation and the electron transfer mediated phosphorylation were diminished. Therefore, if PEPCK were inhibited by CBZ and MBZ it is probable that similar effects would ensue. The results in table 4.3 suggest they do.

It has also been demonstrated that the decreased flow of carbon through the PEP-succinate pathway is accompanied by an increased production of lactate, which would result in increased generation of ATP at the pyruvate kinase reaction. This is probably an attempt at compensation by the parasite as the levels of ATP fall to the extent that phosphorylation of sugars and the biosynthetic processes are greatly reduced. This loss of 'kinetic efficiency' (E.A. Newsholme, personal communication) may over-ride any stimulatory effect caused by allosteric deinhibition of regulatory enzymes as ATP levels fall. It follows, therefore, that when changes in ATP levels become apparent in the presence of the drugs, adaptive mechanisms are overtaxed.

In the lactate pathway only one molecule of ATP is produced per molecule of PEP whereas, in the succinate pathway, at least two molecules of ATP are generated. Therefore, inhibition of flow along the succinate pathway would result in a net decrease in ATP level in the worms.

In *M. expansa* the fumarate reductase reaction is coupled to electron transport mediated phosphorylation (Bryant, 1970, 1972b). Van den Bossche (1972b) reported that benzimidazole anthelmintics inhibited ^{32}P i incorporation in *Ascaris* mitochondria. Incorporation of Pi may be inhibited in two ways: (i) due to inhibition of the FR system, and (ii) due to uncoupling of phosphorylation from the electron transport chain. Uncoupling results in less ATP production but at the same time accelerates substrate utilization, which is no longer limited by the requirement

for ADP to accept the "high-energy" bond. As there is no increase, but rather a significant decrease, in succinate production (table 3.1, Ch. III) it may be assumed that the decreased production of ATP is due, at least in part, to an inhibition, rather than an uncoupling of the FR system by the drugs.

ATP is needed for the active transport of glucose, so a reduced level of ATP might result in diminished uptake of glucose. Following this argument it may be hypothesized that inhibition of ATP generation is the primary effect of CBZ and MBZ, which has resulted in decreased uptake of glucose by the worms.

CHAPTER V

EFFECTS OF CBZ AND MBZ ON GLUCOSE UPTAKE, GLYCOGEN UTILIZATION AND EXCRETION IN *M. EXPANSA*

5.1 Introduction.

The results of the foregoing experiments (Ch. III) revealed that both CBZ and MBZ significantly diminish the glucose pool in *M. expansa*. This effect of the drugs is removed when glucose is excluded from the incubation medium (tables 3.3 and 3.4, Ch. III). On the basis of these findings it is assumed that the drugs exert an inhibitory effect on glucose uptake by the worms. It is also observed (tables 3.1 and 3.2, Ch. III) that inhibition of glucose uptake does not affect the flux significantly, as evidenced by the production of total end-products. This suggests that the glycogen reserves have been utilized to compensate the reduced availability of external glucose. To verify these hypotheses specific experiments on glucose uptake and glycogen utilization by *M. expansa* have been carried out.

5.2 Materials and Methods.

Scolecocytes of *M. expansa* were incubated as described in the preceding chapter. Glucose uptake was determined by assaying the amount of glucose remaining in the media after incubation. Preparation of samples for glycogen determination was done according to the method of Pflleiderer (1963) with some modifications. After incubation the worms were washed in three changes of distilled water, blotted on filter paper, weighed and homogenised in distilled water. The homogenate was digested in 30% KOH (2 ml/ml homogenate) in glass test tubes in a boiling water bath for about 30 mins. Then 96% ethanol was added to the tissue digest (3 ml/ml homogenate)

and heated in the boiling water bath, just bringing to the boil. After cooling down to room temperature (about 25°) the precipitate containing glycogen was centrifuged off, the supernatant discarded, and the precipitate washed twice in 5 ml ethanol. Traces of ethanol were removed from the precipitate by heating the tube in the water bath. Glycogen was hydrolysed to glucose by boiling the precipitate in 2N H₂SO₄ (3.5 ml/ml homogenate) in the water bath for several hours. The sample was then brought to room temperature and neutralised (pH 7.0) with 5N KOH. The final volume of sample was recorded and glycogen was measured spectrophotometrically as glucose equivalents.

5.3 Effects of CBZ and MBZ on Glucose Uptake, Glycogen Utilization and Excretion Under Anaerobic Conditions.

5.3.1 Results.

The results are shown in table 5.1. The table shows that both CBZ and MBZ significantly diminish glucose uptake from the media by *M. expansa*. The drug treatments also result in corresponding decreases in glycogen levels in the worms. There are considerable increases in the internal concentration of lactate in the worms due to CBZ and MBZ treatment. Marked reduction in succinate production, as evidenced by the amount in the worms and the amount in the media, occurs on treatment with both drugs.

5.3.2 Discussion.

(a) Glucose uptake:

Cestodes have an absolute requirement for carbohydrates, without which their growth and reproduction is seriously hampered (Chandler, 1942; Reid, 1942; Read and Rothman, 1957, 1958; Read, Schiller and Phifer, 1958; Read,

Table 5.1 Effects of CBZ and MBZ on Glucose Uptake, Glycogen Utilization and Excretion of Organic Acids by *M. expansa* Under Anaerobic Conditions^a.

		Control	CBZ	MBZ
Glucose uptake	(n = 10)	25 ± 6	12 ± 2 ^b	16 ± 3 ^b
Glycogen in worm*	(n = 4)	87 ± 12	47 ± 7 ^c	67 ± 11 ^e
Lactate in worm	(n = 5)	9 ± 1	15 ± 2 ^b	20 ± 1 ^b
Lactate excreted	(n = 5)	8 ± 2	8 ± 2	9 ± 2
Succinate in worm	(n = 5)	12 ± 0.36	9 ± 2 ^c	7 ± 1 ^b
Succi. excreted	(n = 5)	9 ± 1	6 ± 1 ^b	6 ± 2 ^d

^a The values are means ± SD. The units are μmoles/g wet weight.
 Tests for significance by Student's *t* show b,c,d,e significantly different from control,
 p<0.0005, <0.0025, <0.0125, <0.025 respectively.

* Presented as glucosyl units.

1949, 1957, 1959). Glucose seems to be an important source of energy in cestodes as it is readily absorbed and metabolised (Read, 1956, 1959, 1966; Laurie, 1957; Read and Rothman, 1958; von Brand, 1973).

Lacking an alimentary system, cestodes absorb most of the low molecular weight nutrients through their tegument which is the absorptive surface of cestodes (Lumsden, 1966; Pappas and Read, 1975). Further evidence of its physiological activity is the presence of mitochondria and various enzymes (Erasmus, 1957 a,b; Rothman and Lee, 1963; Lumsden, 1966, 1975.)

In cestodes glucose uptake has been reported to be a process of active transport requiring energy. It has been observed that glucose is accumulated against a concentration gradient by many cestodes (Phifer, 1960b; Fisher, 1965, 1966; von Brand, McMahon, Gibbs and Higgins, 1964; Pappas and Read, 1975). These authors also found that glucose uptake was stereospecific, since uptake was inhibited by structurally similar monosaccharides. Read (1961) found that galactose and several other monosaccharides competitively inhibited the uptake of glucose. Phifer (1960a) reported that the uptake of glucose by *H. diminuta* was nonlinear with respect to concentration. A Pasteur effect has been observed in glucose utilization by *H. diminuta* (von Brand et al, 1964) and *M. expansa* (Behm and Bryant, 1975a).

Sodium ions are necessary for the uptake of glucose in cestodes (von Brand et al, 1964; von Brand and Gibbs, 1966; Pappas and Read, 1975) as in many vertebrate systems (Crane, 1960). Potassium ions do not appear to be essential for glucose uptake in cestodes (Phifer, 1960a; von Brand and Gibbs, 1966).

Erasmus (1957 a,b) observed phosphatase activity in the tegument of *M. expansa* and proposed that phosphorylation of glucose occurs at the time of absorption. More recent studies have shown that phosphatases are not directly involved in the absorption process (Phifer, 1960c; Read, 1966). Read (1966) stated that ATP might be involved in the driving mechanism of active transport.

Structural integrity of the absorptive surface is important, because any disruption or damage to the brush border or to the intracellular organisation will surely disturb absorption and transport of solutes across the membrane. It has been reported that the anthelmintic drug niclosamide penetrates into the body of cestodes by damaging the tegument. One of the effects of the drug is the inhibition of glucose uptake by cestodes (Hawking, 1973). It has also been reported that a lytic process takes place at the level of the tegument which is rich in mitochondria, chiefly the proliferating zone of the neck. Borgers and De Nollin (1975) observed extensive ultrastructural damage to the intestinal cells of *Ascaris suum* after treating infected pigs with MBZ. Similar ultra-structural changes, including loss of microvilli, degeneration and complete necrosis of both the nucleate and anucleate parts of the tegument, have been observed in adult cestodes after MBZ treatment (Verheyen, Borgers, Vanparijs and Thienpont, 1976). The authors stated that the inhibitory effect of MBZ on glucose uptake was due to cellular damage of the absorptive surface.

Therefore, there are at least three ways in which CBZ and MBZ might have inhibited glucose uptake by *M. expansa*;

- (i) damaging the absorptive brush border of the tegument,
- (ii) damaging the intracellular transport mechanism in which mitochondria might be involved, and
- (iii) inhibiting the energy source of absorption and transport systems i.e. inhibition of the generation and/or turnover of ATP. Whereas the first two disturbances could result from local and direct effects of the drugs the third one could be the consequence of a complex chain of events in the metabolic pathway involving several enzyme systems. Therefore, in the case of the third possibility, or even the second one, the inhibition of glucose uptake may not be the primary effect of the drugs as claimed by Van den Bossche and co-workers. Rather, it may be due to reduced generation of high-energy phosphate bonds.

(b) Glycogen Utilization:

Endogenous glycogen in helminths serves as an energy reserve (von Brand, 1973). The polysaccharides stored by cestodes seem to be derived from exogenous carbohydrate, mainly glucose and galactose (Read, 1966; von Brand, 1973). A rapid rate of glycogen synthesis from exogenous glucose has been observed in many species of cestodes (Wardle, 1937; von Brand and Mercado, 1961; Read, 1956; Daugherty, 1956, von Brand, 1973). It has been reported that in *H. diminuta* the *in vitro* glycogen synthesis is dependent on the presence of CO₂ in the environment (Fairbairn, Wertheim, Harpur and Schiller, 1961; Kilejan, 1963). Absence of glucose from the

ambient medium either *in vitro* or *in vivo* results in rapid depletion of glycogen levels in the cestodes (Wardle, 1937; Chandler, 1942; Reid, 1942; Read, 1949, 1956, 1957, 1959; von Brand, 1973). In the cestodes studied so far, there is very little difference in the rate of endogenous polysaccharide utilization whether they are maintained aerobically or anaerobically (von Brand and Bowman, 1961; Agosin, 1957; Read, 1956; von Brand, 1973).

The results in table 5.1 show that there are significant reductions in the glycogen levels in *M. expansa* which have been incubated with CBZ and MBZ. Inspection of the glucose uptake results reveal that there are corresponding decreases of glucose uptake by the worms due to incubation with the drugs. Therefore, it is evident that the inhibitory effects of the drugs on glucose uptake led to increased utilization of reserve glycogen. Further, the results reveal that there has been no statistically significant change in the flux as evidenced by the production of total end-products. This implies that with reduced availability of exogenous glucose, the endogenous glycogen was utilized as an energy source thereby maintaining carbon flux.

