A STUDY OF DRUG ACTION ON

FASCIOLA HEPATICA (TREMATODA)

by

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of

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This thesis is my own work; the experimental work is mine except where specifically acknowledged.

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ABSTRACT

An in vitro study was carried out to examine the effects of three anthelmintics, mebendazole, nitroscanate and rafoxanide, on the glucose metabolism of Fasciola hepatica. Initial incubations showed that flukes utilise glucose and maintain energy production for at least 48 h in vitro. There were indications that accommodation to the new conditions occurred. The three drugs produced marked effects on the adenine nucleotide concentrations when included in the in vitro incubations. There were also effects of the drugs on the concentrations of some of the metabolic intermediates and the excretion of end products.

In vivo studies were carried out with MBZ, NSC and RFX to determine whether they produce similar effects in flukes within their host. Most of the parasites in the drug treated sheep had detached from the bile duct walls and had empty caeca. Many also had necrotic posterior portions. Histological examination showed that the outer tegumental layer was absent from these areas. The adenine nucleotide levels of the flukes were also disturbed, in particular, the ATP concentrations were depressed. Other changes in the glycogen reserves and the internal intermediate levels probably result from the low ATP concentration and the lack of feeding. It is suggested that the drugs have a major effect on the adenine nucleotide metabolism of F. hepatica which is responsible for the detachment of the flukes, the cessation of feeding and their inability to maintain structural integrity.
Some drug-specific effects were apparent following \textit{in vitro} and \textit{in vivo} treatment. RFX caused an increase in succinate excretion which may reflect an increase in formation or a decrease in utilisation. MBZ caused a decrease in the total adenine nucleotide concentration of \textit{F. hepatica} on prolonged contact which may indicate an effect on adenine nucleotide turnover or synthesis.

The effects of three other drugs, bromophenophos, a polymorph of MBZ and a disulphonamide on \textit{F. hepatica} \textit{in vivo} were examined. They also had major effects on the adenine nucleotide levels. It is suggested that adenine nucleotides are good indicators of anthelmintic efficacy and simple \textit{in vitro} and \textit{in vivo} studies, like those described, may be used as an additional means for screening anthelmintics and could provide some information on their mode of action.
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20 SCANNING ELECTRON MICROGRAPH OF THE ORAL SUCKER

21 SCANNING ELECTRON MICROGRAPH OF THE VENTRAL SUCKER
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>DDBA</td>
<td>2,6-dihydroxy-3,5-dichlorobenzoic-4'-chloroanilide</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetate</td>
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<tr>
<td>2,3-diPGA</td>
<td>2,3-diphosphoglycerate</td>
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<td>F6P</td>
<td>fructose 6-phosphate</td>
</tr>
<tr>
<td>FDP</td>
<td>fructose 1,6-diphosphate</td>
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<tr>
<td>GlP</td>
<td>glucose 1-phosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose 6-phosphate</td>
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<tr>
<td>G3P</td>
<td>glyceraldehyde 3-phosphate</td>
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<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
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<tr>
<td>K'</td>
<td>apparent equilibrium constant</td>
</tr>
<tr>
<td>MBZ</td>
<td>mebendazole</td>
</tr>
<tr>
<td>MBZ-Poly C</td>
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<tr>
<td>µCi</td>
<td>microCurie</td>
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<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
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<tr>
<td>OAA</td>
<td>oxaloacetate</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic orthophosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
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<tr>
<td>PGA</td>
<td>phosphoglycerates</td>
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<tr>
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<td>PPI</td>
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<tr>
<td>P/A</td>
<td>propionate/acetate ratio</td>
</tr>
<tr>
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<td>rafoxanide</td>
</tr>
<tr>
<td>TBZ</td>
<td>thiabendazole</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
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<tr>
<td>TP</td>
<td>triose phosphates</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine 5'-diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
<tr>
<td>°</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>ABBREVIATION</td>
<td>NAME USED</td>
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<td>--------------</td>
<td>--------------------------------</td>
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<tr>
<td>ALD</td>
<td>aldolase</td>
</tr>
<tr>
<td>FDPase</td>
<td>fructose 1,6-diphosphatase</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MDH</td>
<td>malate dehydrogenase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PFK</td>
<td>phosphofructokinase</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>SDH</td>
<td>succinate dehydrogenase</td>
</tr>
<tr>
<td>TPI</td>
<td>triose phosphate isomerase</td>
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CHAPTER 1

A. GENERAL INTRODUCTION

THE BIOLOGY OF FASCIOLA HEPATICA

1.1 THE PARASITE

_Fasciola hepatica_ Linnaeus 1758, is a digenetic trematode known as the common liver fluke. It is the most widespread species of liver fluke and is a parasite of the bile ducts of many mammals including sheep, cattle, goats, horses, pigs, marsupials, rodents, carnivores and primates. Human infections with _F. hepatica_ are cosmopolitan in distribution and are of increasing importance in Latin American countries, France and Algeria. Sheep and cattle raising areas are the primary zones in which human infections are found and there is considerable economic loss in these areas due to fascioliasis (Cheng, 1973).

The adult fluke has a flat, often leaf-shaped appearance with the anterior end forming a conical projection. It is 20-30 mm long and 8-13 mm broad. The oral sucker and the mouth are at the tip of the anterior end and the ventral sucker is medially placed at the base of the 'neck'.

The mouth leads into the pharynx then a short oesophagus. The oesophagus forks to form two wide intestinal caeca. Both caeca are extensively branched, producing an elaborate network of diverticula throughout most of the body.
A branched ovary lies toward the right side, a short distance posterior to the ventral sucker, with the coils of the uterus between them. The testes are extensively branched, filling most of the body behind the ovary. Numerous vitelline glands extend backward along the sides of the body from the 'shoulders' to the end of the body where they are confluent behind the testes (Olsen, 1962).

1.2 LIFE CYCLE

The life cycle of *F. hepatica* comprises several morphologically distinct stages and two hosts are involved - a snail and a mammal. Large quantities of eggs are produced by mature flukes and are deposited in the branches of the biliary system of the vertebrate host. They pass through the common bile duct into the duodenum and are carried from the body in the faeces in an unembryonated state. Those falling in water develop and hatch in 9-10 days at summer temperatures. At low temperatures development is retarded and, if temperatures remain too low for development, the eggs are able to survive for several years. Inside the egg the first larval stage develops - the miracidium.

The miracidia are ciliated and have a proboscis-like structure at the anterior end and two semi-lunar eyespots. They are capable of living for up to 24 h in water. Upon finding a suitable snail host they penetrate it and transform into mother sporocysts. The major snail intermediate host in Australia is *Lymnaea tomentosa*.

As the sporocyst grows within the snail, new and different larvae develop inside it; these are the rediae. They are mobile and break free by rupturing the wall of the mother sporocyst. The rediae
feed on the snail liver and give rise to daughter rediae. Each one of these may again repeat the process, growing and producing a further generation of rediae. Eventually they produce still another type of larva, the cercaria, which leaves the snail through the pulmonary cavity 5-7 weeks after the snail becomes infected.

The cercariae are 250-350 μm long and have a long tail. The body wall is filled with cystogenous glands. In the water the cercariae are free-swimming and quickly attach to objects, particularly plants, drop their tails and secrete a cyst about themselves. When encystment is completed they are infective and are called metacercariae.

Infection of the vertebrate host follows ingestion of metacercariae with the herbage. Excystment occurs in the duodenum and the young flukes burrow through the intestinal wall into the coelom and then wander over the peritoneum. Some reach the liver and immediately penetrate it. There is a period of growth and migration in the liver after which they enter the bile ducts and remain there usually for the duration of the host's life. The time taken from infection to production of eggs by mature flukes is about eight weeks in sheep (Olsen, 1962).

1.3 EPIDEMIOLOGY

The epidemiology of fascioliasis is inseparably associated with the life cycle of the intermediate host. Throughout Australia the freshwater snail _L. tomentosa_ is the intermediate host and it may be found in shallow water around the edges of slow running streams or pools, boggy pastures and irrigation channels. It occurs in all states
of Australia except Western Australia and the Northern Territory.

The occurrence of *Lymnaea columella* has recently been reported in New South Wales (Ponder, 1975). It is assumed that the snail is a recent introduction to the Sydney area (and presumably to Australia) because the freshwater molluscan fauna has been extensively sampled regularly. *L. columella* is an eastern North American species and is a well known intermediate host of *F. hepatica*. It has been introduced into Central America, Cuba, west North America, South Africa and Europe and has recently been recognised in New Zealand (Pullan, 1969). There has been a reported increase in the number of liver fluke infections in New Zealand following the introduction of *L. columella*. Its rapid spread has greatly complicated fluke control programmes because its different ecological requirements enable it to colonise areas in which *L. tomentosa* is absent. *L. columella* is usually submerged whereas *L. tomentosa* prefers to be at least partly exposed (Pullan, 1969; Ponder, 1975).

1.4 HABITAT

The physico-chemical conditions of a habitat - e.g. pH, oxygen tension, carbon dioxide concentration, redox potential, temperature - are of major importance in determining whether it is a suitable environment for a parasite. Information about the exact location of a parasite and the conditions experienced by it is necessary for a full understanding of the host-parasite relationship and, in particular, the way in which the parasite is able to exploit the host in order to survive.

Adult *F. hepatica* inhabit the bile ducts of their hosts.
The majority of flukes are found in the larger bile ducts, usually firmly attached and with their oral suckers embedded in the bile duct walls. The flukes are usually in close proximity to each other and are often interfolded. In the larger bile ducts they are in contact with brown detritus containing large amounts of eggs and bile (own observations).

1.4.1 OXYGEN TENSION

The oxygen tension of sheep bile is 0-30 mm Hg (Moss, 1970; Cheng, 1973). Thus, there appears to be very little oxygen available to flukes in the larger bile ducts. However, the oxygen content of the small tributary bile ducts has not been measured. It is possible that the oxygen tension in these may be higher due to diffusion of oxygen from the arterial blood supply.

The determination of the overall oxygen tension of a given organ does not necessarily indicate the precise tension prevailing in the immediate vicinity of a parasite. The local tension may be influenced by various factors such as the distance from an arterial capillary, inflammatory processes or reaction to toxins. The rat intestine is a particularly interesting example. Until recently it has been widely accepted that the intestinal environment is relatively anaerobic, the luminal contents are anoxic, and the region close to the mucosa contains measurable amounts of oxygen (Rogers, 1949; Read, 1950; Crompton, Shrimpton & Silver, 1965; Arme & Read, 1969). This has been found to be an oversimplification. Recent studies (Mettrick & Podesta, 1974; Podesta & Mettrick, 1974, 1975) have shown that a considerable amount of oxygen is dissolved in the aqueous phase of the luminal contents of the rat intestine and the oxygen tension is 40-50
mm Hg. In intestine infected with *Hymenolepis diminuta*, it was found that the presence of the parasite helps to maintain the level of oxygen, due to reduced fluid absorption by the gut and also due to a decrease in the weight of the parasitised mucosa. This reduces the barrier to the diffusion of oxygen across the gut wall. As the parasitised intestine also displays reduced substrate transport and glucose metabolism, less energy is expended within the epithelial cells, less oxygen is utilised and the oxygen tension is effectively raised. This example shows how important it is to establish the conditions of the micro-environment of a parasite when examining the host-parasite relationship.

1.4.2 pH

The pH range of bile from sheep is 5.9-6.7 (Dittmer, 1961; Cheng, 1973). However, parasites of the bile ducts may not be exposed to this level. For example, Podesta and Mettrick (1974, 1975) reported that the pH of the bulk aqueous phase in the small intestine of rats is lowered by the presence of *H. diminuta*. A hydrogen ion secretory mechanism in the worm tissue acidifies the intestinal contents. They also pointed out that, since an increase in hydrogen ion concentration is accompanied by electron uptake, and since a decrease in pH elevates redox potential, less oxygen is consumed by oxidising agents in the luminal contents of the more acidic, parasitised intestine. Another effect observed as a result of an increased hydrogen ion concentration is a decrease in absorption by the rat intestine but stimulated absorption of salts, water and glucose by the parasite. *F. hepatica* is known to excrete acidic end products of metabolism. A similar situation, therefore, may exist in the bile ducts with respect to a
change in the pH affecting other parameters.

1.4.3 CO₂ CONCENTRATION

There are substantial quantities of NaHCO₃ in bile - approximately 40 mEq/l in most mammals (Dittmer, 1961). Studies in rats revealed that the partial pressure of carbon dioxide (pCO₂) in the lumen of the intestine parasitised by *H. diminuta* is significantly greater than that of the uninfected gut (Mettrick & Podesta, 1974; Podesta & Mettrick, 1974, 1975). It is suggested that excretion of hydrogen ions by the parasite shifts the equilibrium of the following reaction to the right:

\[ \text{H}^+ + \text{HCO}_3^- \rightarrow \text{CO}_2 + \text{H}_2\text{O}. \]

The fixation of CO₂ is an important step in the anaerobic metabolism of all helminths so far examined (reviewed by von Brand, 1973 and Bryant, 1975), including *F. hepatica* (Prichard & Schofield, 1968c,d). It has been suggested that in the confining habitat of the bile ducts, the acidic metabolic wastes may have deleterious effects on the flukes were it not for the fact that the NaHCO₃ of the bile counteracts the acidic end products produced (Cheng, 1973).

1.5 FEEDING HABITS

1.5.1 NATURE OF NUTRIENTS

The manner in which adult flukes obtain nutrient and its nature have been the subjects of controversy. It has been stated by some (e.g. Jennings, Mulligan & Urquhart, 1956) that they are almost exclusively blood feeders, and by others (e.g. Dawes, 1963a) that they are tissue feeders.

Histological studies carried out by Dawes (1963b) on the
feeding habits of migrating juvenile flukes in mice showed that the caecal contents of the parasites derive from epithelial cells, muscle cells, glandular and connective tissue. So, during the first 24 h of infection young flukes display a readiness to feed on several different kinds of tissues and, although some vascular damage is caused during the migration through the intestine, erythrocytes are rarely seen in the caeca.

Once in the liver, it appears that the immature flukes are still tissue feeders. Dawes (1961) has described how the young fluke uses its oral sucker to pinch and burst hepatic cells; the resulting homogenate is aspirated into the pharynx and ingested. As haemorrhage occurs from sinusoids when hepatic cells are ruptured some blood is included in the diet. Dawes and Hughes (1964) contend that the amount of blood ingested is probably insignificant in comparison with the amount of cellular debris taken up. However, the contents of the caeca of adult flukes on removal from the bile ducts contain dark material similar to the degradation products of haemoglobin. Stephenson (1947b) and van Grembergen (1950, cited by Todd & Ross, 1966) confirmed that this material is degraded haemoglobin.

Weinland and von Brand (1926, cited by Pearson, 1963) and Stephenson (1947b) observed flukes feeding on clotted blood in vitro. Most flukes contain blood in their caeca within 30 min. In some individuals almost the entire gut is filled in 3 h. Within the gut, oxyhaemoglobin yields successively haemoglobin and acid haematin. After removal of the flukes to saline, the caecal contents are disgorged within 24 h. There is no information on the time taken in vitro for a feeding cycle of ingestion, digestion, absorption and regurgitation. A few flukes
with empty caeca are always found when specimens are collected from host livers (Weinland & von Brand, 1926, cited by Pearson, 1963; own observations) but the majority have full caeca.

Various techniques involving the use of radioisotopes have been used to examine the blood sucking activities of flukes in vivo. Pearson (1963) and Holmes, McLean, Dargie, Jennings and Mulligan (1967) using $^{51}$Cr-labelled erythrocytes, Jennings et al. (1956) using $^{32}$P-labelled erythrocytes and Sewell (1967) using di-isopropyl-fluorophosphate-$^{32}$P-labelled erythrocytes have shown blood loss in sheep infected with F. hepatica and radioactivity in the flukes. The major criticism of these studies is that the isotopes used may be excreted into the bile and there is also leakage of label from the red cells in the case of $^{51}$Cr (Sinclair, 1967). The presence of radioactivity in the flukes therefore, is not unequivocal evidence of blood sucking.

Baray (1969) has observed the apparent blood sucking of adult flukes in the bile ducts of sheep, guinea pigs and rabbits and has found hyperaemic areas of the epithelium. Baray suggests that removal of blood by flukes is possible both by direct sucking action and by the removal of the hyperaemic epithelium in chronic infections. Symons and Boray (1967) also found that when flukes are removed from the bile ducts the mucosa is missing and the region is occupied by a clot containing whole erythrocytes.

Dawes and Hughes (1970) confirm the findings, described by Symons and Boray (1967), of complete abrasion of hyperplastic epithelium only in rare instances but affirm that a thick layer of fibrotic tissue invariably remains. Ulceration, or blood clots of the
kind described, have never been seen by Dawes and Hughes in hundreds of sections in numerous specimens of infected rats and mice. They state that in the hyperplastic, fibrotic state of the bile duct the acquisition of a blood meal by adult flukes is an almost impossible achievement as most of the superficial blood vessels are occluded.

Histological examinations by Dawes and Hughes (1964) have shown that adult flukes in the bile ducts cause a lot of damage to the hyperplastic epithelium of the bile duct. In some cases it is completely denuded. Regenerative processes can be seen and the simple bile duct epithelium becomes transformed into a thick glandular formation.

In order to determine whether the bile duct enlargement is due to the direct feeding activities of the flukes, Isseroff and Girard (1976) have placed adult *F. hepatica* in fine mesh sacks and implanted them into the abdominal cavities of rats. Three weeks later the bile ducts were examined and it was found that the bile duct luminal perimeter was nearly twice that of the sham transplants. The histology of the ducts resembles that of ducts from rats with 20-40 day *F. hepatica* infections. As the sacks prevent physical contact of the flukes with the bile duct and liver the results suggest that the hyperplasia of the biliary tract is induced by a chemical factor.

Dawes and Hughes (1964) state that the flukes move about in the bile ducts, scraping and browsing, here and there completely removing pieces of the new epithelium. As a result of their histological examinations they conclude that flukes enter the biliary system at a time when continued feeding on hepatic cells would greatly
endanger the life of the host, and that as a result of the inflammatory reaction the flukes are provided with a 'pasture' of tissue on which to feed.

Despite Dawes' denial of blood feeding by adult flukes, there is good evidence that blood is found in fluke caeca (Stephenson, 1947b; van Grembergen, 1950, cited by Todd and Ross, 1966). Todd and Ross (1966) have used conventional analytical techniques to examine the origin of haemoglobin in *F. hepatica*. They carried out the tests on flukes with and without caecal contents, bile, bile duct, liver and blood from abattoir material and experimental infections. They found that the caecal contents of the flukes react exactly as blood, but bile and bile duct tissue give no detectable reactions for alkali haematin and haemochromogen tests. The tests indicate that the caecal contents contain appreciable quantities of haemoglobin, or its breakdown products, although the parasite itself does not. On average, the caecal contents are equivalent to 0.023 ml of blood per adult fluke. A comparison of the iron and copper content of blood, bile, bile duct epithelium, liver and caecal contents also indicates, that the adult flukes feed mainly on blood (Todd and Ross, 1966).

It is possible that the adult flukes do not feed all the time. There has been no study of the frequency of feeding of flukes. If a blood meal is only taken once every 24 h, as this appears to be the length of time taken in vitro from feeding to disgorging, it is unlikely that a histological examination of flukes in the bile ducts will show the sucker of a fluke penetrating through the bile duct wall into the liver tissue or blood vessels. The flukes may move around in the ducts once a meal has been consumed. Therefore, it would be hard
to find flukes actively feeding during an in vivo examination, especially as flukes are often found detached and in the duodenum if the livers are not examined immediately after the death of the host (Boray, 1969; own observations).

It seems then, that the adult fluke does have access to the host's blood, liver tissue and bile duct epithelium. It is not possible to state which of these is the most important in providing nutrients for the parasite in vivo.

1.5.2 UPTAKE OF NUTRIENTS

There are two possible routes of entry of nutrients into flukes: by diffusion or transport through the body surface, and by ingestion followed by absorption from the intestine. There is evidence that both routes are functional but the relative importance of the two has not been determined. Mansour (1959) carried out experiments on flukes which had been ligatured between the anterior and ventral suckers in an attempt to determine whether glucose from the external incubation medium has to reach the alimentary canal of the fluke before it is absorbed or whether it can be absorbed through the tegument. It was found that the glucose uptake of ligatured flukes is almost identical with that of the controls.

Isseroff and Read (1974) have studied the absorption of monosaccharides in ligated and unligated flukes. They showed that the uptake of 3-O-methyl glucose, glucose, fructose, galactose, mannose, glucosamine and ribose across the tegument is linear with respect to concentration, and is stereospecific. 3-O-methyl glucose, an analogue of glucose which is not metabolised by F. hepatica, is not
accumulated; a steady state is attained within about 90 min.
Radioactive glucose is accumulated during two minute incubations, but
the rapid rate of glucose metabolism precludes accumulation during the
short-term incubations. Glucose uptake is Na⁺, phlorizin and ouabain
insensitive. The mechanism of 3-O-methyl glucose and glucose uptake is
considered to be by facilitated diffusion, that is, they move in
relation to the prevailing concentration difference and no energy is
required (Isseroff & Read, 1974).

Based on reciprocal inhibitor studies, Isseroff and Read
(1974) postulate the presence of at least two different monosaccharide
transport systems in F. hepatica. One system, referred to as the
'glucose site', appears to be responsible for 3-O-methyl glucose,
glucose, galactose, mannose and glucosamine transport. The second
system, referred to as the 'fructose site' is inferred from the fact
that fructose uptake is inhibited by the aldohexoses but fructose does
not inhibit the uptake of the same aldohexoses. The data suggest that
glucose, 3-O-methyl glucose and galactose bind unproductively at the
fructose site and inhibit fructose uptake. The inhibition of ribose
uptake by fructose, glucose and galactose suggests that ribose may also
enter through the fructose site.

The uptake of amino acids by F. hepatica has also been studied
by Isseroff and Read (1969). It has been demonstrated that the uptake
of cycloleucine is the same in ligated and unligated flukes. The
uptake rates of cycloleucine, proline, arginine and methionine are
linear with respect to concentration. The data indicate the lack of
mediated amino acid uptake systems in the tegument. It seems that
uptake occurs by diffusion. It was also found that the concentration
of cycloleucine in the flukes seems to be the same as in the blood of the host. Isseroff and Read (1969) suggest that this does not imply that the worms acquire cycloleucine by feeding on blood; rather it may indicate that low molecular weight metabolites in solution in the blood pass readily into the biliary secretions and are then in contact with the fluke tegument.

Further studies of uptake by the tegument (Isseroff & Walczak, 1971) show that pyruvate, succinate, malate and citrate are absorbed by simple diffusion. Acetate absorption appears to occur by a mediated process in a similar manner to monosaccharide uptake (Wright & Isseroff, 1973). A significant proportion of the acetate absorbed is incorporated into higher fatty acids and lipids. The relevance of these findings to the in vivo situation is not known.

An enzyme is present in the gut of *F. hepatica* which readily digests blood albumins to amino acids which are then absorbed (Rijavec, Kurelec & Ehrlich, 1962, cited by von Brand, 1973). Robinson and Threadgold (1975) examined the fine structure of the gastrodermis and found that it consists of a single continuous layer of epithelial cells which show considerable variation in fine structure. The differences in structure are due to different functional states of the cells; absorptive or secretory. Each cell shows a cyclical transformation between the secretory and absorptive forms, but since all the cells in any particular area of the diverticula are not all in the same state, secretion, absorption and digestion are occurring simultaneously and continuously. The main caeca appear to be more concerned with the movement of material back and forth within the lumen of the gut system while the cells of the diverticula are responsible for secretion,
absorption and digestion.

Thorsell and Björkmann (1965) have provided evidence for extraintestinal digestion by the liver fluke. They showed that the secretory granules released by the gut epithelial cells empty their contents into the gut lumen. The lytic effect of unligated flukes on gelatin shows that the acting factor(s) is derived from the gut, since flukes with ligated suckers effect no lysis. It is probable that the secretory granules are responsible for the lytic process.

There is evidence from histological examinations (see 1.5.1) that the flukes abrade the superficial epithelium of the hypertrophied bile ducts; thus it is likely that there are significant levels of low molecular weight nutrients available for absorption from the tissue debris around the flukes. Blood and liver tissue are also probably taken up into the caeca and utilised. However, the relative contribution of these nutrients to the parasite's metabolism in vivo is unknown.
B. METABOLISM OF *F. HEPATICA*

1.6 CARBOHYDRATE METABOLISM

1.6.1 GLYCOGEN DISTRIBUTION

Glycogen is the major carbohydrate storage product in *F. hepatica*. There are many glycogen granules in the parenchymal cells and muscular organs, especially the suckers and cirrus pouch, of flukes. The ovarian eggs contain some glycogen but most of the polysaccharide localised in the female reproductive system is found in the mature vitellarian cells and the uterine ova. Some glycogen is also found in the tonofibrils of the cuticle (von Brand & Mercado, 1961; Pantelouris, 1964).

Histochemical methods (von Brand & Mercado, 1961; Pantelouris, 1964) and direct quantitative measurement of glycogen (Mansour, 1959; Hines, 1969; Threadgold & Arme, 1974) demonstrate that glycogen levels drop when flukes are incubated *in vitro* without a food source. After 12 h starvation the glycogen content of the parenchymal cells decreases (von Brand & Mercado, 1961) and direct measurement shows that about 50% of the glycogen has been utilised (Mansour, 1959; Threadgold & Arme, 1974). After 24 h starvation the parenchymal cells and the muscular organs are almost devoid of glycogen but the amount in the vitellaria and uterine eggs appears to remain unchanged (von Brand & Mercado, 1961). Direct measurement shows that there is a further drop and by 48 h 85% of the original glycogen is lost (Threadgold & Arme, 1974).
Bueding and Orell (1961) found that there is a high and a low molecular weight glycogen fraction and in starved flukes the heavy glycogen practically disappears while the light fraction is still demonstrable. It is thought that the glycogen remaining in the vitellaria and uterine eggs is of the low molecular weight variety.

When starved flukes are returned to media containing glucose, the glycogen reserves are built up to previous levels within 24 h (Mansour, 1959; von Brand & Mercado, 1961; Threadgold & Arme, 1974). It has been found, using $^{14}$C-glucose, that there is preferential conversion of glucose to glycogen rather than incorporation into lipid or protein (Lahoud, Prichard, McManus & Schofield, 1971a).

1.6.2 GLYCOGEN SYNTHESIS AND DEGRADATION

The synthesis of glycogen from glucose is shown in the following diagram:

Glucose is phosphorylated to glucose 6-phosphate (G6P). The phosphate group is transferred to the $C_1$ atom by the action of phosphoglucomutase,
and the glucose 1-phosphate (G1P) so formed reacts with uridine 5''-triphosphate (UTP) to form uridine 5''-diphosphate (UDP)-glucose. The glucose residue is then transferred from the nucleotide onto the end of an uncompleted chain in the glycogen molecule to increase the length of the chain by one glucose residue (Newsholme & Start, 1973). Phosphoglucomutase activity has been reported in *F. hepatica* (Prichard & Schofield, 1968a).

The degradation of glycogen proceeds by a separate pathway from glycogen synthesis. Glycogen phosphorylase catalyses the phosphorolytic cleavage of glycogen to give G1P:

\[
\text{Glycogen} + \Pi \xrightarrow{\text{phosphorylase}} \text{G1P} + \text{Glycogen}
\]

The G1P is converted to G6P via the action of phosphoglucomutase, and is then further metabolised by the glycolytic sequence of reactions (Newsholme & Start, 1973).

1.6.3 GLYCOLYSIS

All cells, whether aerobic or anaerobic, obtain energy from oxidation - reduction reactions. The passage of hydrogen (electrons and protons) from reducing agents to oxidising agents is accompanied by energy yield in a biologically useful form such as adenosine 5''-triphosphate (ATP) or Coenzyme A (CoA) derivatives. The electrons lose energy as they pass from a molecule with low electron affinity to one with a higher electron affinity. In most oxidation reactions a foodstuff molecule reduces a pyridine nucleotide cofactor - nicotinamide adenine dinucleotide (phosphate) (NAD or NADP). If the foodstuff oxidation process is to continue, a resupply of oxidised cofactor is
obviously necessary; that is, the redox balance must be maintained. The cofactor molecules are cyclically reoxidised. The electrons donated to the cofactors by the foodstuff molecule are ultimately donated to another molecule, a terminal electron acceptor, which has a higher electron affinity than the cofactors.

The degradation of glucose into the 3-carbon compound, lactate, by the glycolytic sequence of reactions seems to be a universal system for liberation of energy, some of which is conserved as ATP. ATP production occurs at two sites; the reactions catalysed by 3-phosphoglycerate kinase and pyruvate kinase (PK). These are termed substrate-linked phosphorylations. NADH is produced at the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase and is reoxidised at the reaction catalysed by lactate dehydrogenase (LDH). Lactate, the end product of glycolysis, has the same level of oxidation as glucose. Thus, glycolysis provides anaerobic energy production and at the same time maintains the redox balance.

The wide distribution of many glycolytic enzymes and the demonstration of phosphorylated glycolytic intermediates in parasitic helminths indicate that they possess typical glycolytic sequences at least to the level of phosphoenolpyruvate (PEP), and many produce lactate (Ward & Schofield, 1967; Bueding & Saz, 1968; Barrett & Beis, 1973a; Köhler & Hanselmann, 1973; Hutchinson & Fernando, 1974; Behm & Bryant, 1975a).

All the enzymes of glycolysis have been demonstrated in *F. hepatica*. Mansour (1962) detected phosphofructokinase (PFK), fructose 1,6-diphosphatase (FDPase) and aldolase in fluke homogenates.
PFK was studied in greater detail by Mansour and Mansour (1962) and Stone and Mansour (1967). The properties of FDPase were studied by Prichard and Schofield (1969). De Ley and Vercoysse (1955) measured the activities of a NADP-dependent G6P-dehydrogenase and gluconate 6-phosphate dehydrogenase. Pennoit-de-Cooman and van Grembergen (1942, cited by Prichard and Schofield, 1968a) found dehydrogenase activity with lactate and α-glycerophosphate as substrates. Prichard and Schofield (1968a) demonstrated the activities of all the glycolytic enzymes except PK, using the methods designed for the measurement of the enzymes in rat liver. It was also found that LDH activity is low in F. hepatica compared with rat liver.

It has since been shown that PK is present in flukes but has different properties from the mammalian enzyme (Sturm, Hirschhäuser & Zilliken, 1969; Lee & Vasey, 1970; Prichard, 1976). Maximal activity occurs with Mn++ rather than with Mg++.

1.6.4 TRICARBOXYLIC ACID (TCA) CYCLE

The major fuels of aerobic animals (carbohydrates, fatty acids and amino acids) are ultimately oxidised by the series of reactions known as the TCA cycle. In the presence of oxygen the molecules are completely oxidised to CO₂ and water and the electrons from the reduced cofactors are progressively transferred to oxygen by the electron transport system. ATP is generated during electron transport and the redox balance is maintained.

In spite of the fact that all parasitic helminths investigated so far are capable of consuming oxygen (reviewed by von Brand, 1973), none of them is capable of the complete oxidation of substrates to CO₂ and water. Instead the end products of metabolism are only partly
oxidised before excretion. Such end products include lactate, succinate, acetate, propionate, butyrate and ethanol (reviewed by von Brand, 1973).

There are processes which require molecular oxygen in parasites, and oxygenases are found in them. The direct oxidation of amino acids, egg production (Le Jambre & Whitlock, 1967), tanning of egg-shell proteins (Smyth & Clegg, 1959) and collagen synthesis (Paik & Bennoitin, 1963; Fujimoto & Prockop, 1969; Chvapil, Boucek & Ehrlich, 1970) require oxygen. These are not, however, respiratory processes; they do not result in the net synthesis of energy - they probably expend it.

Bryant and Williams (1962) reported that $^{14}$C-glucose is incorporated by F. hepatica into some of the intermediates of the TCA cycle; succinate, fumarate, malate and citrate. It was suggested that the TCA cycle is present and functional in F. hepatica (Bryant & Williams, 1962; Bryant & Smith, 1963). Thorsell (1963) showed that there are appreciable quantities of citrate, aconitate, succinate, fumarate and malate in F. hepatica. All the enzymes of the cycle have been demonstrated and there is an active citrate synthase; however, the low activities of aconitase and isocitrate dehydrogenase suggest that the cycle may be of minor importance (Prichard & Schofield, 1968b). It is possible that these two enzymes were not assayed under optimal conditions, in which case they may be more active in vivo.

However, the degradation of U-$^{14}$C, L-$^{14}$C and 6-$^{14}$C glucose by F. hepatica confirms the low activity of the TCA cycle as CO$_2$ formation comes primarily from decarboxylation reactions, giving rise to acetate and propionate, rather than from the TCA cycle or the pentose phosphate
cycle (Buist & Schofield, 1971).

An examination of the TCA cycle in other parasitic helminths has shown that, in some cases not all the enzymes are present and, in others, that the metabolism of radioactive precursors does not result in the liberation of CO$_2$ from the cycle or the incorporation of radiocarbon into all the intermediates of the cycle (Oya, Kikuchi, Bando & Hayashi, 1965; Schiebel & Saz, 1966; Davey & Bryant, 1969; Köhler & Hanselmann, 1973; Hutchinson & Fernando, 1975).

1.6.5 CO$_2$ FIXATION AND SUCCINATE FORMATION

It was found by Prichard and Schofield (1968c,d) that, as with many other parasites, part of the TCA cycle functions in reverse (reviewed by Saz, 1969; Ward, Castro & Fairbairn, 1969; von Brand, 1973). Normal glycolysis proceeds to PEP formation. PEP is converted to pyruvate by the action of PK or it is metabolised to oxaloacetate (OAA) by the CO$_2$ fixation of phosphoenolpyruvate carboxykinase (PEPCK). OAA is then converted to malate via malate dehydrogenase (MDH) activity and then to fumarate by a dehydration reaction catalysed by fumarase. Fumarate is then metabolised to succinate by a NADH-linked succinate dehydrogenase (SDH). This enzyme is more commonly termed fumarate reductase in parasites as it has higher affinity for fumarate and is less sensitive to succinate accumulation. Fumarate reduction by NADH is achieved by an electron transport system in helminths (reviewed by Bryant, 1970).

It appears from a study of some helminths that fumarate reduction is linked with a phosphorylation step. Incorporation of $^{32}$Pi has been demonstrated anaerobically in Ascaris (Seidman & Entner, 1961; Saz & Lescure, 1969; Saz, 1971; Van den Bossche, 1972a), H. diminuta
(Schiebel, Saz & Bueding, 1968; Saz, Berta & Kowalski, 1972) and *F. hepatica* (de Zoeten & Tipker, 1969). One phosphorylation step is probably involved, as de Zoeten and Tipker (1969) measured a ratio of NADH consumed / ATP generated of 1.0 in *F. hepatica* and Saz (1971) found 0.5 mole ATP / mole of malate supplied in *Ascaris*. The latter is consistent with what would be expected in *Ascaris* where malate is dismutated in the mitochondria to succinate and acetate in a 1:1 ratio.

1.6.6 END PRODUCTS OF CARBOHYDRATE METABOLISM

It was stated in 1.6.4 that the end products of parasitic helminths are only partly oxidised before excretion. In *F. hepatica* the major end products of carbohydrate metabolism are acetate and propionate (Mansour, 1959; Lahoud *et al.*, 1971a). Lactate and succinate are also excreted (de Zoeten, Posthuma & Tipker, 1969) as well as small quantities of isobutyrate, isovalerate and 2-methylbutyrate (Lahoud *et al.*, 1971a; Lahoud, Prichard, McManus & Schofield, 1971b).

Acetate is derived from pyruvate oxidation by pyruvate dehydrogenase activity and acetyl CoA synthetase, presumably. Pyruvate dehydrogenase has been reported in *F. hepatica* (Prichard & Schofield, 1968c) but acetyl CoA synthetase activity has not been measured. NADH is produced by the combined reactions. The pyruvate is either derived from PEP by the reaction catalysed by PK or else it can be produced from malate by the action of malic enzyme. Prichard and Schofield (1968c) reported that *F. hepatica* has an active malic enzyme. NADH is produced in this reaction also.

Propionate is produced from succinate via methylmalonyl-CoA...
and propionyl-CoA. De Zoeten et al. (1969) demonstrated the presence of the enzymes methylmalonyl-CoA mutase and propionyl-CoA carboxylase, which catalyse the reactions.

The rate of propionate/acetate (P/A ratio) excreted in vitro by *F. hepatica* is affected by the gas phase used (Lahoud et al., 1971a). The ratio changes from 2.5 in anaerobic conditions to two in air and to one in oxygen and so appears to be a function of oxygen tension. With radioactive glucose in the medium, the specific activities of both acetate and propionate remain equal in spite of changes in the absolute amounts of propionate produced (Lahoud et al., 1971a).

It is likely that, under high concentrations of oxygen, a substantial proportion of NADH is oxidised by oxygen rather than by fumarate in the fumarate reductase reaction. Hence, less succinate is produced, thus decreasing the amount of propionate formed (Lahoud et al., 1971a). Under complete aerobiosis, propionate is still produced and, in air, the rate of propionate production is only slightly less than that under anaerobic conditions. This suggests that fermentative metabolism occurs in vivo (Lahoud et al., 1971a).

De Zoeten and Tipker (1969) have shown in *F. hepatica* that when fumarate is reduced to succinate with the consequent reoxidation of one molecule of NADH, one molecule of ATP is formed. In the formation of succinate from glucose or glycogen, redox balance is maintained; the two molecules of NADH formed during glycolysis are reoxidised at the MDH and LDH reactions. Van Vugt, Kalaycioglu and Van den Bergh (1976) have studied the fate of NADH produced during acetate production in *F. hepatica*. In isolated fluke mitochondria they
have found that malate utilisation is tightly coupled to phosphorylation as it is adenosine 5'-diphosphate (ADP)-dependent and can be stimulated by the addition of an uncoupler. Determination of phosphorylation efficiencies under anaerobic and aerobic conditions suggests that NADH formed during the oxidation of malate can be reoxidised either by fumarate or oxygen. In the latter case more ATP is produced. Under aerobic conditions succinate can act as substrate for the production of ATP. Prichard and Schofield (1969) have shown that succinate can be incorporated into glycogen. Thus, it is likely that under aerobic conditions oxygen can act as an electron acceptor, succinate can be metabolised to produce ATP and glycogen stores can be built up. Whether this occurs in vivo is not known.

1.6.7 ELECTRON TRANSPORT SYSTEMS

The classical mammalian electron transport system consists of an acceptor, coenzyme Q, followed by a series of cytochromes, b, c, a, and a3, which are electron-transferring molecules. The last one, cytochrome a3 or cytochrome oxidase, can donate electrons to molecular oxygen. The overall reaction for the oxidation of NADH by molecular oxygen is:

\[
\text{NAD}^{\text{reduced}} + \frac{1}{2}\text{O}_2 + 2\text{H}^+ \rightarrow \text{NAD}^{\text{oxidised}} + \text{H}_2\text{O}.
\]

At three points along the electron transport system there is a large drop in free energy as a pair of electrons move from one carrier to the next. At these sites phosphorylation of ADP to ATP occurs. Such phosphorylation in the electron transport system is called oxidative phosphorylation. The complete equation for electron transport is:
NAD$_{\text{red.}}$ + 3ADP + 3Pi + 2H$^+$ + $\frac{1}{2}$O$_2$ $\longrightarrow$ NAD$_{\text{ox.}}$ + 3ATP + 4H$_2$O.

Oxidative phosphorylation can be inhibited by certain poisons in such a way that electron transport still continues but the linked phosphorylation of ADP to ATP does not. Under these conditions the energy of oxidation of glucose is dissipated completely as heat and none is recovered as ATP. Such poisons are called uncoupling agents.

Functional cytochromes have been found in the mitochondria of many parasitic helminths (Cheah & Bryant, 1966; Cheah, 1967a,b; Cheah & Chance, 1970; Bryant & Nicholas, 1966). In some parasites the conventional mammalian-type cytochrome system has been found (Cheah, 1968; Cheah & Chance, 1970). However, there is evidence that alternative systems operate. For example, in Moniezia expansa there seems to be a branched electron transport system containing two terminal oxidases (Cheah & Bryant, 1966; Cheah, 1967a,b; Cheah, 1968). The minor one seems to have cytochrome a$_3$ as the terminal oxidase and part of a classical cytochrome chain, capable of oxidising succinate, NADH and glycerol 3-phosphate. The other system has 'cytochrome 552, 556 (M. expansa)', an o-type pigment similar to that found in bacteria and algae, as the terminal oxidase. Electrons are transferred to either oxygen (forming hydrogen peroxide) or fumarate (forming succinate) from NADH, succinate or glycerol 3-phosphate.

Studies on the electron transport system of F. hepatica have shown that the classical mammalian cytochromes are present as well as a b-type of cytochrome component containing a carbon monoxide reactive o-type cytochrome (de Zoeten & Tipker, 1969; Cheah & Prichard, 1975).
It is suggested that *F. hepatica* contains a branched electron transport system, including the classical system and at least two b-type cytochromes, with the involvement of two terminal oxidases (de Zoeten & Tipker, 1969; Cheah & Prichard, 1975). Presumably, under aerobic conditions, both systems can operate with oxygen and fumarate acting as electron acceptors. Under anaerobic conditions the classical system involving oxygen as the terminal electron acceptor no longer functions. Crompton *et al.* (1965) point out that an oxygen tension of 5 mm Hg is required for the satisfactory functioning of cytochrome oxidase. It is not known whether the oxygen tension in the sites occupied by *F. hepatica* in the host experience such low levels of oxygen or whether the parasite utilises both systems *in vivo*.

1.6.8 ENERGY LEVELS AND REDOX BALANCE

Two measurements are often used as indicators of the energy status of a tissue or organism; ATP/ADP ratio and the adenylate energy charge. The ATP/ADP ratio is an index of the high-energy phosphorylation state of the tissue or organism while the adenylate energy charge \( \frac{[\text{ATP} + \frac{1}{2}\text{ADP}]}{[\text{ATP} + \text{ADP} + \text{AMP}]} \) is a measure of the relative amount of energy stored in the adenylate system (Atkinson & Walton, 1967). Tissues can have quite variable ATP/ADP ratios but need to maintain a constant adenylate energy charge to ensure a balance between anabolic and catabolic processes (Atkinson, 1971). The energy charge has been proposed as a fundamental control parameter affecting the activity of regulatory enzymes in anabolic and catabolic sequences, e.g. citrate cleavage enzyme, PFK (Atkinson & Walton, 1967; Shen, Fall, Walton & Atkinson, 1968), and it has been shown for *Escherichia coli* that growth only occurs when the adenylate energy charge is \( \geq 0.8 \). Viability is
maintained when it is between 0.5 and 0.8 and death occurs below 0.5 (Chapman, Fall & Atkinson, 1971). Animal tissues normally maintain their adenylate energy charges around 0.85, plant tissues are generally lower and microorganisms show considerable variation depending on culture conditions (Chapman et al., 1971).

It is essential for the continued production of energy that redox balance is maintained. When an aerobic tissue becomes anaerobic, the major effect is an increase in the level of reduction of the tissue, since NADH is not reoxidised. In neonatal rats kept in air or nitrogen for 25 min, liver cytosolic NAD/NADH ratios drop from 614 in the controls to 139 in the anoxic rats; mitochondrial ratios decrease from 29.7 to 2.4. At the same time the adenylate energy charge drops from 0.82 to 0.598 (Ballard & Philippidis, 1971). However, parasitic helminths examined so far can maintain both high NAD/NADH ratios and high adenylate energy charges in the absence of oxygen. For example, *Ascaris lumbricoides* muscle maintained anaerobically has a cytosolic NAD/NADH ratio above 1,000 (Barrett & Beis, 1973b) and an adenylate energy charge of 0.86 (Barrett & Beis, 1973c).

In *F. hepatica* NAD is present mainly in the oxidised form (NAD/NADH is 3.4) and NADP is mainly in the reduced form (NADP/NADPH is 0.57). Also, the NAD content is higher than the NADP content (Barrett & Beis, 1973c). These results are consistent with the role of NAD in energy metabolism while NADP is involved in reductive synthetic pathways (Barrett & Beis, 1973c).

The ATP/ADP ratio in *F. hepatica* is around 1.26 (Barrett & Beis, 1973c), which is lower than those reported in vertebrate tissues (Helmreich, Danforth, Karpatkin & Cori, 1965; Williamson, 1965). The
adenylate energy charge is about 0.61, which is also lower than vertebrate tissues. Despite the fermentative carbohydrate metabolism of *F. hepatica* energy levels and redox balance are maintained.