(c) Excretion of End-products:

Lactate and succinate are the excretory products of carbohydrate metabolism in *M. expansa* (Behm and Bryant, 1975a). The route and mechanism of excretion in cestodes is not adequately known. It has been speculated that the protonephridial system, also called the flame-cell system,

of cestodes has an excretory function (Hyman, 1951; Wardle and McLeod, 1952). Howells (1969) questioned the excretory role of the 'nephridial' system of *M. expansa* and suggested that the system could function in the movement of nutrients from one part of the worm to another. Webster and Wilson (1970) found lactate and Webster (1972) found both lactate and succinate in the protonephridial canal fluid of *H. diminuta*. The authors stated that the protonephridial system plays an excretory role in cestodes.

Table 5.1 shows that there are very high levels of lactate accumulated in the worms due to incubation with CBZ and MBZ. The drugs have no effect on the excretion of succinate as evidenced by the ratios between the internal pools and the excreted pools. This situation implies that in *M. expansa* the excretion of the two end-products may be accomplished by different routes, and that only the excretory mechanism of lactate is impaired by the drugs.

5.4 Effects of CBZ and MBZ on Glucose Uptake, Glycogen Utilization and Excretion Under Aerobic Conditions.

5.4.1 Results:

The results are presented in table 5.2. The table shows that CBZ and MBZ significantly inhibit glucose uptake by the worms. Significant decreases in glycogen levels in the worms incubated with the drugs also occur. It is also observed that under aerobic conditions glucose uptake is decreased in all groups of worms, compared with the anaerobic situation.

Table 5.2 Effects of CBZ and MBZ on Glucose Uptake, Glycogen Utilization and Excretion of Organic Acids by

M. expansa Under Aerobic Conditions^a.

	Control (n = 5)	CBZ (n = 4)	MBZ (n = 4)
Glucose uptake	14 ± 2	7 ± 1 ^b (5)	9 ± 2 ^b (5)
Glycogen in worm*	95 ± 7 (4)	73 ± 4 ^c	81 ± 2 ^d
Lactate in worm	11 ± 4	8 ± 3	9 ± 2
Lactate excreted	10 ± 2	8 ± 3	8 ± 2
Succinate in worm	17 ± 4	11 ± 4 ^e	11 ± 2 ^e
Succinate excreted	3 ± 1	6 ± 2 ^e	4 ± 1

^a The values are means ± SD. The units are μmoles/g wet weight.

Tests for significance by Student's *t* show b,c,d,e significantly different from control, p<0.0005, <0.0025, <0.005, <0.025 respectively.

Figures in parentheses refer to the number of experiments in each category.

* Presented as glucosyl units.

There is no significant change in the production and excretion of lactate due to the drugs. There are significant decreases of the internal pools of succinate in the worms treated with the drugs whereas only CBZ causes a significant increase in succinate excretion.

5.4.2 Discussion:

The effects of CBZ and MBZ on the glucose uptake and glycogen utilization in *M. expansa* are similar under both anaerobic and aerobic conditions. Behm and Bryant (1975a) reported that *M. expansa* absorbed more glucose under anaerobic conditions than under aerobic conditions. A similar Pasteur effect is observed whether the worms are incubated with the drugs or not.

Under aerobic conditions there is no change in the excretion of lactate and only CBZ increases the excretion of succinate to a significant level. This situation again suggests that the end-products are excreted by different mechanisms. In *M. expansa* lactate is produced in the cytosol whereas succinate is produced in the mitochondria, so it is probable that their excretion kinetics are different and may vary under different experimental conditions. The mechanism of end-product excretion in cestodes is speculative and not fully known; therefore, from these results, it is not possible to conclude whether the drugs interfere with the excretion of end-products in *M. expansa*.

Effects of CBZ and MBZ on the production of succinate and lactate have been described and interpreted earlier (Ch. III).

CHAPTER VI

ACTIVITIES OF SELECTED GLYCOLYTIC ENZYMES AND THE EFFECTS OF CBZ AND MBZ

6.1 Introduction.

The results of the foregoing experiments reveal that incubation of *M. expansa* scoleces in media containing CBZ and MBZ causes marked alterations in carbohydrate metabolism. The drugs have been found to inhibit glucose uptake, diminish the synthesis and/or turnover of adenine nucleotides and disturb the formation of end-products.

In this cestode, glycolysis is a complex of relationships and interdependences between the various substrates, enzymes and co-factors involved in the pathway. Moreover, the branched pathways are also dependent on each other. As a result it becomes enormously difficult to ascertain which are the primary secondary and tertiary effects of the drugs. A closer examination of the activities of the enzymes involved in the process may provide sufficient clues to permit a conclusion. Effects of CBZ and MBZ on the activities of some isolated enzymes of *M. expansa* are investigated in this section.

Hexokinase, PFK and PK were selected because of their possible regulatory role in glycolysis. LDH was chosen for its role in the production of one of the end-products, and MDH was selected for the role it plays in maintaining the redox balance of the cytosol by virtue of its very high catalytic activity. Due to the technical difficulties involved in their assay systems, the spectrophotometric assays for the activities of PEPCK, fumarase and fumarate reductase could not be performed. However, the

activities of PEPCK and FR have been determined with the help of ^{14}C labelled substrates (Ch. VII). The other enzymes of glycolysis were not considered for the study of drug action, as they are equilibrium enzymes with much higher activities and, with the possible exception of enolase (Ch. IV), there is no evidence that either CBZ or MBZ affects them.

6.2 Materials and Methods.

6.2.1 Preparation of Supernatant:

- (a) Scoleces of *M. expansa* were separated, rinsed several times first in KRP and then in homogenising buffer, blotted, weighed and used immediately. The homogenising buffer was the buffer to be used in the relevant enzyme assay. The scoleces were homogenised in 1:1 (w/v) buffer using an all-glass Dounce-type homogeniser (Kontes Glass Co.). The homogenate was centrifuged at $100,000 \times g$ for 20 mins at $0 - 4^\circ\text{C}$ in a Beckman Model L ultracentrifuge. The supernatant was separated and assayed immediately with or without adding drugs.

The supernatant was also filtered through Membrane-filters (Amicon) by centrifuging at $750 \times g$ for 30 mins at $0 - 4^\circ\text{C}$ with two washings with 5 ml buffer and assayed for enzyme activity with or without adding the drugs. No differences in the activities of the enzymes were observed whether the supernatant was filtered through the Amicon membrane or not. Therefore, $100,000 \times g$ supernatant was used directly for assays.

- (b) Homogenate of *M. expansa* was incubated with or without the drugs (0.04%) for 30 mins at 35°C in a shaking water bath (Labmaster) and then centrifuged at $100,000 \times g$ for 20 mins at $0 - 4^\circ\text{C}$. The supernatant was filtered

through Whatman No. 1 filter paper to remove drug particles, and used immediately.

- (c) Worms were incubated in KRP containing 10 mM glucose with or without the drugs (0.08%). After incubation the worms were rinsed several times in buffer, blotted, weighed and supernatant prepared as described above. The 100 000 x g supernatant was assayed for HK and PFK activities with or without adding extra amount of the drugs (0.1 mM) to the assay mixture.

6.2.2 Enzyme Assays:

Activities of the enzymes were assayed spectrophotometrically at 25°C using a Gilford 2400 recording spectrophotometer with a light path of 1 cm in glass cuvettes. Enzyme activity was calculated from the extinction coefficient of $6.2 \times 10^6 \text{ cm}^2/\text{mole}$ published in Bergmeyer (1963). Assays were performed according to Behm and Bryant (1975a) with some modifications as required.

The assay mixture:-

(i) Hexokinase:

Triethanolamin-hydrochloride buffer 0.1 M containing 1 mM EGTA, pH 7.4, 1.75 ml; NADP 0.4 mM; MgCl_2 5 mM; ATP 0.2 mM; G6PDH 30 μg ; drug (when added) 0.1 mM; glucose 0.3 mM; supernatant preapration and distilled water to 2 ml final volume.

Reaction was started by adding either ATP or glucose depending on the assay condition desired. Five concentrations of ATP (0.1 mM to 0.3 mM) and glucose (0.15 mM to 0.6 mM) were tested for comparison of the effects of the drugs.

(ii) Phosphofructokinase:

Triethanolamin-HCl buffer 0.1 M containing 1 mM EGTA and 0.1 M $(\text{NH}_4)_2\text{SO}_4$, pH 8.0, 1.7 ml; NADH 0.1 mM; MgCl_2 5 mM; G3PDH 50 μg ; TPI 5 μg ; aldolase 20 μg ; drug (when added) 0.1 mM; ATP 0.1 mM; F6P 3.75 mM; sample and distilled water to 2 ml final volume. The reaction was started by adding either ATP or F6P depending on the assay condition desired. Five concentrations of ATP (0.075 mM to 0.25 mM) and F6P (1.25 mM to 5 mM) were tested to compare the effects of the drugs.

(iii) Pyruvate kinase:

Triethanolamin-HCl buffer 0.1 M pH 6.5, 1.7 ml; KCl 100 mM; ADP 0.33 mM; NADH 0.15 mM; MgCl_2 5 mM; LDH 5 μg ; drug (when added) 0.1 mM; PEP 0.5 mM; sample and distilled water to 2 ml final volume. The reaction was started by adding PEP to the reaction mixture.

(iv) Lactate dehydrogenase:

Triethanolamin-HCl buffer 0.1 M containing 1 mM EGTA, pH 7.4, 1.8 ml; NADH 0.15 mM; Na-pyruvate 0.25 mM; drug (when added) 0.1 mM; sample and distilled water to 2 ml final volume.

The reaction was started by adding pyruvate, 0.1 M potassium phosphate buffer was tested instead of triethanolamin-HCl buffer. No difference in LDH activity was observed.

(v) Malate dehydrogenase:

0.1 M potassium phosphate buffer pH 7.4, 2.9 ml; NADH 0.1 mM; neutralised OAA 1.7 mM; drug

(when added) 0.1 mM; sample and distilled water to 3 ml final volume.

The reaction was started by adding OAA.

In all the assays, the reaction mixture (except the starting substrate) was incubated at 25°C to allow any nonspecific reaction to cease - until the drift became linear. Blanks and standards were always run to check any reaction due to contamination of enzymes and substrates. Blank assays did not contain any sample and control assays did not contain the drugs in reaction mixtures.

6.3 Results.

6.3.1 Effects of the Drugs on the Activities of Enzymes in Cell-Free Extract of *M. expansa*:

The results are presented in table 6.1. Enzyme activities are expressed as U/g wet weight of worm tissue instead of U/mg protein. However, the protein content in *Moniezia scolex* is 22 mg/g wet weight (range 19 mg to 24 mg) as determined by the method of Lowry et al (1951).

Table 6.1 shows that CBZ significantly inhibits the activities of HK and PFK, but MBZ has no effect on the enzymes. The activities of PK, LDH and MDH are not affected by the presence of CBZ and MBZ in the reaction mixture.

6.3.2 Effects of the Drugs on the Activities of the Enzymes in the Homogenate of *M. expansa*:

The results are presented in table 6.2. The table shows that both CBZ and MBZ significantly inhibit the activities of HK and PFK. No other enzyme is found to be affected by the drugs.

Table 6.1 Effects of CBZ and MBZ on the Activities of Selected Enzymes of Carbohydrate Metabolism in

M. expansa (n = 7)^a.

Units: $\mu\text{moles/min per g wet weight}$.

	Control	CBZ	MBZ
Hexokinase	0.094 $\pm$ 0.008	0.064 $\pm$ 0.012 ^b	0.082 $\pm$ 0.012
Phosphofructokinase	0.258 $\pm$ 0.026	0.113 $\pm$ 0.025 ^b	0.228 $\pm$ 0.041
Pyruvate kinase	0.316 $\pm$ 0.035	0.283 $\pm$ 0.018	0.279 $\pm$ 0.037
Lactate dehydrogenase	6.141 $\pm$ 0.514	6.686 $\pm$ 0.890	5.069 $\pm$ 1.020
Malate dehydrogenase	29.09 $\pm$ 4.12	26.44 $\pm$ 2.37	27.25 $\pm$ 2.49

^a 100,000 x g supernatant preparation assayed directly with or without the drugs (0.1 mM) in assay mixtures.