1.6.9 ENERGY PRODUCTION DURING CARBOHYDRATE METABOLISM BY *F. HEPATICA*

The metabolism of one molecule of G6P by glycolysis to the level of PEP results in the net formation of one molecule of ATP and the reduction of two molecules of NAD. If PEP is metabolised via PEPCK to succinate a molecule of GTP is produced in the PEPCK reaction. This can presumably be converted to a molecule of ATP by the action of a nucleoside diphosphokinase. NADH is reoxidised during the conversion of OAA to malate, catalysed by MDH. The fumarate reductase reaction provides one molecule of ATP per NADH molecule oxidised. Propionate formation from succinate results in a further production of one ATP molecule.

Conversion of PEP to lactate via PK and LDH activity results in the formation of one molecule of ATP and the reoxidation of one molecule of NADH per molecule of PEP metabolised.

Acetate production from pyruvate via pyruvate dehydrogenase activity results in the formation of one molecule of ATP and the generation of one molecule of NADH. The conversion of malate to pyruvate by malic enzyme also results in the reduction of a molecule of NAD.
1.6.10 THE GLYOXALATE CYCLE AND GLYCOMEGENESIS

The glyoxalate cycle is shown in the diagram:

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glucose

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<th>glucose</th>
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<td>succinate</td>
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<td></td>
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<td>glyconeogenesis</td>
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It has been reported to be functional in developing eggs of *A. lumbricoides* at the time of conversion of fatty acids to carbohydrate (Barrett, Ward & Fairbairn, 1970) and in four free-living nematodes (Rothstein & Mayoh, 1964, 1965, 1966). The overall equation of the series of reactions is:

\[
2 \text{Acetyl-CoA} + \text{NAD} \rightarrow \text{succinate} + \text{NADH} + 2 \text{CoA}.
\]

The enzymes of the cycle have been demonstrated in *F. hepatica*.
Supernatant fractions of fluke homogenate produce $^{14}\text{C}$-glyoxalate from $^{14}\text{C}$-1, 5-citrate, and $^{14}\text{C}$-malate is produced from $^{14}\text{C}$-glyoxalate. Addition of an acetyl-CoA generating system results in greater incorporation of radiocarbon in malate. Significant levels of radiocarbon are also detected in succinate, although this may be derived from radioactive malate or $\alpha$-ketoglutarate.

In view of the presence of PEPCK and the relatively high activity of FDPase in *F. hepatica* (Prichard & Schofield, 1968a,d, 1969) it is suggested by Prichard and Schofield (1969) that the glyoxalate cycle is important for providing succinate, or its metabolites derived from glucogenic or ketogenic precursors, for glyconeogenesis. The incorporation of $^{14}\text{C}$-succinate into glycogen does occur, indicating that the glyconeogenic pathway can operate in *F. hepatica* (Prichard & Schofield, 1969). Glyconeogenesis is likely to be important in the fluke as glycogen makes up 15-21% of its dry weight and the eggs are very rich in glycogen (Wilson, 1967).

### 1.6.11 REGULATION OF CARBOHYDRATE METABOLISM

#### 1.6.11. (a) REGULATORY ENZYMES

The flux through a metabolic pathway is controlled by the slowest reaction in the sequence and this is catalysed by a non-equilibrium enzyme, unless the supply of one of the reactants governs the rate of the reaction (Krebs, 1969). An advantage of control of a pathway by a non-equilibrium enzyme is that regulation may be governed by factors not directly related to the reaction, thus allowing a greater degree of flexibility in control and coordination of metabolic pathways (Newsholme & Start, 1973).
A comparison of the mass-action ratio for a reaction with its apparent equilibrium constant allows the reaction of a pathway to be classified as 'equilibrium' or 'non-equilibrium' \textit{in vivo} (Rolleston, 1972). The non-equilibrium reactions are those most likely to play a regulatory role in a pathway. Behm and Bryant (1975a) carried out the comparison of mass action ratios with equilibrium constants of most of the glycolytic reactions in \textit{M. expansa}. They found that hexokinase, PFK, aldolase and PK are significantly out of equilibrium. Aldolase may be disregarded as a regulatory enzyme as it has a high catalytic activity \textit{in vitro} (Behm & Bryant, 1975a) and it is likely that there is an interaction between it and triosephosphate isomerase (TPI) (as found for perfused rat heart; Williamson, 1965) so that the combined reaction is close to equilibrium.

The 'crossover' technique, originally developed by Chance and coworkers (Chance, Holmes, Higgins & Connelly, 1958) to identify sites of interaction in the electron transport chain, has been used by Rolleston (1972) to determine sites of regulation in metabolic pathways. Sites of regulation can be recognised when the concentration of the substrate of the reaction changes in the opposite direction to the change in flux through the pathway as a whole. Behm and Bryant (1975a) have used this technique to examine the metabolic intermediate levels of the glycolytic pathway in \textit{M. expansa} under different experimental conditions. Consistent crossover points are apparent in \textit{M. expansa} at hexokinase, PFK and PK again indicating a possible regulatory role for them. Although hexokinase is implicated as a regulatory enzyme in \textit{M. expansa} it may not limit the flux through the entire glycolytic sequence as a major contribution to the G6P pool.
(the product of the reaction) comes not from glucose (the substrate of the reaction) but from glycogen (Behm & Bryant, 1975a).

Behm and Bryant (1975a) also suggest that PEPCK may be a regulatory enzyme in *M. expansa* as it is sufficiently out of equilibrium and has a relatively low activity *in vitro*. The regulatory properties of PEPCK, PK and PFK have been examined in detail by Behm and Bryant (1975b,c).

The crossover technique has also been used in the study of the effect of antimonials on PFK of *Schistosoma mansoni* (Bueding & Mansour, 1957; Bueding & Fisher, 1966). It was found that intact adult *S. mansoni* incubated with potassium antimonyl tartrate show increased levels of fructose 6-phosphate (F6P) and a decrease in fructose 1,6-diphosphate (FDP). This evidence indicates that PFK activity is inhibited, because an increase in substrate concentration is associated with a decrease in flux through the pathway.

PFK activity in *F. hepatica* has been examined in detail (Mansour, 1962; Mansour & Mansour, 1962; Stone & Mansour, 1967). It has been found to limit the rate of glycolysis and exhibits sigmoid kinetics with F6P. Activators of the enzyme include 3',5'-cyclic AMP, calcium ions and 5-hydroxytryptamine, although the last two require the presence of a heavy particulate fraction from the fluke.

1.6.11. (b) THE PK/PEPCK BRANCHPOINT

It has been suggested that an important control point in the respiration of parasitic helminths exists at the level of PEP (Saz, 1971) and that the nature of the end products formed by a parasite depends on the competition for PEP by the two enzymes PK and PEPCK.
Thus, the PK/PEPCK ratio has been correlated with the proportion of lactate and succinate produced by a parasite. Bryant (1975) has reviewed this concept and found that in many cases the hypothesis is substantiated. However, he also criticises it as, in some cases, the ratios are not correct because the enzymes have not been assayed under optimal conditions, consideration has not been given to the regulation of the enzymes by various effectors, and sometimes there is more than one form of the enzyme. For example, PK is regulated in mammals by a number of allosteric effectors including FDP and adenine nucleotide levels (Newsholme & Start, 1973). Bryant (1972a,b) has shown that in *M. expansa* there are at least two forms of PK and different PK/PEPCK ratios can be calculated under different conditions. A further complication is that malate has been found to inhibit FDP-activated PK in *M. expansa*. Similarly, Carter, Watts and Fairbairn (1972) describe five isoenzymes of PK in *H. diminuta*; two are modulated by FDP and ADP and most of the activity is associated with the non-modulated isoenzymes.

Another criticism of PK/PEPCK ratios acting as indicators of the importance of the two pathways is that PK is not essential for lactate production. Malic enzyme catalyses the formation of pyruvate from malate, so if a parasite has both malic enzyme and LDH, lactate formation is possible. This is the case for *A. lumbricoides* (Bueding & Saz, 1968; Saz & Lescure, 1969), *Haemonchus contortus* larvae (Ward & Schofield, 1967; Ward, Schofield & Johnstone, 1968), *Trichinella spiralis* larvae (Ward, Castro & Fairbairn, 1969), *H. diminuta* (Bueding & Saz, 1968; Saz, Berta & Kowalski, 1972), *F. hepatica* (Prichard & Schofield, 1968a,c) and possibly *Moniliformis dubius* (Körting &
Fairbairn, 1972). Malic enzyme has not yet been assayed in many parasites.

In some parasites the production of lactate and succinate varies according to experimental conditions. For example, *M. expansa* produces 51% more lactate under anaerobic conditions than under aerobic conditions. Succinate production remains unchanged (Bryant & Behm, 1976). It is suggested that under aerobic conditions succinate or NADH may be oxidised by an electron transfer system using oxygen as the terminal electron acceptor. Malate accumulates within the mitochondria and in the cytosol. PK is inhibited by high malate concentrations, hence less pyruvate is produced and therefore, less lactate is excreted. Under anaerobic conditions the activity of the aerobic system is suppressed due to the lack of oxygen and less malate is produced. PK activity is enhanced and lactate production increases.

In *H. diminuta* the kinetic behaviour of PEPCK and PK have been examined (Mettrick, Mustafa, Podesta, Moon & Hulbert, 1976; Moon, Mustafa, Hulbert, Podesta & Mettrick, 1977). PEPCK has an acid pH optimum and is activated by HCO$_3^-$; PK is inhibited by HCO$_3^-$ and has a low affinity for PEP at acid pH. Hence, high CO$_2$ concentrations in the environment of the parasite favour the PEPCK reaction. PEPCK from *M. expansa* has a similar acid pH optimum (Behm & Bryant, 1975c) and it is likely that a CO$_2$-rich environment favours the PEPCK reaction.

The concentration of CO$_2$ in the parasitic environment seems to be an important consideration in the regulation of carbohydrate metabolism. As mentioned in 1.4.2 it has been found that the pH is lower in rat intestine parasitised by *H. diminuta* (Podesta & Mettrick,
1974, 1975). This elevates the pCO₂ resulting in the diffusion of CO₂ into the worm tissue. Within the tissue the equilibrium of the CO₂/HCO₃⁻ system favours the production of H⁺ and HCO₃⁻. The presence of HCO₃⁻ within the worm probably leads to buffering problems. Thus, the utilisation of CO₂ by parasites in habitats of high pCO₂ may be a response to overcome the toxic effects of CO₂ rather than a response to lack of oxygen.

1.6.11. (c) THE PK/PEPCK BRANCHPOINT IN F. HEPATICA

In the early studies of the enzymes involved in carbohydrate metabolism in F. hepatica PK activity could not be found (Prichard & Schofield, 1968a) or was found to be very low in activity (Sturm et al., 1969). As lactate is not one of the major end products of metabolism in flukes and as PEPCK activity was demonstrated (Prichard & Schofield, 1968d), it was thought that CO₂ fixation into PEP and subsequent metabolism to propionate is the major pathway involved in carbohydrate metabolism. Lactate was thought to be produced from pyruvate by the action of malic enzyme.

PK has since been demonstrated in F. hepatica (Lee & Vasey, 1970). The reason for its non-activity in earlier assays is due to the use of the assay system normally used for measuring mammalian PK. The fluke enzyme operates under different conditions. A more detailed study on the regulation of PK and PEPCK has since been carried out (Prichard, 1976), however, the work is incomplete and in some cases is subject to severe criticism.

Prichard (1976) carried out the work on fluke PK and PEPCK on a supernatant fraction with no attempt at purification, despite his reference to the work of Bryant (1972a,b) on M. expansa and of Köhler
(1974) on Dicrocoelium dendriticum. Both these authors partially purified the enzyme preparation and found more than one form of PK. Apart from the fact that there may be different isoenzymes of PK in F. hepatica the lack of purification of the sample means that other enzymes, substrates and cofactors will be present and may interfere with the assays. For example, in the determination of the Km for PEP of PK, there are probably other enzymes present which are capable of metabolising PEP in the supernatant fraction used; PEPCK and enolase. There may even be PEP generating systems within the impure enzyme fraction. Thus the Km for PEP found by Prichard (1976) for PK may not be accurate.

Prichard (1976) states that there is a marked activation of fluke PK activity when FDP is added and when PEP, K⁺ and Mn⁴⁺ are present. However, the appropriate control has not been included - i.e. the addition of FDP without substrate (PEP) present. If this is done there is a rapid rate of FDP-dependent NADH oxidation (Behm, 1976, personal communication). This activity is probably due to contamination of the enzyme fraction with 3-glycerol phosphate dehydrogenase. So, the activation of PK activity by FDP is equivocal in F. hepatica.

PEPCK activity was also supposedly measured by Prichard (1976). It is stated that PEPCK is assayed radioisotopically by measuring the incorporation of NaH¹⁴CO₃ into the acid stable fraction. However, there is nothing in the assay mixture to ensure that the ¹⁴C-OAA produced by PEPCK is converted to an acid stable form. OAA spontaneously decarboxylates. Usually, OAA is converted to malate by including MDH and NADH in the assay mixture (Behm & Bryant, 1975a).
The treatment of the experimental vials at the completion of the experiment liberates the acid unstable radiocarbon. The results of Prichard's experiments suggest that a lot of radiocarbon is lost by this treatment, perhaps due to decarboxylation of OAA. When NADH is added to the assay mixture there is a marked increase in the amount of radiocarbon recovered in the acid stable fraction. Behm (1974) found that even after purification of *M. expansa* PEPCK there was sufficient MDH present to convert OAA to malate. However, NADH addition is still required to give total conversion and thus, an accurate measure of PEPCK activity. Prichard (1976), using a crude supernatant fraction, probably had sufficient MDH present but not NADH. The results he has reported for PEPCK activity in *F. hepatica* are probably very low as all the OAA produced by the reaction may not have been converted to acid stable products. Also, other enzymes in the enzyme fraction used may have metabolised PEP or malate. For example, malic enzyme may convert some malate to pyruvate. This is a decarboxylation reaction, the molecule of CO$_2$ released being the same one that is fixed in the PEPCK reaction. Thus, even if there is sufficient NADH to convert OAA to malate some of the radiocarbon may be released due to the activity of malic enzyme, i.e. the PEPCK activity will appear to be low.

Thus, the control of the PK/PEPCK branchpoint and the regulation of carbohydrate metabolism in *F. hepatica* are still not fully understood.

1.7 NITROGEN EXCRETION AND AMINO ACID METABOLISM

Despite the controversy regarding the food source of *F. hepatica in vivo*, a substantial part of it must be proteinaceous
and therefore has a high carbon:nitrogen ratio (Moss, 1970). Thus, if the fluke is to utilise proteins it must be capable of metabolising it to a useable form and excreting the nitrogenous wastes. The excretory metabolism of *F. hepatica* *in vitro* has been examined by Moss (1970) who found that nitrogen is excreted in the form of ammonia, urea and amino acids. When compared with the rat tapeworm, *H. diminuta*, it is found that flukes produce ten times more ammonia. Moss (1970) suggests that this may reflect a difference in diet, the fluke feeding mainly on tissue proteins of the host and the tapeworm mainly on the carbohydrates from the food materials in the gut of its host. The addition of glucose to the media in which the flukes are cultured produces a marked decrease in the ammonia and an increase in the lactate excreted. Moss (1970) suggests that flukes are not obligatory protein feeders and that they may utilise carbohydrate when it is available.

It is usually concluded that when urea is found as an excretory product it is produced by the ornithine-urea cycle. This cycle is shown in the following diagram:
Flukes contain a highly active arginase (Janssens & Bryant, 1969; Kurelec, 1975a) but only small amounts of ornithine transcarbamylase (Campbell & Lee, 1963; Kurelec, 1964b, cited by Coles, 1975a; Janssens & Bryant, 1969), carbamoylphosphate synthetase, arginosuccinate lyase (Janssens & Bryant, 1969) and arginine synthetase are present (Kurelec, 1964a, cited by Coles, 1975a). Experiments carried out by Janssens and Bryant (1969) using radioactive intermediates failed to demonstrate a functional urea cycle. It is probable that the enzymes of the cycle which have been found have a different function.
Metabolism of amino acids to precursors of the TCA cycle occurs in *F. hepatica*. Transamination reactions have been demonstrated between α-ketoglutarate and amino acids (Daugherty, 1952; Connelly & Downey, 1968; Sturm *et al.*, 1969; Janssens & Bryant, 1969; Coles, 1975a). Glutamine synthetase and glutamate dehydrogenase have also been found (reviewed by Coles, 1975a).

Kurelec and Rijavec (1966) examined the amino acid pools of *F. hepatica*. Their findings demonstrate that there are high amino acid concentrations in flukes; the total sum of free amino acids exceeds 3 mg/g wet weight. All the amino acids usually found in proteins are found in the free state in *F. hepatica*. Histidine, alanine and proline account for 50% of the free amino acid nitrogen.

Isseroff, Tunis and Read (1972) investigated the amino acid concentrations in the bile of infected animals to determine whether the free amino acids of host bile are altered following the pathological changes of the bile ducts. It was found that most biliary amino acids are increased in fascioliasis. Proline shows the greatest increase in rat, rabbit and cattle bile. Some of the amino acids which are present at very low levels in the bile of uninfected animals show large increases in infected animals, e.g. methionine, tyrosine, alanine, histidine and proline.

Analysis of the host tissues and fluke tissue shows that the concentration of proline in the worm is always greater than in the bile and host tissues have proline concentrations similar to those in uninfected controls (Isseroff *et al.*, 1972). Experiments revealed that the proline is produced and excreted by the fluke (Moss, 1970). It has
been shown that the enzymes involved in proline biosynthesis (ornithine-6-transaminase and Δ'pyrroline-5-carboxylic reductase) are present in *F. hepatica* and have greater activity than the enzymes in rat and rabbit liver. Also, proline oxidase, the enzyme which breaks down proline, could not be demonstrated in flukes (Ertel & Isseroff, 1974; Isseroff & Ertel, 1976).

The major precursor of proline is ornithine (Ertel & Isseroff, 1974; Kurelec, 1975a). The experiments carried out by Kurelec (1975a) using radioactive arginine and ornithine suggest the operation of the pathway shown in the diagram:

All the experimental data support this series of reactions. It seems that both proline and urea are end products of arginine metabolism. The sequence of reactions described explains the peculiarities of the amino acid pool of the fluke, the production and excretion of proline and alanine observed *in vitro* and *in vivo* and clarifies the nature of urea production and excretion.
The function of the pathway is not clear. Hochachka and Mustafa (1972) suggested that proline could act as a potential energy source during anaerobic conditions in helminths and molluscs. However, after the observation that proline is excreted by *F. hepatica* it was suggested that proline production may be involved in the maintenance of redox balance due to the regeneration of NAD in the final reaction catalysed by Δ-pyrroline-5-carboxylic reductase (Hochachka, Fields & Mustafa, 1973). Prichard suggests (cited by Kurelec, 1975b) that the arginine/proline pathway does not regenerate any more NAD than would be reduced in providing the precursors necessary to maintain the pathway. However, as Kurelec (1975b) adds, for maintenance of the overall pathway:

\[
\text{arginine (exogenous) + pyruvate + NADH} \rightarrow \text{proline + alanine + NAD}
\]

only arginine is required. The mitochondrion possesses an active glutamate-pyruvate transaminase which would convert alanine to pyruvate. Thus, the pyruvate can be recycled.

The set of reactions described provide a means for eliminating ammonia. Arginine has four amino groups: the first two amino groups are eliminated in the form of urea which is less toxic than ammonia, the third is eliminated in the form of proline and the fourth as alanine (Kurelec, 1975a).

The other amino acid which has a high concentration in *F. hepatica* is histidine. Kurelec, Rijavec and Klepac (1969), using radioactive histidine, found that there is an active histidine decarboxylase in *F. hepatica* which converts histidine to histamine. They consider that due to the high levels of histidine in fluke tissue certain aspects of toxicology of *F. hepatica* are due to the release of
1.8 FATTY ACID OXIDATION

In mammals, lipids are hydrolysed to free fatty acids and glycerol before oxidation and energy production. The process is referred to as β-oxidation because the oxidation occurs at the β-carbon atom of the chain. It leads to the step-wise removal of acetic acid equivalents from the carbon chain. The products of the process are eventually oxidised to completion by the reactions of the TCA cycle.

Lipids are not utilised by anaerobically or aerobically maintained helminths such as *M. expansa* (von Brand, 1973), *H. diminuta* (Fairbairn, Wertheim, Harpur & Schiller, 1961), and *A. lumbricoides* (von Brand, 1973).

In adult *F. hepatica* lipid constitutes about 12-13% of the dry weight (Weinland & von Brand, 1926). Histologically it appears to be mainly associated with the excretory system (Erasmus, 1972). Moss (1970) has reported that neutral triglycerides are present in the protonephridial system.

Barrett and Körting (1976) examined the enzymes of β-oxidation in *F. hepatica* and the ability of the parasite to oxidise fatty acids. It was found that all the enzymes necessary for β-oxidation are present but no guanosine 5'-triphosphate (GTP) specific acyl-CoA synthetase could be detected. Some of the enzymes have low activities compared with mammalian tissues which have an active β-oxidation pathway, e.g. 3 hydroxyacyl-CoA dehydrogenase, while the level of acyltransferase is higher. *F. hepatica* resembles
A. lumbricoides in the relative activities of the enzymes (Ward & Fairbairn, 1970), although the specific activities of the enzymes in F. hepatica are much lower.

The functioning of the β-oxidation pathway was also examined by Barrett and Körting (1976) using radioactive palmitate. It was found that there is no significant production of radioactive CO₂ although palmitate is taken up and incorporated into the neutral and phospholipid fractions. Thus, adult F. hepatica does not seem to have a functional β-oxidation pathway despite a full complement of the enzymes required. This may be due to the TCA cycle being of minimal importance in the oxidative metabolism of F. hepatica. It is suggested by Barrett and Körting (1976) that the enzymes of the β-oxidation pathway might be involved in the metabolism of volatile acids, as has been suggested in adult A. lumbricoides by Ward and Fairbairn (1970), or they may be involved in the malonyl-CoA dependent elongation of long chain fatty acids (Seubert, Lamberts, Kramer & Ohly, 1968). Alternatively the enzymes may be concentrated in the developing eggs in preparation for the free-living miracidial stage.
2.1 PATHOGENICITY OF F. HEPATICA

The pathogenicity of fluke infection depends upon the number of organisms which invade the liver and mature in the bile ducts. When large numbers of metacercariae are ingested over a short period the immature flukes cause extensive destruction of the liver parenchyma and produce many haemorrhagic tracts as they migrate. If excessive numbers of these tracts occur, an acute, traumatic, haemorrhagic hepatitis may result with rupture of the liver capsule and fatal haemorrhage into the peritoneal cavity (Soulsby, 1965; Lapage, 1968).