^b Tests for significance by Student's *t* show significantly lower than control ($p < 0.0025$).

Table 6.2 Effects of CBZ and MBZ on the Activities of Selected Enzymes of Carbohydrate Metabolism in the Homogenate of *M. expansa*^a.

Units: $\mu\text{moles/min per g wet weight}$.

		Control	CBZ	MBZ
Hexokinase	(n = 7)	0.069 $\pm$ 0.001	0.042 $\pm$ 0.002 ^c	0.053 $\pm$ 0.007 ^d
Phosphofructokinase	(n = 7)	0.239 $\pm$ 0.040	0.103 $\pm$ 0.015 ^b	0.126 $\pm$ 0.035 ^b
Pyruvate kinase	(n = 5)	0.217 $\pm$ 0.054	0.256 $\pm$ 0.036	0.209 $\pm$ 0.043
Lactate dehydrogenase	(n = 5)	5.936 $\pm$ 1.036	6.142 $\pm$ 0.683	6.033 $\pm$ 0.921
Malate dehydrogenase	(n = 5)	24.52 $\pm$ 3.884	23.40 $\pm$ 3.960	24.29 $\pm$ 3.333

^a Homogenate of *M. expansa* was incubated with or without the drugs (0.04%) and 100,000 x g supernatant assayed for enzyme activity.

Tests for significance by Student's *t* show b,c,d significantly lower than control, $p < 0.0025$, $p < 0.005$, $p < 0.01$ respectively

6.3.3 Effect of CBZ and MBZ on the Activity of Hexokinase in Different Preparations at Various Concentrations of ATP and Glucose.

The results are presented in table 6.3. The table shows that CBZ significantly inhibits the activity of hexokinase when added to the assay mixture containing the supernatant prepared from fresh worms. The drug also inhibits the enzyme when homogenate of the worm is incubated with it. The inhibition is constant at all concentrations of ATP (0.1 mM to 0.3 mM) and glucose (0.15 mM to 0.60 mM) tested. When worms are preincubated with CBZ and the enzyme is subsequently extracted, it is not found to be affected. However, when an extra amount of CBZ (0.1 mM) is added to the assay mixture, the activity of HK is significantly inhibited.

MBZ has no effect on the activity of HK in any preparation at any concentration of the substrates and addition of extra amount of the drug (0.1 mM) to the assay mixtures has no effect.

6.3.4 Effects of CBZ and MBZ on the Activity of PFK in Different Preparations at Various Concentrations of F6P and ATP.

The results are presented in table 6.4. The table shows that CBZ significantly inhibits the activity of PFK when added to the assay mixture irrespective of the concentration of F6P and ATP tested. The activity of the enzyme is also inhibited when homogenates of the worm are incubated with the drug. CBZ had no effect on the activity of PFK extracted from worms preincubated with it. However, when an extra amount of the drug (0.1 mM) is added to the

Table 6.3 Effects of CBZ and MBZ on the Activity of Hexokinase from *M. expansa* (n = 20)^a

Preparation	Substrate Concentration		Activity (μmoles/min/g wet weight)	
	Glucose	ATP	Control	MBZ
Supernatant without preincubation.	0.3 mM	0.3 mM*	0.094 ± 0.008	0.064 ± 0.012 ^b 0.082 ± 0.012
	0.6 mM**	0.2 mM	0.099 ± 0.012	0.075 ± 0.021 ^b 0.096 ± 0.006
Supernatant from pre-incubated homogenate.	0.3 mM	0.3 mM*	0.069 ± 0.001	0.052 ± 0.004 ^c 0.060 ± 0.007
	0.6 mM**	0.2 mM	0.071 ± 0.010	0.048 ± 0.005 ^c 0.062 ± 0.005
Supernatant from preincubated worms ^d .	0.3 mM	0.3 mM*	0.071 ± 0.011	0.056 ± 0.008 0.061 ± 0.007

* The effect of CBZ was constant over a range of ATP concentrations (0.1 mM - 0.3 mM).

** Inhibition by CBZ was constant over a range of glucose concentrations (0.15 mM - 0.60 mM).

^a Values are means ± SD. Tests for significance by Student's t show b,c significantly different from control p<0.005, <0.01 respectively.

^d Addition of CBZ (0.1 mM) to this supernatant resulted in significant inhibition (p<0.025).

Addition of MBZ had no consistent effect.

Table 6.4 Effects of CBZ and MBZ on the Activity of Phosphofructokinase from *M. expansa*^a.

Preparation	Substrate Concentration.		Activity (μmoles/min/g wet weight).	
	F6P	ATP	Control	CBZ
Supernatant - no preincubation.	3.75 mM	0.25 mM*	0.25 ± 0.09	0.11 ± 0.04 ^b
	1.25 mM**	0.10 mM	0.18 ± 0.04	0.08 ± 0.03 ^b
Supernatant from preincubated homogenate,	2.5 mM**	0.10 mM	0.25 ± 0.02	0.11 ± 0.03 ^c
				0.12 ± 0.02 ^c
Supernatant from preincubated worms. ^d	2.5 mM**	0.10 mM	0.23 ± 0.01	0.21 ± 0.01
				0.22 ± 0.04

* Effects of CBZ was constant over a range of ATP concentrations (0.075 mM - 0.25 mM).

** Effects of CBZ was constant over a range of F6P concentrations (1.25 mM - 5.00 mM).

^a The values are means ± SD. Tests for significance by Student's t show b, c significantly different from control p<0.025, <0.0005 respectively (n = 20).

^d Addition of CBZ (0.1 mM) to this supernatant produced inhibition of about 50% (p<0.0005, n = 24). Addition of MBZ had no effect.

assay mixture the activity of the enzyme is significantly inhibited.

MBZ only inhibits the activity of PFK when homogenates of the worm are incubated with it.

The effects of CBZ and MBZ are consistent over a range of F6P concentrations (1.25 mM to 5.00 mM).

6.3.5 Effects of CBZ and MBZ on the Activities of Selected Commercial Enzymes (Boehringer).

The activities of a number of enzymes of carbohydrate metabolism, obtained from Boehringer, were assayed (table 6.5). The reaction mixtures were the same as described except that known concentration of commercial enzymes were used instead of preparations from the worm.

The table shows that neither CBZ nor MBZ affects the activities of the enzymes from the commercial source.

6.4 Discussion.

The results of the assays for enzyme activity show that CBZ inhibits the activities of both HK and PFK whenever it is added to reaction mixtures containing the supernatant either prepared from fresh worms or from preincubated homogenates of the worm. However, when whole worms are preincubated with CBZ for periods which have been shown, earlier in this thesis, to be sufficient to allow metabolic disturbance, the enzymes are not found to be affected. Further addition of CBZ is necessary before inhibition is apparent.

Two explanations are possible. First, the intact worm may interpose a barrier between the enzyme and the drug. When the worms are homogenised, added CBZ is not prevented by this barrier from reaching the enzymes. Alternatively, the

Table 6.5 Effects of CBZ and MBZ on the Activity of Selected Commercial Enzymes (Boehringer).

Enzyme*	$\mu\text{moles/min per mg. protein (n = 5)}$		
	Control	CBZ	MBZ
Hexokinase (15431)	137 $\pm$ 11	135 $\pm$ 10	126 $\pm$ 14
Phosphofructokinase (15103)	56 $\pm$ 6	52 $\pm$ 7	55 $\pm$ 6
Pyruvate kinase (15744)	195 $\pm$ 9	202 $\pm$ 19	198 $\pm$ 15
Lactate dehydrogenase (15371) ,	493 $\pm$ 29	490 $\pm$ 13	490 $\pm$ 17
Malate dehydrogenase (14048)	987 $\pm$ 124	1007 $\pm$ 205	994 $\pm$ 173

* Hexokinase from yeast, malate dehydrogenase from pig heart (mitochondria), and the rest are from rabbit muscle.

Figures in parentheses refer to Boehringer catalogue number (1973/74).

interactions between the drug and the enzymes are loose. The process of homogenising the preincubated worms and the necessary dilution which it entails, may break down the enzyme-CBZ association or may dilute the drug below its level of effectiveness. As it is probable that CBZ does enter the parasite, the alternative explanation is more likely.

With MBZ, it is observed that the activities of HK and PFK are affected only when the homogenate is preincubated with the drug. There are various factors which must be considered in this situation. MBZ is an insoluble compound and was used as a suspension. Insolubility may therefore be the explanation for its failure to affect the activities of the enzymes. The maximum concentration of MBZ which could be used, without displacing the recording pen of the spectrophotometer out of scale, was 0.1 mM. This concentration of the drug may not be sufficient to cause inhibition *in vitro*. Moreover, the temperature (25°C) at which the supernatant was incubated in the reaction mixture with the drug may not permit interference with the enzymes. The homogenate was incubated with 0.04% (1.3 mM) MBZ at 35°C, and even in that case the inhibition of HK is not great (table 6.2). On the other hand the same concentration of CBZ at the same temperature had much greater effect. Therefore, it may be assumed that MBZ is a less potent inhibitor of the enzymes than CBZ because of its greater insolubility.

It might be expected that inhibition of HK activity would cause accumulation of glucose within the worm. However, this effect (if it occurs) is masked by the fact (tables 3.1 and 3.2, Ch. III) that glucose uptake is inhibited by the drugs.

It is still impossible to state that the various enzyme effects observed *in vitro* are responsible for anthelmintic efficacy. Indeed, Van den Bossche (1972a) noted that MBZ did not affect the activity of HK in *Ascaridia galli* (intestine) or in *Syphacia muris* when infected hosts were treated with the drug. These observations render it likely that the effects on HK and PFK are two of multiple effects exerted by the drugs under ideal *in vitro* conditions and that they may have a relatively unimportant role to play *in vivo*. The greater effect of CBZ *in vitro* is almost certainly related to its greater solubility, when compared to MBZ, but this may not be the critical factor *in vivo*.

Inhibition of PFK activity could be fatal to the parasite, because it controls the rate of glycolysis (Bueding and Mansour, 1957; Bueding and Fisher, 1966; Behm and Bryant, 1975b). It has been reported (Bueding and Mansour, 1957) that trivalent organic antimonials inhibited the activity of PFK in *Schistosoma mansoni* and that this inhibition was evidenced by an accumulation of the substrate, F6P, and a decrease in the product, FDP, which led to decreased rate of glycolysis in the worm. Bueding and Fisher (1966) stated that antimony probably exerts its inhibitory effect on PFK by competing with F6P for a binding site. The authors also reported that inhibition of PFK could be reversed by increasing the concentration of F6P in the reaction mixture.

Both CBZ and MBZ inhibit the activity of PFK in the homogenates of *M. expansa*, but in the whole animal these drugs have no effect on the enzyme. Enzyme extracted from parasites preincubated with the drugs is susceptible to inhibition by CBZ.

Increased concentration of F6P has no effect on this inhibition which suggest that *in vitro* effect of CBZ and MBZ on PFK activity is different in nature from that observed by Bueding and Fisher (1966). Moreover, there is little or no evidence for drug-induced changes in the concentrations of F6P and FDP in the worms (Ch. III). The inhibition of PFK activity observed *in vitro* may not be a consistent effect of the drugs *in vivo*.

In vitro, the drugs come into direct contact with the active centres of enzymes, whereas in intact worms the permeability factors and the intra-cellular compartmentation may prevent effective interaction between the enzymes and the drugs. It thus seems likely that the inhibitory effects of CBZ and MBZ on the activities of the enzymes, observed under different *in vitro* conditions, are essentially the results of artificial conditions created in the homogenates. It is of interest, however, that under none of the conditions tested was it possible to detect an effect of CBZ and MBZ on HK and PFK from commercial sources. It seems probable that there exists a real difference between enzymes of parasite, and mammal and microbial origin (Behm and Bryant, 1975b). Although the present study leads to the reluctant conclusion that the inhibitions exerted by the benzimidazoles on HK and PFK activities have little significance *in vivo*, it does not exclude the possibility that anthelmintics can be formulated to exploit the difference between host and parasite enzymes.

CHAPTER VII

EFFECTS OF CBZ AND MBZ ON THE INCORPORATION OF RADIOCARBON FROM LABELLED SUBSTRATES BY *M. EXPANSA* AND ITS CELL-FREE PREPARATIONS

7.1 Introduction.

Experimental results have revealed that both CBZ and MBZ seriously interfere with energy metabolism in *M. expansa* (Ch. III, IV, V). The drugs do not appear to affect the activities of the enzymes involved in the pathway from glucose to lactate in the worm (Ch. VI), but they may exert an effect on the PEP-succinate pathway, which plays a very important role in the energy requirement of the parasite. The reactions in the PEP-succinate pathway depend on CO₂ fixation by PEPCK. The function of this enzyme in helminth parasites is so different from that of mammalian host that it is a potential site for chemotherapeutic attack.

Results in chapter III (table 3.1) show that succinate production in *M. expansa* is greatly diminished by CBZ and MBZ. It is also evident that the drugs reduce the synthesis and/or turnover of adenine nucleotides and generation of ATP in the cestode (table 4.3, Ch. IV). These findings may be taken as *prima facie* evidence for some interference in the PEP-succinate pathway. However, due to lack of reliable techniques for the quantitative measurement of the intermediates of the pathway, it could not be ascertained which reaction(s) may have been affected by the drugs.

Distribution of radiocarbon from labelled substrate into various components of carbohydrate metabolism may provide valid criteria for the determination of the effects of the drugs. Bearing this in mind, experiments on ¹⁴C-labelled substrate

utilization by intact scoleces and cell-free preparations from *M. expansa* were carried out.

The experiments and the results obtained are described in this section.

7.2 Materials and Methods.

7.2.1 Experiment with ^{14}C -glucose:

Scoleces of *M. expansa* were rinsed several times in KRP, blotted, weighed and incubated in pre-gassed KRP containing 10 mM glucose, to which D[U- ^{14}C]- glucose (sp. act. 3 mCi/mmmole) was added at a rate of 1 $\mu\text{Ci/ml}$ incubation medium. Test incubations contained 0.08% CBZ or MBZ. Incubation was carried out in conical flasks placed in a shaking water bath (Gallenkamp) at 37°C for 30 mins.

After incubation the worms were rinsed in KRP and then in distilled water (3 quick changes of each), and dropped into 80% ethanol. The worms were then minced with scissors and homogenised in 1:2 (W/v) 80% ethanol. The homogenates were centrifuged at 12,000 x g for 20 mins and the supernatant (ethanolic extract) was removed. 10 μl of the supernatant was taken in a counting vial containing 20 μl 2N HCl, evaporated to dryness in a fume cupboard and counted with 10 ml PCS in a Packard liquid scintillation counter.

An amount of ethanolic extract containing 100,000 cpm was spotted on chromatography paper (Whatman No. 1, 46 x 57 cm) and run in two directions: first in phenol-water and then, after drying, in propanol-butanol water as described by Smith (1969). The dried chromatographs were exposed on X-ray film (Kodirex, 35.6 x 43.2 cm) for 3 weeks in a light proof box. The films were then developed and fixed in chemicals

supplied by ILFORD according to the manufacturer's directions.

The spots, located by autoradiography, were cut out, placed in counting vials and counted with 15 ml PCS in a Packard liquid scintillation counter. The identity of the radioactive intermediates was established by co-chromatography with standards.

7.2.2 Experiments with $\text{NaH}^{14}\text{CO}_3$:

(a) Whole animal.

Scolecemes of *M. expansa* were processed and incubated as described above except that the incubation media contained $\text{NaH}^{14}\text{CO}_3$ (sp. act. 0.1 mCi/mmol) instead of labelled glucose. The procedures for preparation of ethanolic extracts, chromatography, autoradiography and counting were as described above.

(b) PEPCK experiment (cytosol).

About 5 g of *M. expansa* scolex was homogenised in 1:1 (w/v) 0.1 M imidazole buffer, pH 6.6 and centrifuged at 100,000 x g for 20 mins. The supernatant was incubated with Na-PEP and $\text{NaH}^{14}\text{CO}_3$, and OAA formed was trapped as malate using MDH and NADH. The reaction mixture contained:

0.1 M imidazole buffer, pH 6.6, 0.65 ml; NADH 2.5 mM; GSH 2 mM; MnCl_2 2.5 mM; Na-IDP 1.0 mM; MDH 10 μg ; 1 μCi $\text{NaH}^{14}\text{CO}_3$; drug (when added) 2.5 mM; enzyme preparation and distilled water to 1 ml final volume. PEP concentration was 2.5 mM.

The reaction was started by adding Na-PEP and incubation carried out at 37°C for 15 mins in a shaking water bath under either N_2 or O_2 according to the experimental conditions desired. 100 μl aliquot from each incubation was taken in counting vials containing 200 μl 2N HCl, evaporated in fume cupboard and counted with 10 ml PCS in a

Packard liquid scintillation counter.

7.2.3 Fumarate Reductase Experiment:

(a) Preparation of Mitochondria.

Mitochondrial fraction from *M. expansa* was prepared according to the method described by Cheah (1971). The composition of media "A" and "B" are given in appendix VI. All operations were carried out at 0-2°C.

Scolecies of *M. expansa* (about 20 g) were minced with scissors in medium A (2.5 ml/g), homogenised with an all-glass Dounce-type homogeniser and centrifuged for 5 mins at 850 x g. The supernatant was filtered through 3 layers of surgical gauze and recentrifuged at 14,000 x g for 15 mins. The pellet was suspended in 10 ml of medium B and centrifuged at 850 x g for 5 mins. The supernatant was recentrifuged at 7000 x g for 10 mins and pellet was washed twice more in 10 ml of medium B without BSA. The final pellet was resuspended in 10 ml of 0.25 M sucrose solution and used immediately.

(b) The reaction mixture (Prichard, 1973).

0.1 M potassium phosphate buffer pH 7.0, 20 µl;
NADH 2.0 µM; MgCl₂ 2.5 µM; mitochondrial
preparation 0.1 ml (0.6 mg protein); drug (when
added) 2.6 mM; [2,3-¹⁴C]-fumarate 1 µCi (sp.act.
21.8 mCi/mmol); distilled water to 0.5 ml final
volume.

The reaction mixture was incubated for 60 mins at 37° under N₂ in a shaking water bath. The reaction was

stopped by the addition of 0.5 ml absolute ethanol. 0.5 ml aliquot was taken in a small vial and evaporated to approximately 0.1 ml in a fume cupboard. The evaporated sample was then spotted on Whatman No. 1 chromatography paper with two washings of the vial with 30 μ l of ethanol. The rest of the procedures for chromatography, autoradiography and counting were as described in section (i).

In all the experiments described above, samples for counting were dark adapted for 18 hours at 4° after adding PCS, to avoid chemiluminescence. Counting of samples was done at 1 to 2% preset error. Blank portions of the chromatographs were also counted using the same scintillant. No further correction for the counting efficiency was made.

Statistical analysis of the results was performed using a one-tailed unpaired Student's *t* test.

7.3 Results.

7.3.1 Effects of CBZ and MBZ on the Metabolism of D[U-¹⁴C]-Glucose by *M. expansa* Under Anaerobic Conditions.

The results are presented in table 7.1. The table shows that CBZ and MBZ significantly inhibit incorporation of ¹⁴C into glucose, malate, succinate and alanine pools in the worms. Both the drugs increase incorporation into lactate, but diminish the total incorporation. Only MBZ significantly increases incorporation into fumarate.

7.3.2 Effects of CBZ and MBZ on the Metabolism of D[U-¹⁴C]-Glucose by *M. expansa* Under Aerobic Conditions.

The results are presented in table 7.2. The table

Table 7.1 Effects of CBZ and MBZ on the Incorporation of Radiocarbon from D[U-¹⁴C]-Glucose by *M. expansa*

Under Anaerobic Conditions (n = 4)^a.

Component	cpm x 10 ³ /g wet weight		
	Control	CBZ	MBZ
Glucose	406 ± 16	145 ± 13 ^b	143 ± 14 ^b
Malate	40 ± 2	17 ± 2 ^b	29 ± 6 ^d
Fumarate	42 ± 8	56 ± 11	58 ± 5 ^d
Succinate	507 ± 43	229 ± 19 ^b	212 ± 28 ^b
Lactate	248 ± 41	402 ± 39 ^c	489 ± 46 ^b
Alanine	14 ± 4	6 ± 1 ^d	6 ± 1 ^d
Total radioactivity*	1478 ± 98	866 ± 104 ^b	913 ± 114 ^b

^a Values are means ± SD. The results are radioactivity incorporated in 30 min at 37°C.

Tests for significance by Student's t show b,c,d significantly different from control, p<0.0005, p<0.0025, p<0.01 respectively.

* Radioactivity found in the ethanolic extract of the worms.

Incubation media contained 10 mM non-radioactive D(+) glucose.

Table 7.2 Effects of CBZ and MBZ on the Incorporation of Radiocarbon from D[U-¹⁴C]-Glucose by *M. expansa* Under Aerobic Conditions (n = 5)^a.

Component	cmp x 10 ³ /g wet weight.		
	Control	CBZ	MBZ
Glucose	244 ± 34	122 ± 18 ^b	144 ± 26 ^b
Malate	32 ± 4	36 ± 5	34 ± 6
Fumarate	54 ± 7	53 ± 8	60 ± 14
Succinate	290 ± 40	211 ± 37 ^d	211 ± 23 ^d
Lactate	223 ± 27	191 ± 25	203 ± 26
Alanine	9 ± 1	11 ± 2	8 ± 2
Total radioactivity*	905 ± 124	529 ± 80 ^b	602 ± 137 ^c

^a Values are means ± SD. The results are radioactivity incorporated in 30 min at 37°C. Tests for significance by Student's t show b,c,d significantly different from control; p<0.0005, p<0.0025, p<0.005 respectively.

* Radioactivity found in the ethanolic extract of the worms.

Incubation media contained 10 mM non-radioactive D(+) glucose.

shows that both CBZ and MBZ significantly diminish incorporation of ^{14}C into glucose and succinate pools, and that the total radioactivity incorporated is also decreased.

7.3.3 Effects of CBZ and MBZ on $^{14}\text{CO}_2$ Fixation by *M. expansa* Under Anaerobic Conditions.

The results are presented in table 7.3. The table shows that CBZ and MBZ significantly inhibit total $^{14}\text{CO}_2$ fixation and incorporation of radiocarbon into succinate. Both the drugs significantly increase incorporation into lactate.

7.3.4 Effects of the Drugs on CO_2 Fixation by *M. expansa* Under Aerobic Conditions.

The results are presented in table 7.4. The table shows that both CBZ and MBZ significantly diminish total fixation and incorporation of radiocarbon from $^{14}\text{CO}_2$ into succinate. CBZ also diminishes incorporation into malate and increases incorporation into fumarate.

7.3.5 Effects of CBZ and MBZ on the Activity of PEPCK (cytosolic) from *M. expansa*.

The results are presented in table 7.5. The table shows that CBZ significantly inhibits $^{14}\text{CO}_2$ fixation by PEPCK both anaerobically and aerobically. MBZ has no effect on the activity of the enzyme under either condition.

7.3.6 Effects of CBZ and MBZ on the Fumarate Reductase System in the Mitochondrial Preparation of *M. expansa*.

The results are presented in table 7.6. The table shows that CBZ significantly diminishes formation of radioactive succinate, citrate and alanine, and MBZ diminishes formation of radioactive succinate and citrate. Both the drugs increase the production of malate.