With lower infection rates the host survives the first onslaught and as the flukes gain the bile ducts and mature, the chronic form of the disease supervenes. This is characterised by anaemia, thickening of the bile ducts and marked cirrhosis of the liver. It may occur to such an extent that there appears to be little or no normal liver tissue left (Soulsby, 1965; Lapage, 1968).

Superimposed on these injuries, secondary bacterial infections may occur. In sheep the tissue damage caused by the migrating flukes provides a suitable environment for the rapid multiplication of the spores of the bacterium Clostridium oedematiens, lying dormant in the liver. Hepatic necrosis and toxaemia occur leading to the notorious 'black disease' and early death (Lapage,
Concurrent infections with the abomasal nematode, *Ostertagia ostertagi*, which causes diarrhoea, complicates the clinical picture in bovine fascioliasis (Armour, 1975).

### 2.2 ECONOMIC IMPORTANCE OF *F. HEPATICA*

It is difficult to assess accurately the losses sustained by livestock as the result of fluke infection, but there is no doubt that over the years the economic loss from fascioliasis has been large. Losses are due not only to the deaths caused by the parasite, which in some years reaches as high as 25% (Sneddon, 1967), but the deterioration in wool and mutton value of the surviving sheep is considerable (Sneddon, 1967).

In adult cattle the liver fluke is responsible for a sub-clinical disease with only minor haematological changes but a lowered productivity reflected by inadequate food conversion rates, poor carcass formation and reduced milk yields. Loss of revenue also results from the condemnation of fluke infected livers at the abattoir (Armour, 1975).

Olsen (cited by Cheng, 1973) has reported, as the result of an 11 year study in the United States, that the livers of 1,400,000 cattle and 60,500 calves have been condemned due to fascioliasis. The annual loss represents nearly $1 million. The economic importance of fascioliasis in the United Kingdom is currently estimated to cost around £50 million per annum (Coles, 1975a). In Australia it is estimated that 25% of the sheep and cattle population are grazing on potentially endemic pastures and there are severe economic losses due to mortality, condemned livers, reduction of meat and milk production,
secondary bacterial infection and wastage through frequent anthelmintic treatment (Boray, 1969).

2.3 CONTROL OF THE INTERMEDIATE HOST

The elimination of the snail intermediate host would result in the elimination of fascioliasis; however, many factors operate against such an eventuality. The snails aestivate in mud or marshy ground through long periods of drought. They are also prolific breeders and a single snail may give rise, in the course of two generations, (i.e. normally one year) to 25,000-160,000 individual snails. If attempts to destroy the snails leave only one individual alive, this snail may produce in a few months enough snails to replace those that have been destroyed (Sneddon, 1967; Boray, 1969).

Ideally, the best approach to the control of fascioliasis is by an integration of the available control methods. These are:
1. improved drainage to eliminate the habitats of the snail,
2. the use of molluscicides to limit snail populations, and
3. the strategic use of anthelmintics to reduce pasture contamination with fluke eggs.

Improved drainage represents the best long-term approach to fluke control. However, some areas are not easily drained for topographical reasons. A reduction in the snail population is possible in limited areas with repeated and regular use of molluscicides.

Copper and sodium pentachlorphenate, N-trityl-morpholine (trade name, 'Frescon'), and 5,2'-dichloro-4'-nitrosalicylic anilide (trade name, 'Bayluscide') have been used successfully as molluscicides (Sneddon, 1967; Boray, 1969; Armour, 1975).

*Shell Chemical (Aust) Pty Ltd
†Farbenfabriken Bayer, Germany
Throughout Australia the areas and conditions where snail control can be effectively carried out are limited. It is necessary to treat stock regularly with a fasciolicide to reduce the incidence of infection in the stock and the intermediate host.

2.4 CHEMOTHERAPY OF FASCIOLIASIS

Despite the fact that *F. hepatica* has been a continuous problem to animal health for many years, no compound is yet available which is completely satisfactory for the treatment of fluke infection. In the majority of cases the useful preparations are active against the late immature and the mature stages of the parasite but have little or no effect against the earlier immature stages.

2.4.1 EARLY TREATMENTS

One of the earliest drug treatments used was an extract of male fern, *Aspidium filix-mas*. Montgomerie (1926a) cites Perroncito (1913) as being the first to report its efficiency against flukes when, in 1881, in trial usage for the treatment of hookworm infestation in man, he observed it also expels liver flukes. In the same publication observations made in 1886 are referred to showing that male fern cures fluke infections in sheep and cattle.

In 1917 an extract, containing the active principle filicic acid, was marketed in Hungary under the name of Distol and in Britain as Danistol. Two other anthelmintics, kouso and kamala, contain similar substances and their toxic action was well recognised; male fern was used many years previously to produce abortions in addition to the expulsion of tapeworms.

Montgomerie (1926a) carried out numerous trials with the
extract with highly satisfactory results against adult flukes. The drug is inactive against immature stages thus greatly reducing its value during serious outbreaks of fascioliasis. At such times the heavily infected sheep die before the majority of flukes have reached the stage of development at which they are susceptible to drug action.

2.4.2 CHLORINATED HYDROCARBONS

Chlorinated hydrocarbons have been used extensively for many years as fasciolicides and they are still in use in many countries. Carbon tetrachloride (CCL$_4$) is the most common one. Montgomerie (1926b) was one of the first to report its efficacy against fluke. Its efficiency is not greatly superior to male fern extract, but being a simple manufactured chemical, its constitution and its activity are much more dependable. Furthermore, it is cheaper, less toxic, readily available throughout the world and can be administered orally by capsule, in solution or in suspension. In Australia it is customary to mix CCL$_4$ with liquid paraffin in a ratio of 1:4; a dose of 5 ml of the mixture is given to sheep.

There are two major drawbacks to the use of CCL$_4$; it may occasionally induce serious toxic symptoms in sheep and death in cattle, and it is ineffective against immature flukes (reviewed by Kendall & Parfitt, 1962). The pathology of CCL$_4$ poisoning is liver damage with central necrosis and fatty change, causing severe liver dysfunction. It also interferes markedly with kidney function (Setchell, 1959; Boyd, 1962; Kearney, Connolly & Downey, 1967; Anwer, Engelking, Gronwall & Klentz, 1976).

Some of the other chlorinated hydrocarbons which have been used against *F. hepatica* are hexachloroethane (C$_2$C$_6$L$_6$).
tetrachlorofluoroethane (Freon 112) and hexachloroparaxylol (Hetol). These drugs also have no effect on immature flukes and often cause deaths and toxic symptoms in the host (reviewed by Kendall & Parfitt, 1962; Lämmler, 1968; Burrows, 1973). \( \text{CCl}_4 \) has an advantage over \( \text{CCl}_6 \) as it has a greater safety margin in cattle. Hetol is less toxic than both \( \text{CCl}_4 \) and \( \text{CCl}_6 \).

The flukes affected by Freon 112 show ascending necrosis of the body and in some cases the whole body, except the oral and ventral suckers, is affected (Baray & Pearson, 1960a,b). A strong contraction of the uterus is also observed and all the eggs in it are extruded. Eggs from treated flukes are adversely affected in their development and hatching.

2.4.3 OTHER FASCIOLICIDES

Tables 2.1 and 2.2 list some of the successful fasciolicides with their chemical formulae and structures, dose rates and some of the effects that have been observed in the flukes and hosts.

It is not possible to get a general picture of the effectiveness of these fasciolicides from reviewing the available literature, as most of them have been tested under conditions which do not allow a direct comparison of efficacy. For example, some are tested on natural infections, others on experimental infections; the mode of administration may not be similar; conditions of pastures and the season of the year are not standardised. Until all the drugs have been tested and compared under comparable conditions it is difficult to evaluate them.

Boray (1969) discusses the application of standardised
<table>
<thead>
<tr>
<th>Compound</th>
<th>Common Name(s)</th>
<th>Chemical Formula</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon tetrachloride</td>
<td></td>
<td>CCl$_4$</td>
<td><img src="image" alt="Cl_4 structure" /></td>
</tr>
<tr>
<td>Hexachloroethane</td>
<td>Fasciolin</td>
<td>C$_2$Cl$_6$</td>
<td><img src="image" alt="hexachloroethane structure" /></td>
</tr>
<tr>
<td>Tetrachlorodifluoroethane</td>
<td>Freon 112, Areton</td>
<td>a mixture of 2 isomers; 1,2 difluorotetrachloroethane and 1,1 difluorotetrachloroethane</td>
<td><img src="image" alt="tetrachlorodifluoroethane structure" /></td>
</tr>
<tr>
<td>Hexachloroparaxylol</td>
<td>Hetol</td>
<td>1,4-bis-trichloromethyl-benzol</td>
<td><img src="image" alt="hexachloroparaxylol structure" /></td>
</tr>
<tr>
<td>Hexachlorophene</td>
<td>Bilevon</td>
<td>2,2'-methylene bis(3,4,6-trichlorophenol)</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Bithionol</td>
<td>Bitin, Actamer</td>
<td>bis(3,5-dichloro-2-hydroxyphenyl) sulphide</td>
<td></td>
</tr>
<tr>
<td>Bithionol sulphoxide</td>
<td>Bitin-S</td>
<td>bis(3,5-dichloro-2-hydroxyphenyl) sulphoxide</td>
<td></td>
</tr>
<tr>
<td>Niclofolan</td>
<td>Menichlopholan, Bayer 9015, Bilevon M</td>
<td>2,2'-dihydroxy-3,3'-dinitro-5,5' dichlorophenyl</td>
<td></td>
</tr>
<tr>
<td>Bromophenophos</td>
<td>Acedist</td>
<td>4,4',6,6'-tetrabromo-2,2'-biphenyldiolmono(dihydrogen phosphate)</td>
<td></td>
</tr>
<tr>
<td>COMPOUND</td>
<td>COMMON NAME(S)</td>
<td>CHEMICAL FORMULA</td>
<td>STRUCTURE</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hilomid and Diaphene</td>
<td></td>
<td>Hilomid is a 1:1 mixture of a &amp; b Diaphene is a 3:1 mixture of a &amp; b a is tribromo-3,4'-5-salicylanilide b is dibromo-4',5-salicylanilide</td>
<td><img src="hilmid.png" alt="Diagram" /> <img src="diaphene.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Clioxanide</td>
<td>Tremerad</td>
<td>4'-chloro-2-hydroxy-3,5-diiodobenzanilide acetate</td>
<td><img src="clioxanide.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Oxyclozanide</td>
<td>Zanil</td>
<td>2,2'-dihydroxy-3,3'-5,5'-6-penta chloro-2,2'-dihydroxybenzanilide</td>
<td><img src="oxyclozanide.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Compound</td>
<td>Common Names</td>
<td>Chemical Structure</td>
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</tr>
<tr>
<td>-------------------</td>
<td>------------------------------------</td>
<td>---------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Rafoxanide</td>
<td>Ranide, Flukanide</td>
<td><img src="image" alt="Rafoxanide" /></td>
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<tr>
<td>Bromoxanide</td>
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<tr>
<td>Disophenol</td>
<td>Ancylol, DNP, Disofen</td>
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<tr>
<td>Nitroxynil</td>
<td>Trodax, Dovenix</td>
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<tr>
<td>Diamphenethide</td>
<td>Coriban</td>
<td><img src="image" alt="Diamphenethide" /></td>
<td></td>
</tr>
</tbody>
</table>

Chemical structures for Rafoxanide, Bromoxanide, Disophenol, Nitroxynil, Diamphenethide.
TABLE 2.2

Fasciolicides and the dose in mg/kg required to remove 90% of *F. hepatica*, the safety index (S.I.), the maximum tolerated dose (MTD) and the effects observed on the flukes and hosts.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>ADULTS</th>
<th>JUVENILES</th>
<th>MTD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose</td>
<td>S.I.</td>
<td>Dose</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>80</td>
<td>10</td>
<td>640</td>
</tr>
<tr>
<td>Hexachloroethane</td>
<td>300&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4</td>
<td>1800</td>
</tr>
<tr>
<td>Freon 112</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hetol</td>
<td>150</td>
<td>4</td>
<td>1200</td>
</tr>
<tr>
<td>Hexachlorophene</td>
<td>15</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Bithionol</td>
<td>4</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Niclofolan</td>
<td>2.7</td>
<td>4.4</td>
<td>9</td>
</tr>
<tr>
<td>Bromophenophos</td>
<td>10</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Hilomid</td>
<td>20</td>
<td>3</td>
<td>120</td>
</tr>
<tr>
<td>Clioxyanide</td>
<td>15</td>
<td>6.7</td>
<td>135</td>
</tr>
</tbody>
</table>
PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS

1. liver and kidney damage in host\textsuperscript{b}
2. lipid solvent action on cell membranes\textsuperscript{c}

- \textsuperscript{d}

1. ascending necrosis of fluke's body; egg development and hatching affected\textsuperscript{e}

- \textsuperscript{f}

1. inhibits SDH activity in flukes\textsuperscript{f}
2. early onset of rigor mortis in mice; contraction of phrenic nerve-diaphragm; stimulation of ATP'ase activity in rat mitochondria\textsuperscript{g}
3. stimulation of oxygen uptake by flukes\textsuperscript{h}

1. inhibits SDH activity in flukes\textsuperscript{f}
1. inhibits SDH activity in flukes\textsuperscript{f}
2. early onset of rigor mortis in mice; contraction of phrenic nerve-diaphragm; stimulation of ATP'ase activity in rat mitochondria\textsuperscript{g}
1. retarded, abnormal and reduced fluke egg production\textsuperscript{j}
2. early onset of rigor mortis in mice; contraction of phrenic nerve-diaphragm; stimulation of ATP'ase activity in rat mitochondria\textsuperscript{g}

MODE OF ACTION

unknown
unknown
unknown
unknown
possibly an uncoupler of phosphorylation
unknown
possibly an uncoupler of phosphorylation
possibly an uncoupler of phosphorylation
unknown
unknown
<table>
<thead>
<tr>
<th>COMPOUND</th>
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<th>MTD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose</td>
<td>S.I.</td>
<td>Dose</td>
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<tr>
<td>Oxyclozanide</td>
<td>15</td>
<td>4</td>
<td>135</td>
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<tr>
<td>Rafoxanide</td>
<td>6.7</td>
<td>8.9</td>
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<td>Bromoxanide</td>
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<td>30</td>
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<tr>
<td>Disophenol</td>
<td>15</td>
<td>2.7</td>
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<tr>
<td>Nitroxynil</td>
<td>6.7</td>
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<td>30</td>
</tr>
<tr>
<td>Diamphenethide</td>
<td>120</td>
<td>3.3</td>
<td>80</td>
</tr>
</tbody>
</table>
### PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS

<table>
<thead>
<tr>
<th>EFFECT</th>
<th>MODE OF ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Early onset of rigor mortis in mice; contraction of phrenic nerve-diaphragm; stimulation of ATP'ase activity in rat mitochondria.</td>
<td>Possibly an uncoupler of phosphorylation.</td>
</tr>
<tr>
<td>1. Retards development of surviving flukes; incomplete development of vitelline follicles; spermatogenesis disrupted.</td>
<td>Unknown.</td>
</tr>
<tr>
<td>2. Stimulates oxygen uptake by <em>H. diminuta</em> mitochondria; inhibits it at high concentrations.</td>
<td>Unknown.</td>
</tr>
<tr>
<td>3. Inhibits malate-induced $^{32}$Pi incorporation into ATP in <em>Ascaris</em> mitochondria.</td>
<td>Unknown.</td>
</tr>
</tbody>
</table>

### MODE OF ACTION

- Possibly an uncoupler of phosphorylation.
- Unknown.
REFERENCES FOR TABLE 2.2

a Boray & Happich (1968);
b Setchell (1959), Anwer et al. (1976), Kearney et al. (1967);
c Gallagher (1961);
d Lämmler (1968);
e Boray & Pearson (1961a,b);
f Panitz & Knapp (1970);
g van Miert & Groeneveld (1969);
h Corbett & Goose (1971);
i Boray, Happich & Andrews (1967);
j Burrows (1973);
k Edwards & Parry (1972a);
l Armour & Corba (1970), Boray (1969), Mrozik, Jones, Friedman, Schwartzkopf, Schardt, Patchett, Hoff, Yakstis, Riek, Ostlind, Plishker, Butler, Cuckler & Campbell (1969);
m Stammers (1975);
n Yorke & Turton (1974);
o Van den Bossche (1972b);
p Theodorides, Parish, Fuschsman & Lee (1974);
q Stammers (1976);
r Edwards & Parry (1972b).
techniques when anthelmintics are tested and compared for efficacy. He has carried out several trials under standardised conditions. Most of the data in table 2.2 on dose rates are taken from these trials and are therefore comparable. The maximum tolerated dose (MTD) is determined from standardised acute toxicity tests and is the maximum dose which is tolerated without serious clinical symptoms. The safety index (SI) is the maximum tolerated dose divided by the dose actually used.

Table 2.2 shows that all the drugs except diamphenethide are more effective against adult than immature flukes. A dose rate of 100 mg/kg of diamphenethide is 100% effective against flukes as young as one day of age (reviewed by Rowlands, 1974). Most of the drugs which are effective against juvenile flukes also have a low safety index. Rafoxanide and diamphenethide seem to be the best choices for treatment of acute fascioliasis.

2.4.3.(a) DIAMPHENETHIDE

Kendall and Parfitt (1975) have found that diamphenethide is less active in cattle than sheep and at doses which are effective in sheep it is inactive against flukes in rats and rabbits (Coles, 1975b). Hayes, Bailar and Mitrovic (1974) also showed that diamphenethide, at a concentration of 0.1%, shows no activity against either immature or mature *F. hepatica* in the rat, when administered in the diet. Harfenist (1973) has shown that there is no effect of the drug on flukes incubated *in vitro*.

It has been found that it is the free amine of diamphenethide, but not diamphenethide itself, that is active against mature flukes in the rat (Coles, 1976). Rat liver extracts are not able to deacetylate the drug to the free amine. This confirms the suggestion of Coles
that failure to deacetylate diamphenethide by the rat is the reason for its inactivity. However, this is not the reason in those species where diamphenethide, at similar concentrations, is not as fully active as in sheep, e.g. the mouse (Coles, 1976), the cow (Kendall & Parfitt, 1975) or the rabbit, in which it is inactive (Coles, 1975b). These hosts are capable of deacetylating diamphenethide (Coles, 1976). It is possible, in these species, that there are high rates of breakdown of the amine to inactive constituents, or rapid excretion of the active moiety, or the relative rates of deacetylation and acetylation may be such that there is a very low concentration of free amine (Coles, 1976). These findings emphasise that activity of potential anthelmintics should always be evaluated in the final fluke host, the sheep or cow.

Another advantage of diamphenethide is the lack of toxicity so far shown. It was thought in the past that, where death of F. hepatica occurs in the liver parenchyma, the resultant pathology may prove detrimental to the host. A study by Armour and Corba (1972) shows this is not the case. They measured various parameters and found that rapid healing of the liver takes place. Hughes, Treacher and Harness (1974) examined the plasma enzyme changes in goats treated with diamphenethide after infection with F. hepatica. They found no rise in plasma enzymes when flukes are killed by the drug one week after infection, probably because the majority of flukes are killed in the body cavity and have not penetrated the liver. If treatment is delayed until three weeks after infection, when liver damage occurs (as shown by an increase in sorbitol and glutamate dehydrogenase activities) the enzyme levels fall rapidly to normal on the death of the flukes. There
is no evidence that progressive liver damage occurs after death of the flukes.

2.4.4 EFFICACY AGAINST IMMATURE VERSUS MATURE FLUKES

Apart from diamphenethide, all the fasciolicides so far produced are more active against the adult flukes than the immature stages. Higher doses must be used to increase the efficacy against juvenile flukes. The reason for this has not been determined. There are several differences between adult and juvenile flukes which may be responsible for, or at least contribute to, the relative insusceptibility of the juvenile flukes; they are different in size and stage of development, they occupy different sites in the host, they probably feed on different tissues and they may have differences in metabolism.

Kendall and Parfitt (1962) suggest that the resistance of the young flukes is due to their different state of development and not to their location in the host. The time spent in the peritoneal cavity by migrating flukes is very short (approximately three days in sheep) (Kendall & Parfitt, 1962). They suggest that once in the liver tissue the flukes are exposed to the drugs that attain a therapeutic concentration in the blood and tissue fluids, no matter what their age. However, they do not consider the feeding habits of the flukes. The adult flukes probably consume a greater concentration of drug due to their greater uptake of blood. The juvenile, migrating flukes appear to feed more on liver tissue (see 1.5.1).

The studies with diarnphenethide support this hypothesis. Adult and immature flukes are not affected by the addition of diamphenethide to the *in vitro* culture medium (Harfenist, 1973). It is
known that diamphenethide must be metabolised to the deacetylated form (see 2.4.3.(a)). When this is added to the culture medium the flukes die very quickly. Also, if diamphenethide is added with liver cells, which still contain deacetylating enzymes, to the culture medium, the flukes die. It is suggested by Harfenist (1973) that the immature flukes in the liver tissue are exposed to a much higher concentration of the drug because of the deacetylation of the compound by the liver cells. The active component is further metabolised and detoxified in the liver so that the concentrations in the blood or other host tissues are never very high; hence the low activity of this drug against the mature flukes. In the case of diamphenethide, it seems that it may be the site occupied by the flukes and probably also their feeding habits which make the juvenile flukes more susceptible.

There is evidence that there are differences in metabolism between the various stages of the life cycle of *F. hepatica* (Bryant & Williams, 1962; Metzger & Düwel, 1974). It is possible that a drug may attack an active pathway in the juvenile flukes which is not as important in the adult flukes.

2.5 BIOCHEMICAL STUDIES ON ANTHELMINTIC ACTION

Only a few studies have been carried out to examine the biochemical effects of fasciolicides on flukes and to attempt to explain their mode of action. The effects of some drugs on various parameters of mammalian hosts have been studied but it is necessary to conduct experiments on the flukes and examine any effects on their metabolism before a mode of action can be postulated.
Van Miert and Groeneveld (1969) have examined the effects of several fasciolicides (hexachlorophene, nitroxynil, niclofolan and bromophenophos) on small mammals and compared their activity with the known uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (DNP). They examined the effects of these compounds on the time of onset of rigor mortis in mice. Decapitation or a lethal dose of sodium pentobarbital results in rigor mortis occurring 60-90 min later. Following treatment with a lethal dose of DNP, post mortem rigidity occurs within 5-8 min. This effect is due to a decrease in the ATP concentration of striated muscle cells. Hexachlorophene (150 mg/kg), niclofolan (50 mg/kg), nitroxynil (50 mg/kg) and bromophenophos (50 mg/kg) cause a similar reduction in the time of onset of rigor mortis.

Another parameter examined by van Miert and Groeneveld (1969) is the phrenic nerve-diaphragm preparation of the rat. This has been used to study the effects of uncoupling agents in vitro on contractions of the striated muscle of the diaphragm (Barnes, Duff & Threlfall, 1955). DNP causes a decrease in contractions; those produced by phrenic nerve stimulation disappear first, followed by the gradual onset of a characteristic contracture. The four fasciolicides examined also show this response; however, bromophenophos is less active than the others.

The effect of these compounds on the stimulation of ATP'ase activity of freshly prepared rat liver mitochondria was compared with that of DNP. They all stimulate ATP'ase activity but again, bromophenophos is less active than the others (van Miert & Groeneveld, 1969).
It is concluded by van Miert and Groeneveld (1969) that these drugs are active uncouplers of oxidative phosphorylation in warm-blooded animals and the toxic symptoms found in the hosts under field conditions are explained by this mode of action.

Veendendaal and de Waal (1974) have carried out a similar study with oxyclozanide. A dose of 75 mg/kg causes post mortem rigidity within 1-3 min. It has a similar effect to DNP on the phrenic nerve-diaphragm preparation of the rat and also stimulates ATP'ase activity of rat liver mitochondria. The effect of oxyclozanide and DNP on the oxygen uptake of rat liver mitochondria was measured poligraphically. Low concentrations (1 µM) of oxyclozanide stimulate oxygen uptake and higher concentrations (10-20 µM) inhibit mitochondrial respiration. Oxyclozanide is more potent than DNP in this respect. It is concluded that oxyclozanide is an active uncoupler of oxidative phosphorylation in rodents.

Panitz and Knapp (1970) used histological techniques to examine the effects of bithionol, bromophenophos and hexachlorophene on the SDH activity of _F. hepatica_ sections. All three cause inhibition of SDH activity. Thorsell (1967) has also reported that hexachlorophene inhibits SDH activity in _F. hepatica_ exposed _in vitro_. However, inhibition of this enzyme _in vitro_ does not mean that this is the mode of action of the compounds _in vivo_. It has been mentioned earlier (1.6.5) that SDH appears to act in the reverse direction (i.e. as fumarate reductase) in the parasites so far studied. It is not known whether this is a different enzyme or whether it is SDH acting in reverse. Further work is needed to establish whether these drugs inhibit respiration in _F. hepatica_ due to inhibition of this enzyme.
By using sections of *F. hepatica* the permeability barriers normally present *in vivo* have been destroyed. The drugs may not reach this site of action under normal conditions.

Some studies have been carried out on the effects of some anthelmintics on fumarate reductase in some parasites. Prichard (1970) examined the effect of thiabendazole (TBZ) on fumarate reductase of *H. contortus*. It was found that 1 mM TBZ completely inhibits the fumarate reductase system. Malkin and Camacho (1972) reported that 0.5 mM TBZ significantly inhibits the fumarate reductase system of TBZ-sensitive *H. contortus* but at a concentration of 1.0 mM it has no effect on the fumarate reductase system of the TBZ-resistant strain. It is possible that the resistant worms are capable of utilising a different system for the reoxidation of NADH.

Düwel and Metzger (1973) have examined the inhibition of the SDH complex of the rat and *F. hepatica* by various substituted 2,6-dihydroxybenzanilides. The results, when compared with the field trials of the drugs against flukes in sheep show that there is no real correlation between the chemotherapeutic index and *in vivo* activity. It is suggested that absorption and metabolism of the drugs by the host may affect the drugs and also, that there are permeability barriers which must be overcome before the drug can get to the mitochondria of the fluke and the SDH/fumarate reductase complex.

Yorke and Turton (1974) examined the effects of oxyclozanide, rafoxanide, nitroxynil and diamphenethide on the oxygen uptake of isolated mitochondria from *H. diminuta*. Oxyclozanide, rafoxanide and nitroxynil, at low concentrations, stimulate oxygen uptake and higher concentrations inhibit oxygen uptake. This is similar to the action of
DNP (Huijing & Slater, 1961), and is consistent with the compounds acting as uncouplers of respiratory chain phosphorylation. The significance of inhibition of aerobic metabolism in parasites is unclear as most seem to live in fairly anaerobic conditions and have an anaerobic metabolism and energy production. Presumably, as electron transport systems are present in parasites, it is likely that uncouplers may disrupt electron flow and thus energy production.

Another biochemical study, by Corbett and Goose (1971) examines the effect of several fasciolicides (hexachlorophene, nitroxynil and oxyclozanide) on flukes. The oxygen uptake by the flukes in the presence of the drugs increases. The measurements were made using Warburg manometry. Three to five flukes were placed in each flask in 3 ml of medium. The results show that oxygen uptake was measured for 24 h which is an unusually long time for using manometric techniques. Flukes excrete acidic end products and the media need to be changed frequently. With 3-5 flukes in only 3 ml of medium for 24 h there will be a large build up of excretory products. These may have deleterious effects on the flukes as bile flow normally removes the waste products in vivo. The results presented by Corbett and Goose (1971) in a table show that the stimulation of oxygen uptake by the drugs is about 30% but in the graphs it appears to be only a marginal increase. The minimum uncoupling concentrations for rat liver mitochondria by hexachlorophene, nitroxynil and oxyclozanide are 0.6-0.8 µM, 27-35 µM and 0.3-0.4 µM respectively.

Corbett and Goose (1971) also measured the concentration of the drugs required to inhibit motility of flukes incubated in vitro within 24 h. However, the results do not include the length of time
control flukes remain capable of movement in the system. Three flukes were incubated in 10 ml of medium for 24 h. The build up of acidic end products may aid in the inhibition of motility. The results of the study by Corbett and Goose (1971) are badly presented and confusing. No conclusions can be drawn from them.

Metzger and Dwel (1973) examined the effects of a series of concentrations of 2,6-dihydroxy-3,5-dichlorobenzoic-4'-chloroanilide (DDBA) on some of the intermediate pool sizes of metabolic pathways and on SDH in flukes following in vitro incubation. The fumarate reductase system is inhibited by DDBA and the changes in the intermediate levels can be explained in terms of this.

It can be seen that only a small number of biochemical studies have been carried out on the effects of fasciolicides on flukes and none of these has led to the determination of their mode of action.

2.6 ANTHELMINTICS USED IN THE PRESENT STUDY

2.6.1 MEBENDAZOLE

Mebendazole (MBZ) is the generic name for methyl 5(6)-benzoyl-2-benzimidazole carbamate. Its structure is shown in figure 2.1. It has a wide spectrum of activity against gastrointestinal parasites of mammals, including Strongyloides, Trichuris, Trichinella, Ancylostoma, Enterobius, Ascaris, Hymenolepis, Moniezia and Toxocara species (Van den Bossche, 1972b). It is also effective against Echinococcus granulosus and Taenia hydatigena infections in dogs (Gemmel, Johnstone & Oudemans, 1975).

It has recently been demonstrated that the micronised form of
FIGURE 2.1

Mebendazole

\[
\begin{align*}
\text{NH - C - O - CH}_3
\end{align*}
\]

Nitroscanate

\[
\text{SCN} \quad \text{O} \quad \text{NO}_2
\]
MBZ is more effective than the coarse powder (Kelly, Chevis & Goodman, 1975). Heath, Christie and Chevis (1975) have reported the efficacy of micronised MBZ against experimental secondary hydatid cysts in mice, *Taenia pisiformis* cysticerci in rabbits and multiplying tetrathyridia of *Mesocestoides corti* in mice.

The first report of a benzimidazole derivative effective against *F. hepatica* is by Delak, Mijatovic and Mikacic (1965, cited by Kelly, Chevis & Whitlock, 1975). They found that a combination of thiabendazole and hexachlorophene is effective against adult *F. hepatica* in sheep. Another trematode, *D. dendriticum*, can be eliminated from sheep by thiabendazole (Guilhon, 1965, cited by Kelly *et al.*, 1975). Kelly *et al.* (1975) carried out a study to confirm their field observations that in sheep, fine-particle MBZ has anthelmintic efficacy against *F. hepatica* and a concurrent nematode infection. They found that single oral treatments have considerable anthelmintic activity against adult *F. hepatica*. At a dose rate of 12.5 mg/kg body weight there is a 42% reduction in faecal output of fluke eggs and a 59% reduction in total fluke numbers. At the highest dose level, 100 mg/kg, two out of five sheep recorded zero counts for fluke egg output (a total reduction of 91%) and there is a 94% reduction in fluke numbers. The 12.5 mg/kg treatment gives a greater than 99% reduction in nematode egg output and this is due to the elimination of adult worms. It is suggested by the authors that MBZ should be considered as an anthelmintic suitable for programmed alternation with traditional fasciolicides in areas where endemic ovine fascioliasis is complicated by concurrent nematode infections.

Thienpont (unpublished, cited by Chevis & Kelly, 1976) has
shown that MBZ, administered to sheep in a medicated ration, prevents the establishment of an artificial infection of *F. hepatica* when the medication is started two days before infection. Chevis and Kelly (1977) found that micronised MBZ has some activity against immature *F. hepatica* at a dose rate of 50 mg/kg in sheep. Its activity is greater against immature flukes in the bile ducts than against the migrating flukes.

Several *in vitro* studies have been carried out in an attempt to determine the mode of action of MBZ in several parasites. Van den Bossche (1972b) has examined the effect of MBZ on the uptake of glucose by some nematodes. It was found that incubation with MBZ interferes with their glucose uptake, decreases their glycogen reserves and also results in depletion of ATP levels. He concludes that the primary effect of the drug is to inhibit glucose uptake. As a result the parasite utilises its glycogen reserves but ultimately these are depleted; therefore there is a decrease in ATP formation. A further study by Van den Bossche and De Nollin (1973) shows that MBZ inhibits the uptake of 3-O-methyl glucose, fructose and amino acids in *Ascaris*.

The uptake of low molecular weight nutrients in *Ascaris* is an active process, i.e. energy requiring. If ATP synthesis is impaired in some way, similar results to those found in the *in vitro* studies would occur. A decrease in the parasite's ATP level would inhibit active transport of nutrients so the storage reserves would be utilised. A similar explanation can be given for the results of De Nollin and Van den Bossche (1973) on the effects of MBZ on *T. spiralis* larvae.

The results of Van den Bossche (1972a) support the hypothesis that MBZ has an effect on the energy metabolism of the parasites. He
found that it inhibits malate-induced $^{32}\text{Pi}$ incorporation into ATP in *Ascaris* mitochondria. Also, McCracken, Campbell and Blair (1976) have examined the effect of MBZ on glucose transport by *H. diminuta*. They found that administration of MBZ to the host has no effect on the velocity of $^3\text{H}$-glucose absorption of the parasite in short-term *in vitro* incubations. Further, MBZ has no effect on the initial rate of glucose absorption at any of the concentrations examined. They conclude that because of the absence of any direct inhibitory effect of MBZ on glucose influx in *H. diminuta*, an inhibition of glucose transport *per se* does not account for its anthelmintic efficacy in this parasite (McCracken, Campbell & Blair, 1976).

Rahman (1976) carried out a detailed study of the effects of MBZ on *M. expansa*, *in vitro* and *in vivo*. It was found that *in vitro*, glucose uptake from the medium is inhibited and the glycogen reserves of the parasite are depleted within a 30 min incubation time. There is also a marked decrease in the ATP levels and the total adenine nucleotides found in the worm, indicating an effect on the synthesis and/or turnover of adenine nucleotides. Other effects include a shift in carbon flow from the succinate producing pathway to the lactate producing one. Inhibition of two of the enzymes in the succinate producing pathway, PEPCK and fumarate reductase, was found. PEPCK was inhibited in the whole animal but not when it was isolated. This probably causes the shift in carbon flow. Similar changes in the intermediate and adenine nucleotide levels were found *in vivo* when infected lambs were treated with MBZ and the worms recovered 3 h and 6 h later. It is suggested by Rahman (1976) that the reduced energy levels found in the parasite following MBZ treatment are responsible
for the decrease in glucose uptake and the depletion of glycogen reserves.

There have been several studies on the cytochemical and ultrastructural changes induced by MBZ in various parasites. Borgers and De Nollin (1975) have described an effect of MBZ on the secretory granules in the intestinal cells of *Ascaris*. In the intestine of untreated *Ascaris* the secretory granules, which are continuously produced in the Golgi apparatus, are transported toward the apical cytoplasm and reach the outer compartment of the microvillus border. Soon after treatment with MBZ the transport of these organelles appears to be blocked; the terminal web area becomes devoid of secretory granules and, at the site of synthesis, there is excessive accumulation, and fusion of the granules occurs. Borgers, De Nollin, De Brabander and Thienpoint (1975a) found that the block in the transport of the secretory granules coincides with the disappearance of cytoplasmic microtubules which are involved in the transport of organelles within the cell. There are two possible effects of a prolonged intracytoplasmic storage of secretory substances:

1. deprivation of the microvillus absorptive surface of the protective coat of carbohydrates (glycocalyx) and of enzymes necessary for digestion and absorption of nutrients, may result in impaired feeding, and,

2. if the secretory substances contain proteolytic and hydrolytic enzymes which become active at the absorptive surface, and are therefore present in an inactive form during transport, they may be activated upon prolonged cytoplasmic storage and cause cellular damage. Cellular autolysis has been seen in *Ascaris* and *Syngamus* intestine
after 24 h and 48 h of treatment, respectively (Borgers et al., 1975a). Either effect will certainly result in the death of the parasite.

A similar effect on microtubules, accumulation of secretory substances in the Golgi areas, 'ballooning' of tegumental cells followed by destruction of the tegument is found in cysticerci of *Taenia taeniaeformis* after MBZ treatment (Borgers, De Nollin, Verheyen, Vanparijs & Thienpont, 1975b), and in *Hymenolepis nana* and *T. taeniaeformis* adults (Verheyen, Borgers, Vanparijs & Thienpont, 1976). It is suggested by the authors that the primary site of action of MBZ is the microtubular system as has been found in the absorptive cells of nematodes (Borgers, et al., 1975a).

The study carried out by Rahman (1976) shows marked effects on the ATP and total adenine nucleotide levels in *M. expansa* within 30 min contact with MBZ *in vitro* and within 3 h *in vivo*. The intracellular effects observed on nematodes (Borgers & De Nollin, 1975; Borgers et al., 1975a) and cestodes (Borgers et al., 1975b, Verheyen et al., 1976) take many hours to become apparent. It is suggested by Rahman (1976) that as ATP is necessary for the maintenance of intracellular structure, degeneration of the microtubules is probably due to the lack of ATP. It follows that the primary effect of MBZ could be on energy production. More work is required to link the biochemical and ultrastructural effects of the drug.

### 2.6.2 NITROSCANATE

Nitroscanate (Lopatol, 4-isothiocyano-4'nitrodiphenyl ether) is a new broad spectrum anthelmintic developed for dogs (Boray, Von Orelli & Sarasin, 1973). Its structure is given in figure 2.1.
Gemmel and Oudemans (1975) found that the drug is effective against *E. granulosus* and *T. hydatigena* in dogs, causing destrobilation and a reduction in worm numbers. A dose rate of approximately 1,000 mg/kg is required to kill *E. granulosus* but this dose is too high for the comfort of the dog. Two treatments of 250 mg/kg however, eliminated all the worms from the ten dogs used in the study. At 250 mg/kg there is some vomiting and diarrhoea and a transient 'tranquilising' effect in some dogs (Gemmel & Oudemans, 1975). A single treatment of 64 mg/kg eliminates *T. hydatigena* and, according to Boray *et al.* (1973), most species of nematodes and *Dipylidium caninum*. Recent work shows that the drug is highly effective against tapeworms and nematodes at 50 mg/kg and side effects are rare when the micronised form is used.

Nitroscanate is also effective against *F. hepatica* and some nematodes in sheep (Boray, personal communication). No biochemical studies have been carried out with this drug and its mode of action is unknown.

2.6.3 RAFOXANIDE

The efficacy of rafoxanide against mature and immature *F. hepatica* is shown in table 2.2 and its structure is given in table 2.1. It is a very effective and well tolerated fasciocide and is also effective against *H. contortus*, *H. nana* and *H. diminuta* and has some activity against *S. mansoni* (Mrozik *et al.*, 1969; Egerton, Yakstis & Campbell, 1970; Snijders, Horak & Louw, 1973).

Several *in vitro* studies have been carried out with rafoxanide, but not on whole parasites. Yorke and Turton (1974) have examined the effect of the drug on the oxygen uptake of isolated mitochondria from *H. diminuta*. It increases the rate of oxygen uptake
at low concentrations whereas at high concentrations it inhibits oxygen uptake. Van den Bossche (1972a) has found that rafoxanide inhibits malate-induced $^{32}$Pi incorporation into ATP in isolated *Ascaris* mitochondria. These two observations are characteristic of the action of uncouplers of oxidative phosphorylation in mammalian mitochondria. It is not known whether uncouplers have the same action in parasites which have mainly anaerobic energy production and different electron transport systems from mammals.

It has been noted (Presidente & Knapp, 1972) that flukes surviving treatment with rafoxanide are considerably shorter than flukes recovered from untreated control sheep. Stammers (1975) has investigated this in flukes recovered from rabbits which were treated with 6.7 mg/kg of rafoxanide two, four, six and eight weeks after infection. The surviving flukes are not as heavy as control flukes suggesting that the growth rate is retarded. Histological examination shows that abnormalities are apparent. Flukes recovered four weeks after treatment show incomplete development of vitelline follicles. Testes smears show that spermatogenesis is also disrupted (Stammers, 1975).

Stammers (1975) suggests that the disruption of spermatogenesis in surviving flukes may be associated with the uncoupling activity of the drug since the amount of ATP available for cell division will be reduced. Indeed, it is likely that the retarded growth rate could also be due to a lack of ATP.

2.7 SCREENING OF ANTHELMINTICS

The most reliable method for producing new anthelmintics at
present is the synthesis of large numbers of compounds which are then screened for activity against various parasites. The development of a new remedy takes about six to ten years. Ten to 20 years ago one could expect that out of every 2,000 newly manufactured chemical compounds one would develop into a marketed preparation. 6,000-8,000 syntheses are required today and the amount of additional work has increased to the same extent (Dewel, 1975).

The testing of anthelmintics involves the grading of individual chemical compounds for their efficiency in experimentally infected animals. This is very costly in terms of material consumption, particularly in the case of large animals, or impractical, as in humans. Tests can be done more quickly and less expensively on small animals but many problems can arise because the animals may not be the normal host of the parasite and chemical substances often behave differently in different hosts. Typical examples of this are the derivatives of salicylic acid which are ineffective against F. hepatica in laboratory animals but have an excellent effect in ruminants (Boray et al., 1967). Also, diamphenethide is active against flukes in the sheep (Kingsbury & Rowlands, 1972; Rowlands, 1974), less effective in the cow (Kendall & Parfitt, 1975) and inactive in the rat and rabbit (Coles, 1975b).

After successful screening the compound is studied further to determine in detail its efficacy and toxicity. The structurally related compounds are also examined fully in an attempt to produce the best combination of efficacy and non-toxicity. As the function of anthelmintics is to remove helminth parasites from their hosts, most work has been done on the activity and efficacy of the compounds, or on
their toxicity to the host (reviewed by Lämmler, 1968; Gibson, 1969; Burrows, 1973). The action of the drug on the metabolism of the parasite or host is generally considered to be of minor importance. The lack of information on the mode of action of anthelmintics is partly due to the difficulty of studying the effects of drugs on a metabolic system where little is known about the normal functioning of that system, as is the case for most parasites.

There is a need for a better understanding of the relationship between the biochemical action of anthelmintics and their chemotherapeutic effects in order to disclose mechanisms essential for the functional integrity of the parasite and also point out which of them are liable to be inhibited by chemical agents. These data in turn should provide the pre-requisites for the rational design of chemotherapeutic agents (Bueding, 1969).

The ideal anthelmintic should affect a process in the parasite which is absent in the host, or at least one of minor importance, to avoid any effect on the host. Saz (1970) has pointed out that there are striking differences between the metabolism of the host and that of the parasite. Such differences range from the levels of protein structure and enzyme kinetics to the levels of metabolic pathways, nutritional requirements and physiological activity of corresponding tissues. It is possible that a knowledge of the reactions of the parasite which are different from the host could result in the development of a simple, systematic screening procedure for new anthelmintics. It would also provide some information for understanding drug action.
The pathways of energy metabolism in parasites are different from their hosts. Common modifications include the excretion of reduced end products, the fumarate reductase reaction and electron transport systems. Anthelmintics which act at these sites will be successful. However, if a drug acts on another essential pathway of the parasite which disturbs the energy producing pathway and the ability of the parasite to maintain its adenine nucleotide levels, it will also be effective. When adverse changes in the energy producing pathway and adenine nucleotide concentrations are detectable the parasite has been stressed beyond its capacity to adapt.

2.8 EXPERIMENTAL STRATEGY AND STUDY PLAN

The purpose of this thesis was to examine the effects of several anthelmintics, known to be active fasciolicides, on flukes maintained in vitro for certain periods. Various parameters were measured in an attempt to find an indicator of anthelmintic effect which could be used as an adjunct to the present unsatisfactory screening techniques and also to provide some information on the mode of action of the drugs.