Table 7.3 Effects of CBZ and MBZ on $^{14}\text{CO}_2$ Fixation by *M. expansa* Under Anaerobic Conditions ($n = 3$).

cpm $\times 10^3$ /g wet weight.

Intermediate	Control	CBZ	MBZ
Malate	150 $\pm$ 7	164 $\pm$ 19	139 $\pm$ 16
Fumarate	31 $\pm$ 9	29 $\pm$ 4	19 $\pm$ 3
Succinate	1281 $\pm$ 161	805 $\pm$ 164 ^d	915 $\pm$ 155 ^e
Lactate	61 $\pm$ 13	112 $\pm$ 9 ^c	107 $\pm$ 13 ^d
Total Fixation*	1860 $\pm$ 122	1066 $\pm$ 19 ^a	1341 $\pm$ 30 ^b

* Total radioactivity found in the ethanolic extract after 30 mins incubation at 37°C.

Tests for significance by Student's t show a,b,c,d,e significantly different from the control, $p < 0.0005$, < 0.0025 , < 0.005 , < 0.0125 , < 0.025 respectively.

Table 7.4 Effects of CBZ and MBZ on $^{14}\text{CO}_2$ Fixation by *M. expansa* Under Aerobic Conditions ($n = 3$).

Intermediate	cpm $\times 10^3$ /g wet weight.		
	Control	CBZ	MBZ
Malate	27 $\pm$ 9	15 $\pm$ 4 ^e	21 $\pm$ 3
Fumarate	5 $\pm$ 1	11 $\pm$ 3 ^c	8 $\pm$ 3
Succinate	928 $\pm$ 119	591 $\pm$ 78 ^b	636 $\pm$ 32 ^b
Lactate	57 $\pm$ 14	53 $\pm$ 9	48 $\pm$ 15
Total Fixation*	1406 $\pm$ 113	817 $\pm$ 90 ^a	896 $\pm$ 305 ^d

* Total radioactivity found in the ethanolic extract after 30 mins incubation at 37°C.

Tests for significance by Student's *t* show a,b,c,d,e significantly different from control, $p < 0.0005$, < 0.0025 , < 0.005 , < 0.01 , < 0.025 respectively.

7.4 Discussion.

The effects of CBZ and MBZ on the uptake of radiocarbon from ^{14}C -glucose, shown in table 7.1 and 7.2, are very similar to the effects observed in chapter III (tables 3.1, 3.2), and can be interpreted in the same way.

It appears that the drugs inhibit ^{14}C -glucose uptake by *M. expansa* both anaerobically and aerobically, which confirms the earlier finding.

Under anaerobic conditions incorporation of radiocarbon from ^{14}C -glucose into malate, succinate and alanine is also diminished, but incorporation into lactate is greatly increased by the drugs (table 7.1). The reduced incorporation into malate may result from an inhibition in the pathway between PEP and malate. The results in table 6.1 (Ch. VI) show that CBZ and MBZ do not affect the activity of MDH, which suggests that inhibition of PEPCK activity by the drugs is probably responsible for lowering the malate pool.

Decreased production of radioactive succinate may result from inhibition of PEPCK or the FR system or both. The way(s) in which partial obstruction to the flow of carbon through the succinate pathway can result in increased lactate production has been described earlier (chapter III).

Alanine is produced through transamination of pyruvate and as this reaction is not a part of the favoured pathway in *M. expansa*, reduced incorporation into alanine may not be of any serious consequence to the parasite.

The results in table 7.2 show that incubation of the parasite under aerobic condition moderates the effects of the drugs. Lack of an effect on malate suggests that, under aerobic conditions, the drugs probably do not inhibit $^{14}\text{CO}_2$ incorporation by PEPCK. Though the

Table 7.5 Effects of CBZ and MBZ on the CO₂ Fixation by Cytosolic Preparation (PEPCK) of *M. expansa*^a.

	cpm x 10 ³		
	Control	CBZ	MBZ
Anaerobic (n = 7)	340 ± 67	188 ± 52 ^b	312 ± 78
Aerobic (n = 7)	267 ± 12	151 ± 19 ^b	256 ± 32

^a Values are means ± SD. The units are radioactivity incorporated in malate from NaH¹⁴CO₃/min per g wet weight of worm.

^b Tests for significance by Student's t show significant inhibition p<0.0005.

effect on succinate indicates reduced incorporation, yet the degree of reduction is not as great as with the anaerobic situation. Moreover, there is no increase in the amount of radioactive fumarate due to the drugs. These facts suggest that under aerobic condition the fumarate reductase system is less important than the oxidation of succinate utilizing molecular oxygen as the terminal electron acceptor (Bryant and Behm, 1976). Therefore, the effects observed anaerobically are less pronounced under aerobic conditions.

It is also evident (tables 7.1 and 7.2) that CBZ and MBZ significantly diminish total incorporation of radiocarbon from ^{14}C -glucose, both anaerobically and aerobically. Due to lack of quantitative data on the amount of radioactivity excreted into the media as lactate and succinate, it was not possible to confirm the observations in chapter V which suggested that the drugs disturbed excretion in *M. expansa*. An attempt to separate lactate and succinate from glucose in the incubation media with the help of ion-exchange resin (Amberlite - MB3, Ajax) proved unsuccessful, because considerable radioactivity due to glucose was found even in the 5th x 5 ml washing of the column. However, the spectrophotometric assays of incubation media (table 5.1 and 5.2, Ch. V) show that, under anaerobic conditions, there is accumulation of lactate within the worms treated with the drugs, but no difference in the amount excreted into the media.

It has also been observed that, because less succinate is produced by the worms treated with the drugs anaerobically, there is a decreased excretion of succinate compared with the control. Under aerobic conditions, however, an increase in succinate excretion is observed in the worms treated with the drugs, though the internal pool of succinate is reduced by the drugs. Therefore, the decreased

Table 7.6 Effects of CBZ and MBZ on the Fumarate Reductase Activity in the Mitochondrial Preparation of

M. expansa (n = 7)^a.

	cpm x 10 ³		
	Control	CBZ	MBZ
Succinate	37 ± 4	15 ± 4 ^b	23 ± 4 ^b
Fumarate	198 ± 29	184 ± 55	138 ± 7 ^b
Malate	81 ± 11	169 ± 24 ^b	224 ± 33 ^b
Citrate	34 ± 7	24 ± 7 ^c	9 ± 2 ^b
Alanine	11 ± 2	2 ± 0.6 ^b	9 ± 2

^a Values are means ± SD. The units are cpm incorporated/min per g wet weight.

Tests for significance by Student's t show b,c significantly different from control p<0.0005, <0.01 respectively.

incorporation of radiocarbon into succinate in the worms, under aerobic conditions (table 7.2), may result from increased excretion, as well as by oxidation of succinate.

The results of the experiment with $\text{NaH}^{14}\text{CO}_3$ (tables 7.3 and 7.4) suggest that both CBZ and MBZ significantly inhibit CO_2 fixation by *M. expansa* as evidenced by the total radioactivity fixed in 30 mins. The results also indicate that in *M. expansa* CO_2 fixation occurs both anaerobically and aerobically. Mettrick et al (1976) reported that anaerobic helminths can efficiently carry out their metabolic activities under aerobic conditions, and that anaerobiosis is influenced more by the pCO_2 of the environment than the pO_2 . As PEPCK is the major CO_2 fixing enzyme in *M. expansa* (Behm and Bryant, 1975a), it is most likely that the drugs inhibit the activity of the enzyme.

Once again, the diminished incorporation of radiocarbon from $^{14}\text{CO}_2$ into succinate in the worms (table 7.3) indicates an inhibition, by CBZ and MBZ, of the FR system under anaerobic conditions. The generally lower incorporation of ^{14}C into succinate found under aerobic conditions suggests increased excretion and/or oxidation of succinate.

Production of labelled lactate indicates some disturbance in the PEP-succinate pathway after the fixation of $^{14}\text{CO}_2$ from $\text{NaH}^{14}\text{CO}_3$ by PEPCK. As all the reactions in the sequence are reversible and as fumarate is a symmetrical molecule, inhibition of the FR system would result in accumulation of fumarate in the pathway, which eventually gives rise to labelled PEP (carboxyl group) and ^{14}C -lactate.

Under aerobic conditions (table 7.4) the decreased incorporation into malate due to CBZ may be the result of decreased fixation of $^{14}\text{CO}_2$, and the increase in fumarate may result from

oxidation of succinate. However, these two effects of CBZ may not be important, because malate and fumarate are linked by an equilibrium enzyme.

Further studies with the isolated PEPCK (cytosolic) reaction reveal that CBZ significantly inhibits $^{14}\text{CO}_2$ fixation both anaerobically and aerobically (table 7.5). This result confirms the observations in the foregoing sections. Table 7.5 also shows that MBZ has no effect on the activity of PEPCK in the reaction mixture. The results of the previous experiments with whole animal show that MBZ significantly inhibits ^{14}C incorporation from ^{14}C -glucose and $\text{NaH}^{14}\text{CO}_3$. Therefore, it is probable that the lack of an effect of MBZ on $^{14}\text{CO}_2$ fixation by cytosolic preparations of *M. expansa* may be due, once again, to the insolubility of the drug. The other possibility is that MBZ exerts its effects elsewhere in the pathway - probably on the FR system.

The results in table 7.6 suggest that both CBZ and MBZ significantly diminish the conversion of fumarate to succinate by the mitochondrial preparation of *M. expansa* and increase the formation of malate. Decreased incorporation of ^{14}C from fumarate into succinate indicates inhibition of the FR system. In *M. expansa*, production of succinate is coupled to the electron transport mediated phosphorylation of ATP in which fumarate acts as the terminal electron acceptor (Cheah and Bryant, 1966; Bryant 1972b). Inhibition of the FR system will therefore result not only in reduced production of succinate but also reduced generation of ATP. It has already been observed (Ch. IV) that incubation of *Moniezia* scoleces with CBZ and MBZ results in decreased production of ATP. Therefore, it is likely that some, at least, of the observed reduction in the conversion of fumarate to succinate in the reaction mixture is due to inhibition of the FR system.

It has been reported that CBZ inhibited the FR system of *Haemonchus contortus* (Malkin and Camacho, 1972; Prichard, 1973). Prichard (1973) stated that though MBZ shares some structural similarities with CBZ, a 2.5 mM suspension of MBZ did not affect the FR system of *H. contortus* possibly because of its insolubility. In *M. expansa* mitochondria, approximately the same concentration of MBZ (2.6 mM) shows significant inhibition of succinate formation. However, the suspension used in this study contained micronised MBZ, and it is known to be much more effective against parasites than the coarse particle one. It is not known whether Prichard used the micronised MBZ in his study. Therefore, there are two possibilities; either the drug used by him was not micronised or the FR system of *H. contortus* is less susceptible to MBZ than is the system in *M. expansa*.

Van den Bossche (1972b) reported that benzimidazole anthelmintics inhibited ^{32}P i incorporation into ATP by *Ascaris* mitochondria. Inhibition of phosphorylation may result from either inhibition of the electron transport system or uncoupling of phosphorylation from the electron transport chain. In case of the latter, decreased generation of ATP would be accompanied by increased rate of substrate utilization; that is, in *M. expansa*, increased formation of succinate. As CBZ and MBZ inhibit the conversion of fumarate to succinate by *M. expansa* mitochondria (table 7.6), it is assumed that the drugs inhibit phosphorylation without uncoupling.

Increased malate formation from radioactive fumarate in the presence of the drugs can be ascribed to the fumarase reaction. In mammalian tissue the enzyme functions in the direction of malate formation as a component of the TCA cycle. Cheah and Bryant (1966) have demonstrated that, in *M. expansa*, the reaction is more active in the direction of fumarate production - i.e. dehydration of malate

to fumarate, but Davey and Bryant (1969) observed that, in the homogenates of *M. expansa*, all the reactions in the PEP-succinate pathway are reversible. An inhibition of the FR system may therefore result in accumulation of NADH₂ and fumarate in the mitochondria, leading to redistribution of carbon along the PEP-succinate pathway, and thereby increased incorporation into malate is possible.

Decrease in labelled fumarate in MBZ treated incubation is reflected in increased label in the malate pool. Fumarase is generally considered to be an equilibrium enzyme, and fumarate is readily converted to malate in the reaction mixture (Prichard, 1973). Therefore, in assessing the inhibition of succinate formation, the total radioactivity in malate and fumarate should be considered together. Table 7.6 shows that there is no difference in the amount of accumulated radiocarbon (fumarate + malate) between CBZ and MBZ treated incubations.

The results in table 7.6 also show that CBZ inhibits incorporation of radiocarbon into citrate and alanine, while MBZ inhibits incorporation into citrate only. Davey and Bryant (1969) reported that homogenates of *M. expansa* incorporated radiocarbon into citrate and alanine from ¹⁴C-succinate and malate. Reduced incorporation into citrate may result from inhibition of any reaction between malate and citrate in the TCA cycle. As the presence of a functioning TCA cycle in *M. expansa* is doubtful (Davey and Bryant, 1969), the inhibition of citrate formation, due to the drugs, must remain problematical.

Alanine can be formed from transamination of pyruvate produced after decarboxylation of malate or OAA. Behm and Bryant (1975a) reported that the activity of malic enzyme was very low. It seems likely that labelled pyruvate is formed, after randomisation

of ^{14}C distribution in the PEP-succinate pathway, either by spontaneous decarboxylation of OAA or by the action of PEPCK. Decreased incorporation of radiocarbon into alanine may result from inhibition of any of these reactions.

The major conclusions to be drawn from this section are that glucose uptake and carbon dioxide fixation are inhibited by both CBZ and MBZ. The subsequent patterns of labelling are consistent with the view that the anthelmintics also inhibit the FR system in *M. expansa*.

CHAPTER VIII

EFFECTS OF CBZ AND MBZ ON THE CARBOHYDRATE METABOLISM OF

M. EXPANSA IN VIVO.

8.1 Introduction.

The results of the *in vitro* experiments show that CBZ and MBZ have marked effects at several sites on the pathway for energy metabolism in *M. expansa*. The *in vitro* effects may or may not be the actual effects *in vivo*, and may or may not be related to the anthelmintic efficacy of the drugs.

Smyth (1969) pointed out that, in biochemical studies on cestodes, one of the most challenging problems is to relate results from studies carried out *in vitro* to processes which actually occur *in vivo*. The physico-chemical conditions used *in vitro*, especially pCO_2 , pO_2 , pH and Eh, are likely to have profound influences on biochemical reactions and may be different from those encountered *in vivo*. Factors dependent upon the presence of the host create further problems which must be solved before it is possible to conclude that the effects observed *in vitro* are responsible for anthelmintic efficacy. Thus, interactions between the host and the parasite metabolism - such as, for example, the utilization of parasitic metabolic products by the host and *vice versa* - could occur *in vivo* (Smyth, 1969), and cannot be predicted from *in vitro* situations. However, whilst admitting this limitation upon results obtained *in vitro*, it should be stressed that such results produce working hypotheses, which may then be tested in the intact host-parasite system.

Therefore, to determine whether the effects exerted by CBZ and MBZ on *M. expansa in vitro* have any relationship to effects

and to anthelmintic efficacy observed *in vivo*, experiments with infected lambs were carried out. The experimental procedures and the results are described in this section.

8.2 Material and Methods.

The lambs used for the *in vivo* experiments had not previously been treated with any anthelmintic. Infected lambs were identified by faecal examinations for the presence of cestode segments. They were immediately transported to the place of experiment and drenched. Food and drinking water was given *ad lib* to all lambs during the course of the experiments.

- (a) MBZ experiment : Sixteen suckling Merino lambs, 10 to 12 weeks old and naturally infected with *M. expansa* were procured from a property near Bringelly, N.S.W. Ten lambs were treated with 'Telmin' (5% MBZ suspension sheep drench) orally at a dose rate of 10 mg/kg body weight. Six lambs were kept as untreated control.

The six control lambs were killed immediately, and *M. expansa* was collected. Five treated lambs were killed after three hours of treatment and five after six hours. Worms were collected by stripping the small intestine and sieving the contents in an 8 mesh sieve. The worms were immediately placed in ice-cold KRP. They were separated into scolex and gravid portions, rinsed in three quick changes of KRP, blotted and snap frozen in liquid nitrogen. The frozen worm tissue was pulverized in an all-steel pulverizer under liquid nitrogen and suspended in preweighed 6% (w/v) HClO_4 kept at -4°C in ice-ethanol. Weight of the worm tissue was recorded, and the tissue was homogenised in an all-glass Dounce-type homogeniser. The rest of the procedures for preparation of

samples and spectrophotometric assays were as described by Behm and Bryant (1975a).

Scolex and gravid portions of *M. expansa* were also taken for glycogen determination. A known weight of worm was homogenised in 1:1 (w/v) ice-cold distilled water and the homogenate processed for glycogen determination as described in chapter V.

In the control and 3 hours treated lambs most of the worms were found in the jejunum, whereas in the 6 hours treated lambs most of the worms were found in the ileum and in one lamb, even in the caecum. All worms used for preparation of extracts were alive (undulating).

- (b) CBZ experiment : Thirteen mixed breed lambs (Dorset Horn, Border Leicester, Merino), 12 to 14 weeks old and naturally infected with *M. expansa*, were obtained from the Bungendore area, N.S.W. Eight lambs were drenched with a 6.05% suspension of CBZ at a dose rate of 20 mg/kg body weight and five lambs were kept as untreated controls. The control lambs were killed immediately and the treated lambs were killed eight hours after treatment. Worms were collected and processed as described above. Four treated lambs and one control lamb were found to be uninfected.

The time course for the MBZ experiments was chosen in consultation with Mr R.A.F. Chevis, Ethnor Pty. Ltd. Due to difficulties in obtaining sufficient numbers of undrenched, naturally infected lambs only one time course for the CBZ experiment was possible. However, a preliminary experiment with three lambs showed that the drug had no significant effect on the metabolism of *M. expansa* at three hours after

treatment.

'Telmin RLT' was supplied by Ethnor Pty. Ltd., N.S.W. and CBZ suspension was supplied by Merck Sharp and Dohme (Aust). Pty. Ltd., N.S.W.

Only those areas of metabolism where *in vitro* effects had been observed were taken into consideration for investigation *in vivo*.

Statistical analysis of the results was performed by using one-tailed unpaired Student's *t* test.

8.3 Results.

8.3.1 Effects of MBZ on the Metabolism in *M. expansa* Scolex :

The results are presented in table 8.1. The table shows that, in worms recovered both at 3 hours and 6 hours after treatment, MBZ significantly decreases glucose, G6P, malate, lactate and succinate concentrations. The effects at 6 hours are generally more pronounced than at 3 hours after treatment. The drug also reduces the glycogen content of the scolex after 6 hours of treatment.

8.3.2 Effects of MBZ on the Metabolism in the Gravid Proglottids of *M. expansa* :

The results are presented in table 8.2. The table shows that MBZ significantly decreases glucose, malate, lactate and succinate pools in the gravid portion of the worm. The effects at 6 hours are, in general, more pronounced than the effects at 3 hours after treatment. The drug has no significant effect on the glycogen content of the gravid proglottids of the worm.

8.3.3 Effects of MBZ on the Energy Metabolism in the Scolex of *M. expansa* :

The results are presented in table 8.3. The table shows

Table 8.1 Effects of MBZ on the Carbohydrate Metabolism in the Scolex of *M. expansa* in vivo^a.

	Control (n=6)	3 Hours (n=5)	6 Hours (n=5)
Glucose	2516 ± 395	1159 ± 337 ^b	181 ± 103 ^b
Glucose-6-phosphate	190 ± 78	103 ± 27 ^d	100 ± 62 ^e
Fructose-6-phosphate	38 ± 30	15 ± 11	18 ± 22
Malate	1985 ± 259	1447 ± 129 ^c	617 ± 220 ^b
Lactate	8818 ± 3260	1899 ± 965 ^c	2304 ± 1180 ^c
Succinate	19371 ± 2080	11805 ± 4744 ^d	8678 ± 3296 ^b
Total end-product	28189 ± 3012	13713 ± 4666 ^b	10982 ± 4008 ^b
Glycogen*	113228 ± 19874	99349 ± 42630	67202 ± 38431 ^e

^a Units are nmoles/g wet weight.

Tests for significance by Student's t show b,c,d,e significantly different from control, p<0.0005, <0.0025, <0.01, <0.025 respectively

* Presented as glucosyl units.

Table 8.2 Effects of MBZ on the Carbohydrate Metabolism in the Gravid Proglottids of *M. expansa* in vivo (n = 5)^a.

	Control	3 Hours	6 Hours
Glucose	2948 ± 1915	516 ± 212 ^e	269 ± 88 ^d
Glucose-6-phosphate	203 ± 65	160 ± 66	156 ± 24
Fructose-6-phosphate	27 ± 8	27 ± 15	26 ± 10
Malate	1745 ± 139	1096 ± 149 ^b	799 ± 268 ^b
Lactate	7054 ± 1636	733 ± 372 ^b	1597 ± 1074 ^b
Succinate	17114 ± 2695	11740 ± 2901 ^e	7887 ± 2585 ^b
Total end-product	24167 ± 1830	12473 ± 2534 ^b	9484 ± 1550 ^b
Glycogen*	150922 ± 71358	127814 ± 59003	101508 ± 51376

^a Units are nmoles/g wet weight.

Tests for significance by Student's *t* show b,d,e significantly different from control, $p < 0.0005$, $p < 0.01$, $p < 0.025$ respectively.

* Presented as glucosyl units.

that MBZ significantly diminishes ATP, ADP and total adenine nucleotides levels in the scolex and gravid proglottids of *M. expansa* both at 3 hours and 6 hours after the treatment. The effects at 6 hours are, generally, more pronounced than those at 3 hours after treatment.

8.3.4 Effects of CBZ on the Metabolism in *M. expansa* :

The results are presented in table 8.4. The table shows that CBZ significantly decreases glucose and succinate pools in the scolex, and glucose, pyruvate and succinate pools in the gravid portion. The drug significantly increases the lactate pool in both the scolex and the gravid portions of the worm, but decreases total end-product only in the gravid segments. CBZ significantly diminishes the glycogen content in both the scolex and the gravid segments.

8.3.5 Effects of CBZ on the Energy Metabolism in *M. expansa* :

The results are presented in table 8.5. The table shows that CBZ significantly diminishes ATP, ADP and total adenine nucleotides levels in both the scolex and the gravid portion of the worm 8 hours after treatment.

8.4 Discussion.

A comparison of the results in chapters III, IV and V with those in tables 8.4 and 8.5 reveals that the effects of CBZ on the metabolism of *M. expansa* are similar *in vitro* and *in vivo*. Also the majority of the *in vitro* effects of MBZ are reproduced *in vivo* (tables 8.1 and 8.3). For a summary, see table 8.6.

With MBZ, the exceptions are decreased production of lactate (with a consequent effect on total end-products), malate and ADP *in vivo*. However, the lactate effect was observed in *in vitro* anaerobic incubations in the absence of external glucose. With CBZ,

Table 8.3 Effects of MBZ on the Energy Metabolism of *M. expansa* in vivo^a.