*F. hepatica* is a suitable parasite to work with as it can be obtained in large quantities from the abattoirs or from experimental infections throughout the year and it is easy to maintain in the laboratory using simple incubation techniques. A lot of information is available on the metabolic pathways of flukes, in particular those of carbohydrate metabolism and energy production. The techniques for measuring most of the intermediates of these pathways have been adapted for use in the tapeworm *M. expansa* (Behm, 1974) and some of
them have been used to measure compounds in *F. hepatica* (Metzger & Düwel, 1973; Prichard, 1976).

The three fasciolicides chosen were mebendazole, nitroscanate and rafoxanide as they are unrelated structurally. Flukes were incubated *in vitro*, samples were taken at various times and many parameters were measured. It was established that the control flukes were able to maintain normal energy metabolism during the incubations. The effects of the drugs on these parameters were examined and provided information about the mode of action. *In vivo* experiments were then carried out to correlate the *in vitro* effects with the action of the drugs on the flukes within the host.
CHAPTER 3

IN VITRO MAINTENANCE OF F. HEPATICA

3.1 INTRODUCTION

There is a need for the development of a simple reliable in vitro system for the evaluation of anthelmintic effect and for the elaboration of new anthelmintics. The present screening programmes are very expensive in time, labour and animals. It is possible that there may be more subtle indicators of anthelmintic efficiency which can be tested relatively simply in a cheap in vitro experimental situation.

Many attempts have been made to culture adult F. hepatica in vitro using different types of media and different methods of preparation of the flukes before culture (Stephenson, 1947a; Dawes, 1954; Rohrbacher, 1957). Taylor and Baker (1963) review the various techniques and conclude that for prolonged survival in vitro it is necessary to obtain the flukes from freshly killed animals and to transfer them immediately to a suitable medium at 37° before attempting culture experiments. Van Noordwijk and de Wolf (1963) reported the successful use of the medium used by Dawes (1954), Hédon-Pleig solution, modified by the addition of benzylpenicillin, streptomycin and 45/1 glucose. For longer incubations sheep serum was added. The flukes (two per 10 ml of medium) remained in good condition, as measured by movement and lack of degeneration, for at least eight days.

The methods used for in vitro culture in the present study...
are very simple, require only a small amount of preparation and attention and many incubations can be carried out simultaneously. It is also easy to change the system, by the addition of an anthelmintic, and to monitor the effects.

If the metabolic differences between a parasite and its host are monitored during *in vitro* incubation of the parasite with an anthelmintic, information on its efficacy and mode of action should be obtained. For the screening of new drugs it would be more worthwhile to monitor a pathway which, when disturbed, will result in the death of the parasite. One such pathway is the major energy producing pathway. If a parasite's capacity to synthesise ATP is irreversibly affected the parasite will be unable to maintain its position within the host and will die.

During carbohydrate metabolism in *F. hepatica*, ATP is generated at the following points: at substrate level in the pathway to PEP; in the reactions catalysed by PEPCK and PK; at the fumarate reductase step; and in the conversion of succinate to propionate. Thus, all but the acetate producing branch of the pathway contribute to the energy economy of the parasite. The acetate producing branch provides reducing equivalents which can be utilised for energy production and which contribute to the redox balance within the parasite. Disturbances in the overall flow of carbon, or in the proportion of flow received by each branch of the pathway, may alter the energy economy of the parasite. Presumably, adjustments are made by the parasite to the *in vitro* condition. It is essential, therefore, to establish that these adjustments fall within the normal capacity of the fluke to adapt. This can be judged by examining the flow through
the pathways, fluctuations in pool sizes, end product formation and, most importantly, levels of adenine nucleotides.

3.2 METHODS

(a) COLLECTION OF FLUKES

Livers of sheep freshly slaughtered at the Canberra Abattoirs were transported to the laboratory. Adult flukes were removed from the ducts and gently washed in warm Hédon-Fleig salt solution (see appendix 1). Some were used immediately for glycogen determinations and intermediate assays and are termed 'O' time. The others were incubated in fresh medium containing glucose, streptomycin and penicillin (see appendix 1) at 37° for several hours. When their caecal contents and eggs had been ejected they were transferred to the appropriate experimental vessel.

(b) INCUBATIONS

The medium used for all incubations described in this thesis was Hédon-Fleig containing glucose, streptomycin and penicillin. Its composition is given in appendix 1. Suppliers of biochemicals are given in appendix 2.

The incubations were carried out at 37° in 100 ml conical flasks containing 50 ml medium. At the appropriate time flukes (approximately 1 g wet weight) were transferred to the incubation vessel. When long incubations were carried out, the media were changed every 8-12 h and samples of the media were taken for analysis of excreted end products and glucose uptake.

(c) PREPARATION OF PERCHLORIC EXTRACTS

At the end of the incubation the flukes were removed, rinsed several times in Hédon-Fleig salt solution and blotted gently. They were then immediately frozen in liquid nitrogen and powdered manually.
under liquid nitrogen using a stainless steel pulveriser. The powder was added to a preweighed beaker containing 5 ml of 6% HClO₄ which was maintained on ice. The beaker was reweighed and then the mixture was mixed thoroughly with a glass rod. The slurry was kept on ice until soft enough to homogenise. Subsequent operations were carried out at 0-2°. The preparation was homogenised with an all-glass Dounce-type homogeniser and then the precipitated protein was removed by centrifugation for 15 min at 8,000 g. The supernatant was neutralised with 5N KOH to approximately pH 6.5 and left on ice for complete precipitation of KC1O₄. The precipitate was removed by centrifugation as above and the final volume was measured.

(d) INTERMEDIATE ASSAYS

Spectrophotometric enzymatic assays of intermediates were carried out at 340 nm using a Gilford 2400 recording spectrophotometer and at 334 nm in an Eppendorf 1101M recording spectrophotometer, with the light paths of 1 cm. The assay methods were the same as described by Behm and Bryant (1975a) which were taken from Bergmeyer (1963 and 1974), and used with little modification except for proportional adjustments for differences in final volume in the cuvette. Standard solutions were assayed to check for reliability and standard samples of intermediates were added to F. hepatica extracts to check that total recovery occurred. Reagent blanks, which had the appropriate enzyme additions, were used in each assay to correct for contamination of reagents with some of the intermediates to be measured (e.g. AMP in NADH and 3PGA in commercial 2,3-diPGA preparations).

(i) Dihydroxyacetone-phosphate (DHAP), glyceraldehyde 3-phosphate (G3P) and fructose 1,6-diphosphate (FDP) were assayed in the same
cuvette by the method of Bücher and Hohorst, in Bergmeyer (1963, p. 246). The sample volume used was 1 ml in a final volume of 2 ml.

(ii) Pyruvate, phosphoenolpyruvate (PEP), 2-phosphoglycerate (2PGA) and 3-phosphoglycerate (3PGA) were determined in the same cuvette by the assay of Czok and Eckert, in Bergmeyer (1963, p. 229). EDTA was omitted from the published buffer recipe because it inhibited PK. The concentration of 2,3-diPGA was reduced to 0.033 mM. The sample volume used was 1 ml in a final volume of 2 ml.

(iii) Adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) were assayed in the same cuvette by the assay of Adam, in Bergmeyer (1963, p. 573). The sample volume was 500 µl in a final volume of 3 ml.

(iv) Glucose, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P) and adenosine 5'-triphosphate (ATP) were determined by a combination of the assays of Lamprecht and Trautschold, Hohorst, and Slein, all in Bergmeyer (1963, pp. 117, 134 and 543). Glucose concentrations for the ATP assay were reduced to 2.5 mM to minimise drift. G6P, F6P and ATP were assayed in the same cuvette with a sample volume of 500 µl. Glucose was assayed separately with a sample volume of 200 µl. The final volume for both assays was 2 ml.

(v) Lactate and malate were determined initially by combination of the methods of Hohorst, in Bergmeyer (1963, pp. 266 and 328). The sample volume was 200 µl in a final volume of 2 ml. In the experiments carried out in chapters 7 and 8, the new method described by Gutmann and Wahlefeld, in Bergmeyer (1974, p. 1464) was used. A sample volume of 300 µl in a final volume of 3 ml was used with the new method.
(vi) Succinate was measured by the method of Williamson and Corkey (1969) and adapted as described by Behm and Bryant (1975a). A sample volume of 200 µl was used in a final volume of 2 ml.

Assay (i) was carried out on the same day as the experiment, before freezing the sample. Assays (ii), (iii) and (iv) were often carried out on the same day or else on the following day after freezing and thawing once. Assays (v) and (vi) were carried out as soon as possible after that, usually within a week of the sample preparation.

(e) GLYCOGEN DETERMINATIONS

Separate incubations were carried out in conjunction with the incubations used for intermediate determinations. At the end of the incubation the flukes were rinsed thoroughly in Hédon-Fleig salt solution, blotted and weighed. They were then homogenised in an all-glass Dounce-type homogeniser in 5 ml ice cold Hédon-Fleig salt solution and the volume was measured.

1 ml of the homogenate was used for glycogen determination according to the method of Pfleiderer, in Bergmeyer (1963, p. 59) with some modifications. 3 ml of 30% KOH was added to the homogenate to ensure complete digestion of the tissue. 5 ml of 96% ethanol was added for complete precipitation of the glycogen. Each tube was just brought to the boil in a boiling water bath. Individual treatment was necessary to avoid boiling over and loss of sample. The rest of the method was adopted without change except for the determination of glucose. 50 µl of the final sample was used in the glucose assay described earlier (d (iv)). The results are expressed as umoles glucosyl units/g wet weight. Hydrolysis of pure glycogen preparations gives values of 90-95% glucose (Pfleiderer and Grein,

(f) MEDIA ASSAYS

Acetate and propionate excreted into the medium were measured by gas chromatography at the Division of Plant Industry, C.S.I.R.O. where the system is set up for routine use. 5 ml samples of media were steam distilled with 1 ml of 10% orthophosphoric acid. 100 ml distillate was collected in a flask containing 5 ml 0.1N NaOH. After evaporation to dryness, the residue was redissolved in 1 ml 10% orthophosphoric acid. 5 µl samples were analysed using a Varian Aerograph 204B gas chromatograph with a hydrogen flame ionisation detector and a 1 mV Rikadenkie recorder. The column was packed with Porapak N, 80-100 mesh. A similar method is described by DuPreez and Lategan (1976). Appropriate standards were measured and standard curves were constructed each time samples were measured.

The concentration of the acetate and propionate internal pool sizes were also determined by gas chromatography. Flukes were rinsed in Hédon-Fleig salt solution after removal from the liver or at the completion of incubations, and were homogenised in 5 ml ice cold distilled water. The homogenate was centrifuged for 15 min at 8,000 g and 2.5 ml of the supernatant was steam distilled and prepared as described above. 10 µl samples were analysed by gas chromatography.

Samples of media for lactate, succinate and glucose assays were deproteinised with 6% HCl to give a final concentration of 3%. The samples were left on ice for at least 15 min then were neutralised to approximately pH 6.5 with 5N KOH and the precipitate was removed by centrifugation for 15 min at 8,000 g. The samples were frozen and stored at -40°C until the assays described earlier (d (iv, v, and vi))
were carried out.

3.3 RESULTS

Tests for significance have been carried out using Student's 't' or the paired 't' statistic throughout the thesis. In most cases the number of determinations (n) is large enough to indicate significant differences where appropriate. Where insufficient numbers have been sampled the tests have been carried out for the benefit of the reader but cannot be relied upon to show significant differences.

Flukes were maintained under the described conditions for up to ten days before the termination of the experiment. There were no signs of necrosis or degeneration of the flukes and no deaths occurred. Thus, the flukes used in the experiments described in this chapter and those used in the control incubations in subsequent chapters were not close to death.

3.3.1 GLYCOGEN LEVELS

Table 3.1 shows the glycogen levels of *F. hepatica* immediately after removal from the host, i.e. 'O' time, and following 12, 24 and 48 h incubations. At 'O' time the amount of glycogen present is higher than in the 12, 24 and 48 h incubated groups. There is no significant difference between the 12, 24 and 48 h group levels.

3.3.2 INTERNAL METABOLIC POOL SIZES

The internal concentrations of glucose and the end products, lactate, succinate, acetate and propionate at 'O' time and following incubations for 24 and 48 h are given in table 3.2. The glucose pool size increases with time of incubation due to the presence of glucose in the incubation medium. The internal concentration of one of the
TABLE 3.1

Glycogen levels of *F. hepatia* after removal from the liver and following incubation *in vitro*.

The results are expressed as μmoles of glucosyl units/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>TIME OF INCUBATION</th>
<th>0</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>192.0 ± 29.2 (10)</td>
<td>82.5 ± 24.6 (16)</td>
<td>75.8 ± 30.1 (24)</td>
<td>96.4 ± 66.2 (12)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that only the '0' glycogen levels are significantly different from the 12, 24 and 48 h levels, p < 0.001.
TABLE 3.2

Internal pool sizes of glucose and end products in *F. hepatica* maintained *in vitro*.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Pool</th>
<th>0</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>2.2 ± 0.6</td>
<td>5.6 ± 1.0(^a)</td>
<td>8.5 ± 1.4(^a)</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 20)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>Succinate</td>
<td>4.6 ± 1.0</td>
<td>3.3 ± 2.0</td>
<td>1.6 ± 0.6(^b)</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 27)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.0 ± 1.5</td>
<td>1.1 ± 0.7</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 27)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.0 ± 0.1</td>
<td>4.5 ± 1.7(^b)</td>
<td>8.4 ± 1.4(^bc)</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 25)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Propionate</td>
<td>3.0 ± 0.2</td>
<td>2.0 ± 0.8</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 25)</td>
<td>(n = 5)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

\(^a\) significantly different from 0, \(p < 0.005\),

\(^b\) significantly different from 0, \(p < 0.025\),

\(^c\) 24 h, \(p < 0.05\).
major end products, propionate, does not change significantly during the incubation periods. The internal acetate pool increases markedly and the internal lactate and succinate concentrations decrease on incubation.

Table 3.3 presents the internal concentrations of some of the metabolic intermediates of the energy producing pathway. The DHAP and G3P concentrations have been added together and termed triose phosphates (TP) as they are both difficult to measure accurately due to their small levels. Similarly, the 3PGA and 2PGA concentrations are added together and termed phosphoglycerates (PGA). There are increases in the G6P, F6P, FDP and malate concentrations and a decrease in the pyruvate pool following incubation.

3.3.3 ADENINE NUCLEOTIDE LEVELS

The adenine nucleotide levels in flukes at 'O' time and following incubation are given in table 3.4. The only change which is significant is the increase in the ATP level of the 24 h incubated group.

3.3.4 END PRODUCT EXCRETION

The excretion of the end products succinate, lactate, acetate and propionate after 3, 12, 20, 36 and 48 h incubation is given in table 3.5. These values have been used to calculate curves (as described by Brownlee, 1965) of excretion against time (figure 3.1). After 48 h, propionate, acetate and lactate have been produced in the relative proportions of 3.8:1.8:1.0 as calculated from table 3.5 and 4.0:1.9:1.0 calculated from figure 3.1.
TABLE 3.3

Intermediate metabolic pool sizes in *F. hepatica* maintained *in vitro*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th>Pool</th>
<th>0</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 9)</td>
<td>(n = 27)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>G6P</td>
<td>891 ± 245</td>
<td>1747 ± 463 b</td>
<td>1692 ± 454 b</td>
</tr>
<tr>
<td>F6P</td>
<td>200 ± 107</td>
<td>353 ± 111 b</td>
<td>388 ± 138 b</td>
</tr>
<tr>
<td>FDP</td>
<td>26 ± 12</td>
<td>54 ± 23 b</td>
<td>84 ± 36 c</td>
</tr>
<tr>
<td>TP</td>
<td>31 ± 19</td>
<td>37 ± 16</td>
<td>46 ± 15</td>
</tr>
<tr>
<td>PGA</td>
<td>318 ± 98</td>
<td>346 ± 155</td>
<td>387 ± 239</td>
</tr>
<tr>
<td>PEP</td>
<td>111 ± 42</td>
<td>113 ± 54</td>
<td>103 ± 62</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>190 ± 80</td>
<td>74 ± 38 b</td>
<td>118 ± 53 c</td>
</tr>
<tr>
<td>Malate</td>
<td>490 ± 202</td>
<td>835 ± 414</td>
<td>751 ± 87 a</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from 0, *p* < 0.05,
b " " " 0, *p* < 0.005,
c " " " 0 & 24 h, *p* < 0.05.
### Table 3.4

Pool sizes of adenine nucleotides in *F. hepatica* maintained *in vitro*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>0 (n = 10)</th>
<th>24 h (n = 25)</th>
<th>48 h (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATP</strong></td>
<td>895 ± 250</td>
<td>1101 ± 221&lt;sup&gt;a&lt;/sup&gt;</td>
<td>930 ± 234</td>
</tr>
<tr>
<td><strong>ADP</strong></td>
<td>884 ± 187</td>
<td>806 ± 123</td>
<td>780 ± 99</td>
</tr>
<tr>
<td><strong>AMP</strong></td>
<td>282 ± 59</td>
<td>278 ± 92</td>
<td>291 ± 66</td>
</tr>
<tr>
<td><strong>Total adenine nucleotides</strong></td>
<td>2061 ± 384</td>
<td>2185 ± 367</td>
<td>2001 ± 297</td>
</tr>
<tr>
<td><strong>ATP/ADP</strong></td>
<td>1.01</td>
<td>1.36</td>
<td>1.19</td>
</tr>
<tr>
<td><strong>Adenylate energy charge</strong></td>
<td>0.65</td>
<td>0.69</td>
<td>0.66</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tests for significance by Student's 't' show that only the increase in ATP levels between 0 and 24 h is significant, *p* < 0.05.
TABLE 3.5

The excretion of end products by *F. hepatica* during incubation *in vitro*.

The results are expressed as µmoles excreted/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>3 h</th>
<th>12 h</th>
<th>20 h</th>
<th>36 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Succinate</strong></td>
<td>2 ± 1 (7)</td>
<td>4 ± 2 (4)</td>
<td>6 ± 4 (4)</td>
<td>15 ± 4 (5)</td>
<td>13 ± 3 (4)</td>
</tr>
<tr>
<td><strong>Lactate</strong></td>
<td>4 ± 2 (7)</td>
<td>19 ± 4 (7)</td>
<td>40 ± 13 (4)</td>
<td>81 ± 20 (5)</td>
<td>113 ± 4 (3)</td>
</tr>
<tr>
<td><strong>Acetate</strong></td>
<td>30 ± 6 (12)</td>
<td>95 ± 33 (19)</td>
<td>113 ± 40 (10)</td>
<td>162 ± 80 (5)</td>
<td>207 ± 25 (4)</td>
</tr>
<tr>
<td><strong>Propionate</strong></td>
<td>41 ± 13 (12)</td>
<td>216 ± 67 (19)</td>
<td>237 ± 42 (10)</td>
<td>305 ± 120 (5)</td>
<td>425 ± 95 (4)</td>
</tr>
<tr>
<td><strong>P/A</strong></td>
<td>1.4</td>
<td>2.3</td>
<td>2.1</td>
<td>1.9</td>
<td>2.1</td>
</tr>
</tbody>
</table>

These values have been used to calculate the curves shown in figure 3.1.
FIGURE 3.1

The excretion of end products by *F. hepatica* during incubation *in vitro.*

Succinate: \( y = 0.73x^{0.77} \), coefficient of determination, \( r^2 = 0.95 \).

Lactate: \( y = 0.98x^{1.22} \), \( r^2 = 1.00 \).

Acetate: \( y = 15.34x^{0.67} \), \( r^2 = 0.99 \).

Propionate: \( y = 20.38x^{0.8} \), \( r^2 = 0.94 \).
### TABLE 3.6

Apparent equilibrium constants ($K'$) and observed mass action ratios for some reactions of the respiratory metabolic pathway in *F. hepatica*.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K'$</th>
<th>Mass Action Ratio (Range)</th>
<th>Mean Mass Action Ratio $K'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>5500*</td>
<td>0.18-0.61</td>
<td>7.3 x 10^{-5}</td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>0.47*</td>
<td>0.20-0.21</td>
<td>2.3</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>1200*</td>
<td>0.11-0.21</td>
<td>1.3 x 10^{-4}</td>
</tr>
<tr>
<td>Aldolase ($x 10^{-6}M$)</td>
<td>68*</td>
<td>0.12-7.41</td>
<td>0.06</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>0.036†</td>
<td>0.29-10.0</td>
<td>143</td>
</tr>
<tr>
<td>ALD x TPI ($x 10^{-6}M$)</td>
<td>2.45</td>
<td>19.37</td>
<td>7.91</td>
</tr>
<tr>
<td>Phosphoglyceromutase</td>
<td>0.17*, 0.10†</td>
<td>0.09-0.13</td>
<td>0.8</td>
</tr>
<tr>
<td>Enolase</td>
<td>1.4*, 4.6†</td>
<td>3.0 -3.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>15,000*</td>
<td>0.89-1.82</td>
<td>9.0 x 10^{-5}</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>0.44††</td>
<td>0.29-0.44</td>
<td>0.84</td>
</tr>
</tbody>
</table>

* Hess (1963)
† Lowry and Passonneau (1964)
†† Eggleston and Hems (1952)

+ ALD x TPI is the product of the mass action ratios for aldolase and triose phosphate isomerase.

Mass action ratios are means determined in individual experiments.
3.3.5 MASS ACTION RATIOS

From the results shown in tables 3.2, 3.3 and 3.4 it is possible to calculate mass-action ratios for some of the enzymatic steps in the pathway. These are presented in table 3.6. The following enzymes are significantly out of equilibrium: hexokinase, phosphofructokinase, aldolase and pyruvate kinase.

3.4 DISCUSSION

The results presented confirm and extend the earlier observations on the carbohydrate metabolism of *F. hepatica*. Glycogen is the major storage product in *F. hepatica*. Hines (1969) and von Brand (1973) report levels of glycogen measured in flukes directly after removal from the liver. Their values are comparable with those presented in table 3.1. The results in table 3.1 show that although the glycogen levels fall after removal from the host, the new level is sustained for at least 48 h. The drop in the glycogen level after removal is probably due to the initial expulsion of eggs from the fluke which was observed during the first media change. Wilson (1967) reported that one third of the dry weight of *F. hepatica* eggs is due to carbohydrate.