Scolex:	Control (n=6)	3 Hours (n = 5)	6 Hours (n = 5)
ATP	899 ± 261	529 ± 236 ^e	339 ± 83 ^c
ADP	700 ± 191	431 ± 91 ^d	410 ± 95 ^d
AMP	181 ± 43	140 ± 72	163 ± 42
Total nucleotides	1780 ± 445	1099 ± 189 ^d	912 ± 179 ^c
Gravid (n = 5):			
ATP	1276 ± 201	715 ± 165 ^c	502 ± 67 ^b
ADP	706 ± 217	364 ± 98 ^d	417 ± 76 ^e
AMP	175 ± 96	109 ± 39	99 ± 41
Total nucleotides	2157 ± 493	1188 ± 276 ^c	1018 ± 101 ^c

^a Units are nmoles/g wet weight.

Tests for significance by Student's t show b,c,d,e significantly different from control, p<0.0005, <0.0025, <0.01, <0.025 respectively.

exceptions are the absence of an effect on the malate pool size and the depressed ADP concentration. The importance of these differences is hard to determine, in view of the multiple effects of the drugs.

The increase in the production of lactate caused by MBZ in anaerobic incubations *in vitro*, when glucose is present, has been explained by a transfer of carbon flux from the succinate to the lactate producing pathway (Ch. III). The carbon flux is apparently sustained from the external glucose source offered to the worm. This source is absent *in vivo*, but even after 6 hours exposure to the drugs *in vivo* there are considerable amounts of glycogen remaining in the worms, though its concentration is depressed compared with the control. It must follow, therefore, that the rate of mobilisation of the glycogen is insufficient to maintain energy metabolism, in the presence of MBZ. This may be an effect exerted directly upon the process of glycogen mobilisation or indirectly, through the effect upon ATP concentrations. If ATP concentrations drop below the levels at which they are required to act as substrates then carbon flux will be reduced. E.A. Newsholme (personal communication) describes this process as a loss of 'kinetic efficiency'.

ATP/ADP ratios and the adenylate energy charge are often good indicators of the energy status of an organism or a tissue. The ATP/ADP ratio indicates the high-energy phosphorylation state of the tissue and the adenylate energy charge (Atkinson and Walton, 1967) is a useful index of the energy stored in the adenylate system. However, the two indicators are only valuable when the total adenine nucleotide levels within the tissue remain unchanged. In the experiments described here not only are total adenine nucleotides decreased by the CBZ and MBZ treatments, but so also are ATP levels (tables 8.3 and 8.5). The ratios are therefore not helpful in

Table 8.4 *In vivo* Effects of CBZ on the Carbohydrate Metabolism in *M. expansa* (*n* = 4)^a.

	Scolex		Gravid	
	Control	Treated	Control	Treated
Glucose	3369 ± 440	1953 ± 321 ^c	3233 ± 626	1820 ± 389 ^d
Glucose-6-phosphate	113 ± 22	133 ± 30	196 ± 2	158 ± 36
Fructose-6-phosphate	23 ± 4	25 ± 11	49 ± 7	39 ± 15
Pyruvate	103 ± 21	138 ± 51	133 ± 21	60 ± 10 ^c
Malate	2536 ± 792	2116 ± 310	882 ± 253	1021 ± 232
Lactate	4445 ± 651	9509 ± 1085 ^b	2803 ± 161	4834 ± 605 ^c
Succinate	14100 ± 2559	6757 ± 1030 ^c	11439 ± 2261	5487 ± 1001 ^c
Total end-product	18545 ± 2229	16281 ± 2005	14242 ± 2212	10321 ± 505 ^e
Glycogen*	89896 ± 6183	45786 ± 8565 ^b	113112 ± 7180	78527 ± 15070 ^d

^a Units are nmoles/g wet weight.

Tests for significance by Student's *t* show b,c,d,e significantly different from control, *p*<0.0005, <0.0025, <0.005, <0.025 respectively.

* Presented as glucosyl unit.

ascertaining the phosphorylation state or the energy status of the treated worms.

The effects on glucose uptake, glycogen utilization, succinate production, ATP and total adenine nucleotides levels in the scolex are similar *in vitro* and *in vivo*. The interpretations given in chapters III, IV and V may therefore be taken as applying to the *in vivo* experiments.

Additional information obtained from the *in vivo* experiments relates to the effects of the drugs on the carbohydrate metabolism in the gravid proglottids of *M. expansa*. Table 8.2 shows that the effects of MBZ on the gravid proglottids of the worm are essentially the same as on the scolex, except that there is no significant decrease in the glycogen level. There exists a certain amount of controversy as to whether a glycogen gradient occurs along the strobila of adult cestodes (von brand, 1973). Though the majority of the authors are of the opinion that the posterior end of the strobila contains less glycogen than the scolex region, an inspection of the values in tables 8.1, 8.2 and 8.4 would indicate that the gravid proglottids of *M. expansa* contain as much as, if not more glycogen than the scolex. The results in table 8.2 suggest that the glycogen reserve in the gravid proglottids has not been used, though there is marked decrease in the glucose pool, in the presence of MBZ.

There is no adequate information as to the size of the glucose pool in different regions of the strobila of *M. expansa*. Goodchild (1961) reported that the glucose concentration was highest in the posterior third, lowest in the middle third and intermediate in the anterior third of *Hymenolepis diminuta*. The findings of the present study (tables 8.1, 8.2 and 8.4) suggest

Table 8.5 *In vivo* Effects of CBZ on the Energy Metabolism of *M. expansa* (n = 4)^a.

	Scolex		Gravid	
	Control	Treated	Control	Treated
ATP	697 ± 37	556 ± 92 ^e	1011 ± 121	665 ± 83 ^c
ADP	476 ± 34	272 ± 80 ^d	605 ± 64	341 ± 96 ^c
AMP	106 ± 53	41 ± 17	84 ± 12	68 ± 21
Total nucleotides*	1279 ± 69	868 ± 167 ^d	1699 ± 184	1074 ± 150 ^b

^a Units are nmoles/g wet weight.

Tests for significance by Student's t show b,c,d,e significantly different from control, p<0.0005, <0.0025, <0.005, <0.025 respectively.

* Total of ATP, ADP and AMP.

that there is no difference between the size of glucose pools in the scolex and the gravid portion of *M. expansa*. Therefore, the reduced glucose pool observed in the gravid proglottids in the presence of the drugs may result from an inhibition of uptake as found in the scolex. The fact that the glycogen reserve has not been utilized is probably because a great proportion of the glycogen reserve in the gravid proglottids is intended for the development and maintenance of the embryos, and is not available for the energy metabolism for the strobila.

In the absence of glycogen mobilisation compensating for the reduced glucose uptake, the flux through the pathway of energy metabolism is diminished, as evidenced by decreased end-products (table 8.2). A decreased malate pool (table 8.2) may be ascribed to an inhibition of CO₂ fixation at the PEPCK level.

In CBZ treatment (table 8.4), glycogen levels as well as the flux through the pathway are significantly decreased in the gravid proglottids. The situation suggests once again that the reduced glucose uptake has not completely been replaced by mobilisation of reserve glycogen, possibly due to lower metabolic capacity of the gravid regions.

The mature segments of cestodes contain large numbers of ova and as they become more 'ripe', they remain loosely attached to the strobila and ultimately get detached from it. It is therefore likely that the gravid proglottids are less active metabolically, which also suggests that the stored glycogen in them is not rapidly mobilised in response to the decreased glucose uptake.

From the results of *in vivo* experiments it may be concluded that inhibition of glucose uptake leads to increased utilization of reserved glycogen in the scolex of *M. expansa*. The

Table 8.6 Comparison Between *in vitro* and *in vivo* Effects of CBZ and MBZ on the Metabolism of *M. expansa*.

	CBZ		MBZ	
	<i>In vitro</i> (N ₂)	<i>In vivo</i>	<i>In vitro</i> (N ₂)	<i>In vivo</i>
Glucose	↓	↓	↓	↓
Lactate	↑	↑	↑	↓
Malate	↓	↔	↔	↓
Succinate	↓	↓	↓	↓
Total end-product	↔	↔	↔	↓
Glycogen	↓	↓	↓	↓
ATP	↓	↓	↓	↓
ADP	↔	↓	↔	↓
AMP	↔	↔	↔	↔
Total adenine nucleotides	↓	↓	↓	↓

↓ = Decreased, ↑ = Increased, ↔ = No change.

drugs also decrease the flow through the succinate pathway, perhaps by inhibiting PEPCK activity and the FR system. As a consequence, CBZ and MBZ diminish the generation of ATP by inhibiting both substrate linked phosphorylation, at the PEPCK reaction, and the electron transport mediated phosphorylation at the FR system. The drugs may also disturb the turnover of adenine nucleotides.

It is also evident that CBZ and MBZ exert similar effects on the carbohydrate metabolism in gravid proglottids of *M. expansa* but MBZ significantly affects the process as early as three hours after treatment of the infected host.

As the *in vitro* effects are largely reproduced *in vivo*, it is concluded that they are associated with the anthelmintic efficacy of the compounds. The *in vivo* effects of CBZ and MBZ are very similar to the effects exerted by the drugs under anaerobic conditions *in vitro*. This provides indirect evidence for the conclusion that the anaerobic metabolic pathway is preferred *in vivo*.

CHAPTER IX

GENERAL DISCUSSION AND CONCLUSIONS

The results of the present study show that CBZ and MBZ have multiple effects on the respiratory metabolism of *M. expansa*. As the processes of energy metabolism are complex, it is difficult to ascertain which are primary, secondary or tertiary effects. It is also difficult to relate these effects to anthelmintic efficacy. It is doubtless true that the combination of effects contributes to anthelmintic efficacy and that only a very simplistic view would demand that a specific, single action be responsible for killing the parasite *in vivo*.

There are logical explanations for many of the effects observed *in vitro* and *in vivo*, but in some areas the available information is not yet sufficient to permit correlation of *all* observed effects. The results do, however, permit the erection of hypotheses, from which tentative conclusions may be drawn.

Inhibition of glucose uptake may or may not be a primary effect of the drugs. Glucose uptake in cestodes is a physiological process requiring energy. If the energy level in the worm is greatly reduced, it follows that there could be impairment of the capacity of the parasite to absorb glucose from the medium in which it is incubated. In living organisms, the source of energy is the hydrolysis of the terminal bond of ATP. If the level of ATP in the worm is reduced below a certain critical concentration (what that concentration is remains as yet unknown) there will be a concomitant reduction in glucose uptake. Not only does ATP represent energy immediately available to the parasite, it is also an allosteric regulator of metabolism and a substrate for some important enzymatic reactions. When ATP concentrations drop to the level that

they are rate limiting in these reactions, 'kinetic efficiency' is lost. On the basis of these arguments, inhibition of glucose uptake could be a secondary effect of the drugs.

On the other hand, the absorption and transport of glucose across the tegument requires not only energy but also the structural integrity of the absorptive surface. It has recently been observed (literature cited earlier) that benzimidazole compounds cause irreparable damage to the cells of the absorptive surface of helminths, including cestodes. This structural damage may result directly in decreased glucose absorption. On the basis of this argument it is possible to conclude that inhibition of glucose uptake is the primary effect of CBZ and MBZ.

However, a further complication arises. Electron microscopy of the absorptive cells of the cestode tegument indicates that microtubules are damaged (Verheyen *et al*, 1976). Microtubules are responsible for intracellular transportation of nutrients. ATP is necessary for the maintenance of this process and for the maintenance of the structural integrity of the microtubules themselves. Degeneration of the microtubules will result from an insufficient amount of available ATP. It then follows that the primary effect of the drugs is on the energy level, not on glucose uptake. However, further thorough studies are needed to arrive at a definite conclusion.

Another major effect of the drugs is the reduced production of succinate. Reduced glucose uptake implies decreased carbon flux in the pathway of energy metabolism and hence decreased succinate production - providing that there is no alternative source of carbon. However, it has been observed (Ch. V) that both CBZ and MBZ diminish glycogen reserves in the worm. It has also been observed that the carbon flux is largely maintained though the amount of glucose available to the worm is greatly reduced. From this, it may be concluded that the flow of carbon

is maintained by increased utilization of carbohydrate reserves to compensate for the diminished glucose uptake. When worms are incubated in glucose-free media, it is still observed that the drugs decrease succinate production (table 3.3, Ch. III). Therefore, it must be that the diminished production of succinate is not due to the inhibitory effect of the drugs on glucose uptake, but rather to decreased flow of carbon through the PEP-succinate branch of the pathway.

Experiments with $^{14}\text{CO}_2$ show that CBZ and MBZ decrease the total amount of radiocarbon recovered in the PEP-succinate portion of the pathway. They also inhibit the formation of ^{14}C -succinate from ^{14}C -fumarate in mitochondrial preparations from *M. expansa*. These observations together suggest that the PEPCK and FR reactions are inhibited. This is partly confirmed by the observation that CBZ inhibits the activity of cytosolic PEPCK, although MBZ has no effect. The lack of effect of MBZ is most probably due to its insolubility (table 7.5, Ch. VII).

Thus, CBZ inhibits both CO_2 fixation and the FR system of *M. expansa*. MBZ also inhibits the FR system, and though, under certain conditions, the drug inhibits ^{14}C incorporation into malate, it is not possible to arrive at the definite conclusion that PEPCK activity is affected by the drug. However, the reduction *in vivo* of the malate pool by MBZ (table 8.1, Ch. VIII) lends a little more support to this hypothesis.

Decreased production of succinate is accompanied by increased formation of lactate (tables 3.1 and 3.4, Ch. III; tables 7.1 and 7.3, Ch. VII). As the drugs have no effect on PK and LDH (tables 6.1 and 6.2, Ch. VI), it appears that inhibition of carbon flow to the succinate producing pathway has resulted in a shift of flow from the PEP-succinate pathway to the PEP-lactate pathway.

The PEPCK and the FR reactions result in synthesis of

nucleoside triphosphates. If these reactions are inhibited, then decreased ATP levels will ensue unless the parasite has an alternative compensating pathway. CBZ and MBZ decrease ATP levels, probably as a result of reduced phosphorylation rather than increased utilization, as ADP levels remain low. Total adenine nucleotides are also depressed which implicates some effect on the turnover of adenylates. All these considerations lead to the conclusion that the effect on adenine nucleotides may be the primary one.

Under anaerobic conditions lactate accumulates in the worms in the presence of CBZ and MBZ, but there is no change in the amount excreted into the media. With succinate, however, the decreased production brought about by the drugs is accompanied by diminished excretion (table 5.1, Ch. V). Under aerobic conditions, the drugs do not affect lactate excretion, but excretion of succinate is increased due to CBZ treatment (table 5.2, Ch. V). An inspection of the data in these two tables shows that the amount of lactate excreted into the media is fairly constant both anaerobically and aerobically. This implies that the lactate excretory mechanism is saturated, and that it cannot cope with the excess production of lactate, under anaerobic conditions, resulting from the inhibition of flow in the succinate pathway caused by the drugs. The net result of these interactions is that the drugs bring about the accumulation of lactate in *M. expansa*. Lactate is a metabolic waste product and may be toxic to the parasite, because it is a reduced product and its accumulation is likely to impair the redox balance of the cytosol. Accumulation of lactate is definitely a bad prognosis for the survival of the parasite.

Decrease in the excretion of succinate under anaerobic conditions, in the presence of the drugs, follows decreased production of succinate. It is, at present, impossible to explain the increased excretion of succinate which occurs in the presence of CBZ under aerobic conditions;

further investigations are necessary. However, it is clear that the excretion kinetics of succinate may be different from those of lactate, because the former is a mitochondrial product whereas the latter originates in the cytosol.

The results in chapter VI show that whenever CBZ is added to the reaction mixtures, it inhibits the activities of HK and PFK. Both CBZ and MBZ inhibit the activities of the enzymes when homogenates are incubated with them, but have no effect when worms are incubated. This observation must cast some doubt on the contribution that inhibition of HK and PFK makes to anthelmintic action *in vivo*. It is an area which requires further investigation. The drugs do not affect the activities of PK, LDH and MDH under any conditions, and have no effect on the activities of the glycolytic enzymes purchased from Boehringer. It follows that HK and PFK of *M. expansa* have different properties from those from the commercial source. Although the effects of CBZ and MBZ on HK and PFK from *M. expansa* are not consistent under all conditions, it is possible that the differences in the characteristics of helminth enzymes may, in future, be exploitable in the formulation of new and effective anthelmintics.

The *in vivo* experiments (Ch. VIII) support the suggestion that CBZ and MBZ reduce the concentration of ATP below its kinetically efficient level by inhibiting phosphorylation. They also show clearly that the pool size of glucose within the worm, and the glycogen reserves, are severely depleted by the drugs. The observations are quite consistent with the *in vitro* effects and offer the possibility that *in vitro* studies may provide an additional tool in the evaluation and development of anthelmintics. It is also apparent from these studies that levels of adenine nucleotides may provide an index for anthelmintic efficacy. Energy metabolism is concerned with the maintenance of ATP

levels; it is absolutely essential for the survival of the organism and possesses many safeguards and control mechanisms. When it is observed that ATP levels are depressed, one may conclude that the organism is stressed beyond its capacity to adapt. However, the stress may be exerted directly upon the pathway of energy metabolism, or at a system remote from it so that its capacity to supply ATP to that system is overtaxed. It is, therefore, hard to arrive at a definite conclusion about "primary" effects of the drugs. Certainly, the effect on ATP synthesis is a possibility; but it is more probable that anthelmintic efficacy derives from a combination of effects, some of which are described in this thesis.

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Appendix I: Soluble Cambendazole (salt).

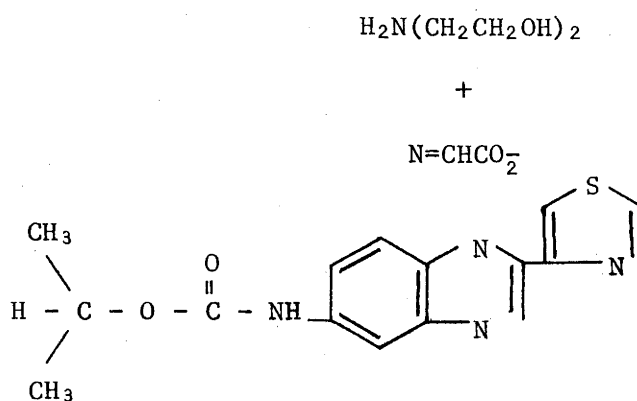
MSD No. L-619, 187

Molecular formula : $C_{20}H_{26}N_6O_6S$

Chemical name :

1-carboxymethyleneamino-5-isopropoxycarbonylamino-2(4-thiazolyl)
benzimidazole, diethanolamine.

Structure :



Properties :

Yellowish, crystalline powder, readily soluble in distilled water.

The compound is unstable when stored for extended periods and at high temperatures. A 50% solution in distilled water has a pH of 7.1 to 7.2.

32 mg of this compound = 20 mg pure cambendazole.

Molecular weight : 478.5

Appendix II: Composition of Incubation Medium.

(1) Krebs-Ringer Phosphate Solution (KRP).

Solution	Mixed in Proportion
0.154 M NaCl	100
0.154 M KCl	4
0.07 M CaCl ₂ *	3
0.154 M KH ₂ PO ₄	1
0.154 M MgSO ₄	1
0.1 M Na ₂ HPO ₄ -HCl, ph 7.4	21
(2) Cambendazole (when added)	0.08%
(3) Mebendazole (when added)	0.08%
(4) 'Tween 80'	0.1%
Final pH: 7.4	

* 0.07 M CaCl₂ was used instead of 0.11 M, because the higher concentration of CaCl₂ resulted in precipitation of calcium phosphate which was found to enhance sedimentation of MBZ particles in the incubation medium. No adverse effect on *M. expansa* metabolism was observed due to this reduction in CaCl₂ concentration.

Appendix III. Effect of DMSO on $^{14}\text{CO}_2$ Fixation by *M. expansa* from $\text{NaH}^{14}\text{CO}_3$ (sp. act. 0.1 mCi/ mmole)

[n = 2].

cpm x 10^6 / g wet weight fixed in 60 mins.

Incubation*	Fraction		Medium (excretion)**
	Soluble	Pellet	
KRP $\bar{c}$ 10 mM glucose	2.318	0.424	0.315
+ 0.1% DMSO	1.550	0.309	0.303
+ 0.1% DMSO + 2.4% MBZ	1.569	0.291	0.264
+ 0.1% DMSO + 2.4% CBZ	1.421	0.261	0.348

* Five scoleces were pooled for each incubation. After incubation the worms were washed in three quick changes of KRP, homogenised in 1:1(w/v) 80% ethanol and centrifuged at 12000 x g for 20 mins. The supernatant (soluble fraction) was removed and the pellet dried in the fume cupboard. Dried pellets were then washed in 80% ethanol. 10 μl aliquots were taken in counting vials containing 20 μl 1N HCl, evaporated to dryness and counted with 10 ml scintillant.

** cpm x 10^6 / ml of incubation medium.

Appendix IV. Effect of 'Tween 80' on $^{14}\text{CO}_2$ Fixation by *M. expansa* from $\text{NaH}^{14}\text{CO}_3$ (sp. act. 0.1 mCi/ mmole)

[n = 3].

cmp x 10^3 /g wet weight fixed in 60 mins

Incubation	Fraction		
	Soluble	Pellet	Medium*
KRP c 10 mM glucose	2241 ± 375	421 ± 103	502 ± 180
+ 1% Tween 80	2240 ± 274	488 ± 73	504 ± 102
+ 1% Tween 80 + 0.08% CBZ	1205 ± 57 ^a	253 ± 63 ^b	403 ± 13
+ 1% Tween 80 + 0.08% MBZ	1370 ± 35 ^c	325 ± 32 ^d	502 ± 70

* units are cpm excreted/ ml of media in 60 mins.

a,b,c,d Percent inhibition, 46, 40, 39, 23 respectively.

Method as described in appendix III.

Appendix V : Suppliers of Biochemicals.Boehringer Mannheim, Germany

All enzymes except those listed below, as $(\text{NH}_4)_2\text{SO}_4$ suspensions

ADP, disodium salt

ATP, trisodium salt

CoA, Grade I

F6P, disodium salt

GDP, trilithium salt

GTH

GTP, trisodium salt

2,3 diPGA.

ITP, trisodium salt

NAD^+ , free acid

NADH, disodium salt

NADP, disodium salt

PEP, potassium salt

Trieth.-HCl buffer

Calbiochem (Australia)

Pronase, B grade

Commonwealth Serum Laboratories, Melbourne

B.S.A., Cohn fraction V

Radiochemical Centre, Amersham

$\text{NaH}^{14}\text{CO}_3$

D[U- ^{14}C]-glucose

^{14}C -U-malic acid

[2,3- ^{14}C] Fumaric acid

[2,3- ^{14}C] Succinic acid

L-[U- ^{14}C] Lactic acid

NCS

PCS

Sigma Chemical Co., St. Louis

FDP, tetrasodium salt

G6P, dipotassium salt

IDP, sodium salt

Imidazole, Grade I

2-PGA, sodium salt

3-PGA, sodium salt

Succinic thiokinase, lyophilised preparation

Pyruvate, sodium salt

'Tween 80'

AMP, sodium salt

EGTA

L-(+) Lactic acid, crystalline, Grade L-I

Malic acid, crystalline

Appendix VI : Media Used in Preparation of Mitochondria.

(from Cheah, 1971)

Medium A (pH 7.4)

KCl	100 mM
Tris-HCl	50 mM
ATP	1 mM
MgCl ₂	5 mM
EDTA	1 mM
Pronase	2 mg/2.5 ml solution

Medium B (pH 7.4)

KCl	100 mM
Tris-HCl	50 mM
ATP	0.2 mM
MgCl ₂	1 mM
EDTA	0.2 mM
BSA	0.5%