The stability of the glycogen levels in *F. hepatica* during incubation agrees with the report of Pantelouris (1964) and Hines (1969) who found that, apart from the initial decrease, *in vitro* incubation with glucose in the medium had no effect on the glycogen levels. Threadgold and Arme (1974), however, claimed a 27% drop in glycogen concentration in *F. hepatica* after 48 h incubation *in vitro*, with glucose present. They used only three replicates in their
determinations. The present study has a larger sample size at three incubation times. The standard deviations are considerable and it is possible that the results of Threadgold and Arme (1974) can be ascribed to the inherent variation in the parasites.

All the enzymes of glycolysis have been measured in F. hepatica (Prichard & Schofield, 1968a; Sturm et al., 1969; Lee & Vasey, 1970) but not all of the intermediates of the pathway have been measured. Metzger and Duwel (1973) have determined the levels of several glycolytic intermediates; G6P + F6P, FDP, TP, PEP and pyruvate. Prichard (1976) has measured FDP, TP, PEP and pyruvate using similar techniques to those in this study, following 7 h incubation of the flukes in media containing glucose. The results in tables 3.2 and 3.3 compare favourably with these and show that all the intermediates of glycolysis have been measured in F. hepatica, except for 1,3-diPGA. The concentration of 1,3-diPGA is too small to measure accurately.

The further metabolism of PEP involves the incorporation of CO₂, catalysed by PEPCK, to form OAA (Prichard & Schofield, 1968c,d; de Zoeten et al., 1969). OAA is then converted to malate via MDH, then to fumarate by a dehydration reaction, catalysed by fumarase. Fumarate is converted to succinate by fumarate reductase. The final product of this part of the pathway is propionate (de Zoeten et al., 1969). In the present study, the internal concentrations of malate, succinate and propionate have been measured (tables 3.2 and 3.3). The other two end products of metabolism, derived from pyruvate, are lactate and acetate. Their internal pool sizes are given in table 3.2.

There are some changes in the concentrations of several
metabolic intermediate concentrations following incubation \textit{in vitro} (tables 3.2 and 3.3). The internal glucose level increases on incubation because glucose is taken up from the medium. Isseroff and Read (1974) have shown that glucose uptake is not an active process but takes place via a specific carrier mainly through the tegument (see 1.5.2). The significant changes in the sizes of the internal concentrations of metabolites are, with three exceptions, increases. This is probably due to the large external source of glucose which allows the intermediate pools to fill.

Of the three exceptions, pyruvate and lactate are components of whole blood (Dittmer, 1961). Flukes at 'O' time have not disgorged their caecal contents, so the high levels at this time may be due to contamination from the host's materials.

The diminished succinate concentration is less easy to explain. It may be depleted because it is utilised in the energy yielding succinate - propionate conversion. Table 3.4 shows that the ATP level increases after the flukes are incubated. The change in the succinate pool may reflect a slightly increased conversion of succinate to propionate which would also result in an increase in the ATP level. Another possibility is that, due to the large amount of glucose available to the parasite, there may be a sparing effect on amino acid metabolism. Thus, if the formation of precursors of the succinate - propionate pathway (e.g. \(\alpha\)-ketoglutarate) from amino acid metabolism decreases, there will be a decrease in the contribution to the succinate pool from sources other than carbohydrate metabolism.

The large increase in the acetate internal pool requires an explanation. Isseroff and Walczak (1971) showed that acetate is
absorbed by *F. hepatica*. Further studies have shown that it occurs by a mediated process in a manner similar to the uptake of monosaccharides (Wright & Isseroff, 1973). Analysis of the acetate which is absorbed indicates that a significant portion is incorporated into higher fatty acids and lipids. According to Meyer, Meyer and Bueding (1970), chain elongation with acetate appears to be the only pathway for synthesis of saturated fats in the platyhelminths examined, which include the blood fluke, *S. mansoni*. A similar role for acetate absorption and utilisation may occur in *F. hepatica*. It is suggested by Wright and Isseroff (1973) that acetate excretion by flukes may be relatively minor *in vivo*. It may be produced as an end product of carbohydrate metabolism and is then utilised in other pathways. If, during *in vitro* incubation, and with a high concentration of glucose available, acetate utilisation diminishes because the other acetate utilising pathways do not continue to operate, the internal acetate pool may increase with a corresponding increase in acetate excretion.

It is also possible that increased glucose utilisation results in a greater rate of acetate formation. The increase in the internal concentration may then be due to saturation of the excretion process. Another explanation could be that acetate is normally excreted *in vivo* and is removed by the flow of bile. *In vitro* the acetate concentration in the medium gradually increases and some absorption then occurs. All three possibilities could explain the increase in the internal acetate pool during *in vitro* incubation.

Barrett and Beis (1973c) measured the adenine nucleotide levels present in *F. hepatica* using similar techniques to those used in this study. Their results are comparable with the values given in
Metzger and Dillwel (1973) also measured the adenine nucleotide levels of *F. hepatica* directly after removal from the liver and following 24, 48 and 72 h incubation *in vitro* in a culture medium containing glucose. The ATP concentration increased during the first 24 h incubation period then dropped during the next 24 h period so that the 48 h level was intermediate between the '0' and 24 h level. A similar effect was found in the present study, as shown in table 3.4. It is not possible to state whether the ATP concentration measured at '0' time reflects the level found *in vivo* as up to 3 h had usually passed from death of the host to freezing of the flukes.

The total adenine nucleotide levels do not change during *in vitro* incubation. The ATP/ADP ratio (which is an index of the high energy phosphorylation state of the animal and therefore its capacity to do work) increases and remains high for 48 h. The adenylate energy charge (a measure of the relative amount of energy stored in the adenylate system, and therefore an index of 'condition' - see 1.6.8) increases during incubation. Thus, for at least 48 h the flukes are able to maintain their adenine nucleotide levels after an initial small increase in the ATP concentration.

The major end products of carbohydrate metabolism in *F. hepatica* are propionate, acetate and lactate (table 3.4 and figure 3.1) (de Zoeten *et al.*, 1969; Lahoud *et al.*, 1971a). It is apparent that succinate forms only a small amount of the metabolites excreted (< 2%) and may be disregarded. Table 3.5 and figure 3.1 show that the excretion of lactate, acetate and propionate increases with time and after about 12 h the increase is practically linear.

Lahoud *et al* (1971a) reported that the relative proportion
of propionate and acetate produced by *F. hepatica* is a function of oxygen tension; the propionate/acetate ratio (P/A) is 2.5 in anaerobic conditions, 2 in air and 1 in oxygen. They show that the ratio changes because the production of propionate decreases with oxygen tension, while acetate production remains independent of oxygen tension. They suggest that under high concentrations of oxygen, a substantial proportion of the NADH formed in the production of acetate is reoxidised by the electron transport system with oxygen acting as the electron acceptor, rather than via the fumarate reductase reaction. Thus, there is less succinate produced and hence less propionate formed and excreted. Under anaerobic conditions the NADH is reoxidised by the fumarate reductase system, hence greater propionate production.

In the present study, the P/A ratio after 3 h incubation is about 1.5 (table 3.5 and figure 3.1) which indicates that there is probably sufficient oxygen available in the media to spare some fumarate reduction and hence, to reduce propionate production. This is probably the case each time the medium is changed. Within 12 h incubation, i.e. before the first medium change, the P/A ratio has increased to about 2.0 (table 3.5 and figure 3.1), suggesting that there is less oxygen present in the medium but the system is not completely anaerobic. At 20, 36 and 48 h incubation times the P/A ratio is about 2.0.

After 36 and 48 h incubation the ratio of propionate:acetate:lactate is very close to 4:2:1. These proportions are similar to the findings of de Zoeten *et al.* (1969). The whole numbers suggest a stoichiometrical relationship between the end products, and figure 3.2 shows how this may be achieved under these circumstances. As suggested by Lahoud *et al.* (1971), the acetate and propionate pathways are linked
FIGURE 3.2
The path of carbon during end product formation in *F. hepatica*.

Figures in brackets are the numbers of carbon atoms. Four molecules of reduced pyridine nucleotides are generated in the conversion of two molecules of malate to acetate. They are reoxidised by the fumarate reductase reaction.
The role of lactate production remains elusive. Our measurements do not reveal any net conversion of pyruvate to lactate, nor does the presence of any lactate production in the medium. Furthermore, there is a greater conversion of lactate to pyruvate. This suggests that at least 48 hours in the medium containing glucose supports the production of lactate.
by the recycling of pyridine nucleotides.

The role of lactate production remains obscure as its formation does not seem to be linked to either acetate or propionate production in the 3, 12 and 20 h incubations. There is a greater relative amount of lactate produced as incubation time increases. This may reflect a change in the pyruvate pool which in turn will depend upon contribution from, and donation to, pathways other than carbohydrate metabolism. For example, transamination of pyruvate to alanine may diminish if amino acid metabolism is reduced due to the high level of glucose available in the medium. Thus, more pyruvate may be converted to lactate via lactate dehydrogenase. The maintenance of the cytosolic NAD/NADH ratio may involve the pyruvate - lactate conversion catalysed by lactate dehydrogenase. The problem of lactate production and excretion probably will not be resolved until the distribution of pyruvate within the cell is known and what pathways contribute to and determine the flux through the pyruvate pool(s).

The enzymes regulating the flow of carbon along the pathways of carbohydrate metabolism include hexokinase, phosphofructokinase and pyruvate kinase. The reactions catalysed by these enzymes are out of equilibrium by several orders of magnitude and favour reactants in *F. hepatica* (table 3.6). These observations are similar to those made on other organisms (e.g. Behm & Bryant, 1975a). Aldolase cannot be regulatory as its equilibrium is displaced in favour of product formation.

The biochemical evidence from this study supports the contention that *F. hepatica* maintains its energy metabolism during incubation for at least 48 h in a simple medium containing glucose. There is evidence of accommodation, possibly in the form of greater
carbohydrate metabolism, but the integrity of the energy producing pathways is maintained. It is probable therefore, that metabolic experiments on *F. hepatica* which are completed within 48 h have validity for the *in vivo* situation and can be used to evaluate the effects of anthelmintics on the energy producing pathway.

There have been a number of *in vitro* studies on the biochemical effect of anthelmintics on parasites. They fall into two groups. In the first, an enzyme system or organelle is isolated from the parasite, or tissue slices are cut, and the drug is tested on them (Van den Bossche, 1972b; Prichard, 1973; Yorke & Turton, 1974; Hanna & Threadgold, 1976). In the second group the effects of the drug on storage products or on the incorporation of isotopes are determined in the whole parasite (Van den Bossche & De Nollin, 1973; Van den Bossche, 1972a). By itself, each approach is unsatisfactory. In the first case, there may be little relevance to the whole animal, as permeability barriers may preclude access of the drug to the enzyme system being tested. In the second case, the studies have not been taken far enough as, ideally, the behaviour of the whole energy producing pathway of the parasite should be monitored.

Exceptions are the work of Metzger and Dhwel (1973) and Rahman (1976). Metzger and Dhwel (1973) reported the effects of a series of concentrations of 2,6-dihydroxy-3,5-dichlorobenzoic acid-4'-chloroanilide on intermediate pool sizes in metabolic pathways in intact *F. hepatica*. Their results show some correlation with the work on subcellular fractions in which they measured the inhibitory effect of this drug on succinate dehydrogenase. However, they did not measure end products or storage product concentrations and used only a single
time interval (4 h), whereas different effects may occur at different times.

The study of Rahman (1976) on the effects of two anthelmintics, mebendazole and cambendazole, on the cestode *M. expansa* is an example of a thorough examination of drug effects on the energy producing pathway of a parasite. Glycogen levels, glucose uptake, intermediate pool sizes, end product excretion and adenine nucleotide levels were measured following *in vitro* incubation with the drugs, under aerobic and anaerobic conditions. Various enzyme activities were examined in the presence and absence of the anthelmintics and finally, *in vivo* experiments were carried out to confirm that the effects found *in vitro* also occur *in vivo*. Rahman concluded that a major effect of the two drugs is the inhibition of phosphorylation, but whether this is the primary effect is not known. However, it is apparent from the study that the levels of adenine nucleotides may provide an index for anthelmintic efficacy.

Chapter 4 describes the effects of three anthelmintics, mebendazole (MBZ), nitroscanate (NSC) and rafoxanide (RFX) on *F. hepatica* maintained *in vitro*. These three drugs were chosen because they are all active against *F. hepatica* in field trials and should show some effects *in vitro*. It is necessary to first test the *in vitro* culture system with drugs of known activity against the parasite before it can be used to test the efficacy of new anthelmintics.
CHAPTER 4

THE EFFECT OF RFX, NSC AND MBZ ON THE ENERGY METABOLISM OF

F. HEPATICA MAINTAINED IN VITRO

4.1 INTRODUCTION

Rafoxanide (RFX) is effective against mature and immature F. hepatica (table 2.2) and is a well tolerated drug. Following oral treatment with RFX (in sheep, cattle, dogs and rats) most of the drug is absorbed, giving high peak plasma levels at 24 h post dose. It is bound to plasma proteins and is not metabolised by the host. Residue studies have shown that the RFX found in the liver is plasma bound (Pollak, 1970). Hence, in vivo, flukes are likely to come in contact with the drug when feeding on the host's blood and tissue. As the drug is not metabolised by the host, incubation of the parasite with the drug in vitro should have some bearing on the in vivo situation.

The in vitro studies already carried out on RFX have been discussed in 2.6.3. The data suggest that it acts as an uncoupler of oxidative phosphorylation but the effect of this on intact flukes has not been determined. There is evidence that, in F. hepatica mitochondria, electrons generated in the oxidation of malate can be transported either to fumarate or to oxygen. One molecule of ATP is produced per pair of electrons transported to fumarate and it appears that more ATP is produced when the electrons are transported to oxygen (Van Vugt et al., 1976). Addition of an uncoupler of oxidative
phosphorylation to this system stimulates malate utilisation which indicates that the phosphorylating process is rate-limiting in the overall dismutation of malate (Van Vugt et al., 1976). It seems then, that uncouplers can disrupt electron transport linked phosphorylation in *F. hepatica* so that ATP production is inhibited and substrate oxidation is increased.

Nitroscanate (NSC) is effective against *F. hepatica* and some nematodes in sheep (Boray, personal communication). Radioisotopic tracer studies have shown that maximum absorption of the drug occurs within 24 h of administration and the radiocarbon appears in the blood of the host. However, these studies have not shown the structure of the compound following absorption. Thus, it is not known whether the drug needs to be metabolised by the host to produce its anthelmintic effect. It is possible that there is some hydrolysis and/or reduction of the molecule by the host. The drug remains in the host for a considerable period and it is available to the flukes, via the blood, for at least 48 h (Boray, personal communication).

NSC was included in the *in vitro* study as it is known to be active against *F. hepatica* in field trials and it is structurally very different from RFX and MBZ (see figure 2.1) so may have a different effect on the energy metabolism of the parasite.

Mebendazole (MBZ) is a drug of negligible toxicity in mammals and birds (Chevis, 1975). Marsboom (1973) tested the effect of single oral doses 32-64 times the therapeutic dose on a variety of laboratory and domestic animals and found no signs of toxicity.

Absorption studies show that most of the drug is passed out
unchanged in the faeces. Only small amounts are absorbed by the host; the levels in blood and tissue are low during the first 32 h period after dosing. Levels in the liver never exceed 4% of the dose. Some MBZ is found in the bile up to 48 h after treatment; the highest concentration occurs between 1 and 5 h after MBZ administration in rats (Janssen Pharmaceutica Report No. N11283, 1976).

MBZ is an effective fasciolicide when administered at a dose rate of 100 mg/kg (Kelly et al., 1975) which is much higher than the recommended therapeutic dose of 12.5 mg/kg for nematodes and cestodes. The high dose may be required because of the very low solubility (Janssen Pharmaceutica Report No. 182, 1972) and absorption of the drug. The concentration of MBZ in the intestine when a dose rate of 12.5 mg/kg is used is sufficient to remove most intestinal parasites. Flukes may be more protected within the liver as they only come into contact with low concentrations of MBZ in the blood, when feeding, and in the bile.

The drug is metabolised to its decarbomethoxylated derivative by the host but this has no anthelmintic properties. Thus, MBZ is suitable for use in in vitro studies as it does not need to be metabolised to produce its anthelmintic effect and it eliminates flukes when administered at high concentrations to the host. Incubation of flukes with MBZ in vitro, followed by an examination of the energy producing pathway of the flukes may provide some information about its mode of action.

The in vitro studies which have been carried out to examine the effects of MBZ on various parasites have been discussed in 2.6.1. It appears that ultrastructural damage occurs in most parasites so far
The concentration of 0.04% was chosen for MBZ because Rahman (1976) showed that it is effective against *M. expansa* in vitro and also forms a uniform suspension. The same concentration was used for RFX and NSC as they also form suspensions and initial experiments showed that this concentration is effective against *F. hepatica* in vitro.
studied after treatment with MBZ and is thought to be responsible for some of the biochemical findings; inhibition of glucose uptake, depletion of glycogen reserves and a decrease in ATP levels (Borgers et al., 1975a). Rahman (1976) has shown that MBZ affects the energy metabolism of *M. expansa* and suggests that a lack of ATP could account for the intracellular changes. More work is required to correlate the ultrastructural findings with the biochemical results.

4.2 METHODS

RFX was provided in powder form by Merck Sharp and Dohme (Australia) Pty Ltd. NSC was supplied in micronised form by CIBA-GEIGY Australia Ltd, and MBZ was provided as a micronised powder by Ethnor Pty Ltd.

The three drugs were used at a concentration of 20 mg in 50 ml i.e. 0.04% (RFX, 0.64 mM; NSC, 1.47 mM; MBZ, 1.36 mM). They are all practically insoluble in water so had to be mixed with a suspending agent. RFX and NSC were suspended with 200 µl of propylene glycol (final concentration 0.04% v/v) and MBZ with 200 µl of Tween 80 (final concentration 0.04% v/v). The drugs were mixed thoroughly with the appropriate suspending agent in the incubation vessel then 50 ml medium was added. The flasks were stirred until the drug was in suspension. During the incubations the flasks were swirled occasionally, to resuspend any of the drug which settled out. Thus, the flukes, which remained near the bottom of the flasks, were probably in contact with the drugs at concentrations greater than 0.04%.

4.2.1 INCUBATIONS

All the incubations were carried out as described in 3.2(a)
except that they contained the appropriate drug and suspending agent. The control incubations also contained the suspending agent.

Initial incubations were carried out to determine whether the drugs killed the flukes during *in vitro* incubation and at what time so that suitable time courses could be constructed for each experiment.

4.2.2 ASSAYS

The metabolic intermediates were measured as described in 3.2(d). The glycogen levels were determined by the method described in 3.2(e) and the media assays were carried out as described in 3.2(f).

4.2.3 $^{14}$C-3-O-METHYL GLUCOSE UPTAKE

$^{14}$C-3-O methyl glucose uptake was measured on individual flukes. Each incubation contained 5 ml Hédon-Fleig salt solution containing methyl glucose (see appendix 1) and some contained the appropriate drug and suspending agent. The incubations were carried out for 3 h at 37°.

To determine whether NSC and MBZ require a longer time to affect methyl glucose uptake flukes were preincubated with NSC for 18 h and MBZ for 36 h before they were transferred to the incubation vials containing $^{14}$C-3-O methyl glucose. The incubations were then carried out for 3 h.

At the completion of the incubation each fluke was removed, washed gently several times, blotted then placed in a preweighed scintillation vial which was then reweighed. 1 ml of NCS tissue solubiliser was added and the tissue digestion was carried out at 50° until the samples appeared homogeneous when shaken. 30 µl of glacial acetic acid was added to each vial to neutralise the sample, then 10 ml
PCS scintillant was added and the samples were counted in a Packard liquid scintillation counter. Internal corrections were made for quenching.

4.3 RESULTS

The initial incubations showed that the three drugs affect flukes incubated in vitro. With RFX present in the incubation the flukes are moribund or dead after 12 h; with NSC 24 h were required for a similar effect and MBZ requires at least 48 h. The incubation times subsequently used were: for RFX - 3, 6, 9 and 12 h; for NSC - 3, 12 and 20 h; and for MBZ - 12, 36 and 48 h.

Tests for significance have been carried out using Student's 't' test and the paired 't' statistic. The number of determinations is large enough in most cases to indicate significant differences where appropriate. Where insufficient samples have been measured the tests have been done for the benefit of the reader but should not be relied upon to show significant differences.

1. GLYCOGEN LEVELS

Tables 4.1, 4.2 and 4.3 show the glycogen levels of F. hepatica incubated with or without the drugs. There are no significant differences between any of the control and drug treated groups.

2. $^{14}$C-3-O-METHYL GLUCOSE UPTAKE

There are no effects of RFX, NSC or MBZ on the uptake of $^{14}$C-3-O-methyl glucose by F. hepatica when incubated with the drugs in vitro for 3 h. Also, preincubation with NSC or MBZ has no effect (tables 4.4, 4.5 and 4.6).
TABLE 4.1

The effect of RFX on the glycogen levels of *F. hepatica* maintained *in vitro*.

The results are expressed as μmoles of glucosyl units/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77 ± 16 (5)</td>
<td>93 ± 33 (6)</td>
<td>82 ± 37 (5)</td>
<td>98 ± 44 (5)</td>
</tr>
<tr>
<td>RFX treated</td>
<td>83 ± 13 (5)</td>
<td>102 ± 26 (5)</td>
<td>78 ± 27 (6)</td>
<td>82 ± 11 (6)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that there are no significant differences between the control and RFX treated groups.
The effect of NSC on the glycogen levels of *F. hepatica* maintained *in vitro*.

The results are expressed as µmoles of glucosyl units/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>3 h</th>
<th>12 h</th>
<th>20 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>74 ± 21 (5)</td>
<td>65 ± 16 (5)</td>
<td>94 ± 50 (11)</td>
</tr>
<tr>
<td>NSC treated</td>
<td>68 ± 15 (5)</td>
<td>71 ± 14 (5)</td>
<td>121 ± 66 (11)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that there are no significant differences between the control and MBZ treated groups.
The effect of MBZ on the glycogen levels of *F. hepatica* maintained *in vitro*.

The results are expressed as µmoles of glucosyl units/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Control</th>
<th>MBZ treated</th>
<th>Time of incubation</th>
<th>Control</th>
<th>MBZ treated</th>
<th>Test for significance by Student's 't' show that there are no significant differences between the control and MBZ treated groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h</td>
<td>64.9 ± 15.6 (5)</td>
<td>79.7 ± 13.3 (5)</td>
<td>36 h</td>
<td>82.3 ± 23.0 (3)</td>
<td>73.7 ± 34.9 (3)</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>74.9 ± 18.5 (4)</td>
<td>51.8 ± 12.8 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that there are no significant differences between the control and MBZ treated groups.
TABLE 4.4

The effect of RFX on $^{14}$C-3-O-methyl glucose uptake by *F. hepatica* during *in vitro* maintenance.

The results are expressed as counts per minute x $10^{-3}$/g wet weight and they are means of three at each time + standard deviation.

<table>
<thead>
<tr>
<th>Time in min</th>
<th>Control</th>
<th>RFX</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>41.3 ± 2.9</td>
<td>47.5 ± 2.9</td>
</tr>
<tr>
<td>20</td>
<td>54.4 ± 0.6</td>
<td>62.4 ± 6.7</td>
</tr>
<tr>
<td>30</td>
<td>64.3 ± 10.7</td>
<td>61.2 ± 3.4</td>
</tr>
<tr>
<td>60</td>
<td>72.5 ± 4.4</td>
<td>77.6 ± 7.5</td>
</tr>
<tr>
<td>120</td>
<td>96.0 ± 3.6</td>
<td>89.1 ± 11.1</td>
</tr>
<tr>
<td>180</td>
<td>96.4 ± 18.3</td>
<td>102.4 ± 9.7</td>
</tr>
</tbody>
</table>

Tests for significance by paired 't' on the individual data show that there are no significant differences between the control group and the RFX treated group.
TABLE 4.5

The effect of NSC on $^{14}$C-3-O-methyl glucose uptake by *F. hepatica* during *in vitro* maintenance.

The results are expressed as counts per minute x $10^{-3}$/g wet weight and they are means of three at each time ± standard deviation.

<table>
<thead>
<tr>
<th>Time in min</th>
<th>Control</th>
<th>NSC</th>
<th>Preincubated control (18 h)</th>
<th>Preincubated NSC (18 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>41.3 ± 2.9</td>
<td>42.0 ± 3.9</td>
<td>36.0 ± 1.8</td>
<td>33.0 ± 1.3</td>
</tr>
<tr>
<td>20</td>
<td>54.4 ± 0.6</td>
<td>56.8 ± 5.6</td>
<td>44.0 ± 0.5</td>
<td>45.5 ± 6.8</td>
</tr>
<tr>
<td>30</td>
<td>64.3 ± 10.7</td>
<td>63.3 ± 8.8</td>
<td>51.3 ± 3.6</td>
<td>45.6 ± 1.9</td>
</tr>
<tr>
<td>60</td>
<td>72.5 ± 4.4</td>
<td>80.6 ± 21.9</td>
<td>46.1 ± 7.5</td>
<td>53.7 ± 1.5</td>
</tr>
<tr>
<td>120</td>
<td>96.0 ± 3.6</td>
<td>99.7 ± 6.6</td>
<td>65.0 ± 2.5</td>
<td>55.8 ± 1.9</td>
</tr>
<tr>
<td>180</td>
<td>96.4 ± 18.3</td>
<td>102.7 ± 22.2</td>
<td>72.8 ± 10.9</td>
<td>61.5 ± 7.2</td>
</tr>
</tbody>
</table>

Tests for significance by paired 't' on the individual data show that there are no significant differences between the control group and the appropriate NSC treated group.
The effect of MBZ on $^{14}$C-3-O-methyl glucose uptake by *F. hepatica* during *in vitro* maintenance.

The results are expressed as counts per minute x $10^{-3}$/g wet weight and they are means of three at each time ± standard deviation.

<table>
<thead>
<tr>
<th>Time in min</th>
<th>Control</th>
<th>MBZ</th>
<th>Preincubated control (36 h)</th>
<th>Preincubated MBZ (36 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>41.3 ± 2.9</td>
<td>40.8 ± 6.3</td>
<td>68.4 ± 16.4</td>
<td>53.4 ± 9.1</td>
</tr>
<tr>
<td>20</td>
<td>54.4 ± 0.6</td>
<td>54.3 ± 3.0</td>
<td>95.3 ± 5.3</td>
<td>72.1 ± 4.2</td>
</tr>
<tr>
<td>30</td>
<td>64.3 ± 10.7</td>
<td>70.3 ± 14.0</td>
<td>87.6 ± 11.0</td>
<td>83.4 ± 4.5</td>
</tr>
<tr>
<td>60</td>
<td>72.5 ± 4.4</td>
<td>83.8 ± 7.3</td>
<td>101.6 ± 21.8</td>
<td>102.7 ± 2.6</td>
</tr>
<tr>
<td>120</td>
<td>96.0 ± 3.6</td>
<td>85.3 ± 10.8</td>
<td>133.2 ± 19.7</td>
<td>115.1 ± 7.6</td>
</tr>
<tr>
<td>180</td>
<td>96.4 ± 18.3</td>
<td>99.8 ± 6.4</td>
<td>122.9 ± 9.1</td>
<td>121.6 ± 3.0</td>
</tr>
</tbody>
</table>

Tests for significance by paired 't', on the individual data, show that there are no significant differences between the control group and the appropriate MBZ treated group.
3. INTERNAL INTERMEDIATE AND END PRODUCT POOL SIZES

Tables 4.7, 4.9 and 4.11 show the internal concentrations of glucose and the end products succinate, lactate, acetate and propionate. The concentrations of some of the intermediates of the energy producing pathways are given in tables 4.8, 4.10 and 4.12.

(a) RFX

The control and RFX treated groups include the samples from the 3, 6 and 9 h incubations (n = 3 from each) as the changes in the drug treated groups are continuous and gradual. Flukes from the 12 h incubations have not been included as they were usually moribund. Tests for significance have been carried out using the paired 't' statistic which works on the null hypothesis that the mean differences from the controls are zero (Ostle, 1963), so it can be used on the pooled samples.

The results in table 4.7 show that the internal glucose concentration in the RFX treated group is lower than in the control group. The succinate and propionate concentrations are increased when RFX is present.

Table 4.8 shows the concentrations of most of the intermediates of the pathway. There are decreases in the G6P, F6P, FDP and malate levels and an increase in the pyruvate concentration when RFX is present.

(b) NSC

Table 4.9 shows that there is less glucose within the flukes after 12 and 20 h incubation when NSC is present compared with the controls. There are increases in the succinate, lactate and acetate
TABLE 4.7

The effect of RFX on the internal pool sizes of glucose and end products of *F. hepatica* maintained *in vitro*.

The results are expressed as μmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Control (μmol/g)</th>
<th>RFX treated (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.2 + 0.8 (9)</td>
<td>3.7 + 0.5 (9)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.2 + 0.9 (9)</td>
<td>5.4 ± 3.5 (9)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.5 + 0.4 (9)</td>
<td>1.6 ± 0.5 (9)</td>
</tr>
<tr>
<td>Acetate</td>
<td>3.9 + 0.9 (14)</td>
<td>4.5 ± 0.9 (14)</td>
</tr>
<tr>
<td>Propionate</td>
<td>1.6 + 0.5 (14)</td>
<td>2.1 ± 0.6 (14)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Tests for significance by paired 't' show that:

- a significantly different from control, p < 0.05,
- b significantly different from control, p < 0.001.
The effect of RFX on some of the metabolic intermediates of \textit{F. hepatica} maintained \textit{in vitro}.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. \( n = 9 \) for all determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RFX treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>1719 ± 352</td>
<td>1203 ± 347&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>F6P</td>
<td>303 ± 76</td>
<td>230 ± 72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FDP</td>
<td>55 ± 15</td>
<td>32 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TP</td>
<td>22 ± 14</td>
<td>19 ± 12</td>
</tr>
<tr>
<td>PGA</td>
<td>380 ± 175</td>
<td>461 ± 175</td>
</tr>
<tr>
<td>PEP</td>
<td>110 ± 63</td>
<td>141 ± 56</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>53 ± 33</td>
<td>320 ± 166&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malate</td>
<td>673 ± 247</td>
<td>385 ± 144&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Tests for significance by paired 't' show that:

- \( a \) significantly different from the appropriate control, \( p < 0.05 \),
- \( b \) significantly different from the appropriate control, \( p < 0.005 \),
- \( c \) significantly different from the appropriate control, \( p < 0.001 \).
TABLE 4.9

The effect of NSC on the internal pool sizes of glucose and end products of *F. hepatica* maintained in vitro.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>3 h Control</th>
<th>3 h NSC</th>
<th>12 h Control</th>
<th>12 h NSC</th>
<th>20 h Control</th>
<th>20 h NSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4.5 ± 2.2 (5)</td>
<td>3.9 ± 1.9 (5)</td>
<td>4.9 ± 0.4 (5)</td>
<td>3.8 ± 0.4 (5)</td>
<td>7.3 ± 1.3 (6)</td>
<td>4.6 ± 0.4 (6)</td>
</tr>
<tr>
<td>Succinate</td>
<td>3.5 ± 2.1 (5)</td>
<td>3.7 ± 2.3 (5)</td>
<td>4.8 ± 1.8 (5)</td>
<td>6.5 ± 2.8 (5)</td>
<td>3.1 ± 2.7 (6)</td>
<td>6.7 ± 5.0 (6)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.2 ± 1.0 (5)</td>
<td>1.1 ± 0.6 (5)</td>
<td>1.1 ± 0.5 (5)</td>
<td>1.2 ± 0.4 (5)</td>
<td>0.6 ± 0.3 (6)</td>
<td>2.1 ± 1.1 (6)</td>
</tr>
<tr>
<td>Acetate</td>
<td>3.7 ± 1.6 (3)</td>
<td>5.1 ± 0.2 (3)</td>
<td>6.4 ± 0.5 (3)</td>
<td>6.4 ± 0.9 (3)</td>
<td>7.6 ± 0.9 (3)</td>
<td>11.1 ± 0.6 (3)</td>
</tr>
<tr>
<td>Propionate</td>
<td>1.7 ± 0.7 (3)</td>
<td>1.6 ± 0.1 (3)</td>
<td>2.4 ± 1.0 (3)</td>
<td>3.5 ± 0.1 (3)</td>
<td>2.9 ± 0.9 (3)</td>
<td>3.4 ± 0.3 (3)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the appropriate control, p < 0.025,
- b " " " " " " , p < 0.005.
TABLE 4.10

The effect of NSC on some of the metabolic intermediates of *F. hepatica* maintained *in vitro*. The results are expressed as nmols/g wet weight and they are means ± standard deviation. n is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>3 h Control</th>
<th>3 h NSC</th>
<th>12 h Control</th>
<th>12 h NSC</th>
<th>20 h Control</th>
<th>20 h NSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>1509 ± 287</td>
<td>1883 ± 464</td>
<td>1682 ± 462</td>
<td>1512 ± 425</td>
<td>2236 ± 517</td>
<td>1447 ± 808a</td>
</tr>
<tr>
<td>6 h</td>
<td>392 ± 54</td>
<td>392 ± 71</td>
<td>372 ± 65</td>
<td>308 ± 161</td>
<td>462 ± 155</td>
<td>308 ± 186a</td>
</tr>
<tr>
<td>12 h</td>
<td>64 ± 16</td>
<td>81 ± 17</td>
<td>42 ± 11</td>
<td>47 ± 29</td>
<td>67 ± 33</td>
<td>51 ± 42</td>
</tr>
<tr>
<td>18 h</td>
<td>58 ± 16</td>
<td>42 ± 6</td>
<td>42 ± 25</td>
<td>39 ± 14</td>
<td>39 ± 29</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>459 ± 147</td>
<td>569 ± 188</td>
<td>296 ± 110</td>
<td>338 ± 153</td>
<td>317 ± 97</td>
<td>419 ± 172</td>
</tr>
<tr>
<td>30 h</td>
<td>163 ± 58</td>
<td>198 ± 90</td>
<td>82 ± 33</td>
<td>108 ± 52</td>
<td>93 ± 34</td>
<td>119 ± 60</td>
</tr>
<tr>
<td>36 h</td>
<td>95 ± 22</td>
<td>156 ± 51b</td>
<td>50 ± 29</td>
<td>230 ± 152c</td>
<td>98 ± 39</td>
<td>570 ± 134c</td>
</tr>
<tr>
<td>42 h</td>
<td>1171 ± 657</td>
<td>1313 ± 659</td>
<td>1016 ± 107</td>
<td>1093 ± 188</td>
<td>516 ± 349</td>
<td>848 ± 412</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the appropriate control, p < 0.05,
- b p < 0.025,
- c p < 0.005.
The effect of MBZ on the internal pool sizes of glucose and end products of *F. hepatica* maintained *in vitro*.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. *(n)* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>12 h Control</th>
<th>12 h MBZ</th>
<th>36 h Control</th>
<th>36 h MBZ</th>
<th>48 h Control</th>
<th>48 h MBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.4 ± 1.3 (10)</td>
<td>4.9 ± 1.3 (6)</td>
<td>7.2 ± 1.9 (5)</td>
<td>5.6 ± 0.6 (5)</td>
<td>8.4 ± 1.9 (5)</td>
<td>6.4 ± 1.2 (6)</td>
</tr>
<tr>
<td>Succinate</td>
<td>4.1 ± 1.6 (10)</td>
<td>2.4 ± 1.1 (6)</td>
<td>2.0 ± 0.3 (5)</td>
<td>1.4 ± 0.9 (5)</td>
<td>1.2 ± 0.4 (5)</td>
<td>1.2 ± 0.5 (6)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.0 ± 0.5 (10)</td>
<td>0.9 ± 0.5 (6)</td>
<td>1.1 ± 0.4 (5)</td>
<td>1.6 ± 0.8 (5)</td>
<td>1.5 ± 1.7 (5)</td>
<td>1.8 ± 1.6 (6)</td>
</tr>
<tr>
<td>Acetate</td>
<td>6.4 ± 0.5 (3)</td>
<td>5.9 ± 0.6 (3)</td>
<td>8.0 ± 2.1 (3)</td>
<td>5.6 ± 0.5 (3)</td>
<td>8.6 ± 1.2 (3)</td>
<td>4.8 ± 0.8 (3)</td>
</tr>
<tr>
<td>Propionate</td>
<td>2.4 ± 1.0 (3)</td>
<td>2.5 ± 0.3 (3)</td>
<td>2.3 ± 1.0 (3)</td>
<td>1.6 ± 0.4 (3)</td>
<td>3.9 ± 0.6 (3)</td>
<td>1.0 ± 0.3 (3)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from control, *p* < 0.05,
b " " " " , *p* < 0.01,
c " " " " , *p* < 0.001.
TABLE 4.12

The effect of MBZ on some of the metabolic intermediates of *F. hepatica* maintained *in vitro*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>12 h Control</th>
<th>12 h MBZ</th>
<th>36 h Control</th>
<th>36 h MBZ</th>
<th>48 h Control</th>
<th>48 h MBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>n</em> = 10</td>
<td><em>n</em> = 6</td>
<td><em>n</em> = 5</td>
<td><em>n</em> = 5</td>
<td><em>n</em> = 5</td>
<td><em>n</em> = 6</td>
</tr>
<tr>
<td>G6P</td>
<td>1659 ± 365</td>
<td>1792 ± 943</td>
<td>1973 ± 210</td>
<td>1361 ± 217&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1829 ± 66</td>
<td>744 ± 253&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>F6P</td>
<td>350 ± 96</td>
<td>331 ± 131</td>
<td>398 ± 176</td>
<td>288 ± 52</td>
<td>379 ± 106</td>
<td>163 ± 34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FDP</td>
<td>40 ± 11</td>
<td>69 ± 38</td>
<td>94 ± 24</td>
<td>102 ± 51</td>
<td>74 ± 45</td>
<td>95 ± 42</td>
</tr>
<tr>
<td>TP</td>
<td>43 ± 13</td>
<td>69 ± 49</td>
<td>52 ± 17</td>
<td>76 ± 14</td>
<td>39 ± 11</td>
<td>50 ± 9</td>
</tr>
<tr>
<td>PGA</td>
<td>289 ± 106</td>
<td>416 ± 347</td>
<td>455 ± 237</td>
<td>679 ± 457</td>
<td>376 ± 293</td>
<td>161 ± 104</td>
</tr>
<tr>
<td>PEP</td>
<td>84 ± 32</td>
<td>115 ± 115</td>
<td>135 ± 56</td>
<td>231 ± 182</td>
<td>72 ± 54</td>
<td>59 ± 35</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>64 ± 29</td>
<td>85 ± 41</td>
<td>147 ± 54</td>
<td>207 ± 103</td>
<td>82 ± 20</td>
<td>109 ± 41</td>
</tr>
<tr>
<td>Malate</td>
<td>935 ± 185</td>
<td>780 ± 351</td>
<td>821 ± 99</td>
<td>578 ± 190&lt;sup&gt;a&lt;/sup&gt;</td>
<td>758 ± 126</td>
<td>536 ± 259</td>
</tr>
</tbody>
</table>

Test for significance by Student's 't' show that:

- <sup>a</sup> significantly different from the appropriate control, *p* < 0.025,
- <sup>b</sup> , *p* < 0.005,
internal concentrations in the NSC treated group following 20 h incubation.

The internal concentrations of most of the glycolytic intermediates are shown in table 4.10. There are only a few differences between the NSC and control groups. The major effect is the increase in the pyruvate concentration which is apparent after 3 h incubation with NSC and increases markedly after 12 and 20 h. There are decreases in the G6P and F6P levels in the 20 h treated group.

(c) MBZ

Table 4.11 shows that there are no significant differences between the control and MBZ treated group after 12 h incubation. After 36 h the only significant effect is the lower glucose pool when MBZ is present. It is also found in the 48 h treated group, where there are also significant decreases in the internal acetate and propionate concentrations.

The concentrations of most of the glycolytic intermediates of *F. hepatica* following incubation with MBZ are given in table 4.12. After 36 h incubation with MBZ there are decreases in the G6P and malate concentrations. The depressed G6P level is also apparent after 48 h incubation and there is a decrease in the F6P pool size at this time.

4. ADENINE NUCLEOTIDE LEVELS

(a) RFX

Table 4.13 shows the effects of RFX on the adenine nucleotide levels of *F. hepatica* during incubation. Both the RFX and control groups again include the 3, 6 and 9 h incubations. The results show
The effect of RFX on the adenine nucleotide levels of *F. hepatica* maintained *in vitro*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* = 9 for all determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RFX treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1193 ± 204</td>
<td>789 ± 277&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>821 ± 51</td>
<td>986 ± 94&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMP</td>
<td>291 ± 73</td>
<td>573 ± 144&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>2304 ± 169</td>
<td>2347 ± 249</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.45</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Tests for significance by paired 't' show that:

<sup>a</sup> significantly different from control, p < 0.001.
that the ATP concentration is depressed in the RFX treated group whereas the ADP and AMP levels are increased. The total adenine nucleotide levels are similar for both groups but the ATP/ADP ratio is lower in the RFX treated group due to the decrease in ATP and increase in ADP concentration.

(b) NSC

The effects of NSC on the adenine nucleotide levels of *F. hepatica* maintained *in vitro* are given in table 4.14. The only significant effect of the drug is the decrease in the ATP level and the increase in the AMP concentration after 20 h incubation. However, at all incubation times the ATP/ADP ratio is lower in the NSC treated groups.

(c) MBZ

The effect of MBZ on the adenine nucleotide levels of *F. hepatica* is presented in table 4.15. With MBZ present after 12 h incubation there is no difference between the control and treated group. After 36 h incubation there is a marked drop in the ATP concentration and an increase in the AMP level in the MBZ treated group. After 48 h incubation with MBZ the ATP level has dropped further and is only 28% of the total adenine nucleotide concentration. There are also decreases in the ADP level and the total adenine nucleotide concentration at this time. The ATP/ADP ratio drops markedly in the MBZ treated groups during the period of incubation due to the drop in the ATP concentration.

5. END PRODUCT EXCRETION

(a) RFX

The amount of end products excreted during the incubations
TABLE 4.14

The effect of NSC on the adenine nucleotides of *F. hepatica* maintained *in vitro*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>3 h Control</th>
<th>3 h NSC</th>
<th>12 h Control</th>
<th>12 h NSC</th>
<th>20 h Control</th>
<th>20 h NSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>n</em> = 7</td>
<td></td>
<td><em>n</em> = 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>892 ± 176</td>
<td>940 ± 147</td>
<td>1062 ± 157</td>
<td>933 ± 81</td>
<td>1229 ± 223</td>
<td>631 ± 387&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>745 ± 112</td>
<td>875 ± 154</td>
<td>832 ± 117</td>
<td>881 ± 101</td>
<td>870 ± 157</td>
<td>846 ± 148</td>
</tr>
<tr>
<td>AMP</td>
<td>265 ± 56</td>
<td>308 ± 80</td>
<td>287 ± 77</td>
<td>406 ± 152</td>
<td>340 ± 154</td>
<td>590 ± 163&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total adenine</td>
<td>1902 ± 263</td>
<td>2123 ± 370</td>
<td>2181 ± 319</td>
<td>2220 ± 89</td>
<td>2439 ± 425</td>
<td>2067 ± 392</td>
</tr>
<tr>
<td>nucleotides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.20</td>
<td>1.07</td>
<td>1.28</td>
<td>1.06</td>
<td>1.41</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

*<sup>a</sup>* significantly different from appropriate control, *p* < 0.01,

*<sup>b</sup>* similarly, *p* < 0.025.
TABLE 4.15

The effect of MBZ on the adenine nucleotide levels of *F. hepatica* maintained *in vitro*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>12 h Control</th>
<th>12 h MBZ</th>
<th>36 h Control</th>
<th>36 h MBZ</th>
<th>48 h Control</th>
<th>48 h MBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>n = 10</td>
<td>n = 6</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 6</td>
</tr>
<tr>
<td>ATP</td>
<td>1031 ± 176</td>
<td>950 ± 209</td>
<td>985 ± 210</td>
<td>668 ± 117&lt;sup&gt;a&lt;/sup&gt;</td>
<td>877 ± 216</td>
<td>364 ± 178&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>782 ± 116</td>
<td>797 ± 113</td>
<td>773 ± 132</td>
<td>781 ± 81</td>
<td>754 ± 48</td>
<td>585 ± 185&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMP</td>
<td>251 ± 66</td>
<td>250 ± 91</td>
<td>245 ± 28</td>
<td>368 ± 49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>311 ± 78</td>
<td>347 ± 79</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>2067 ± 307</td>
<td>1997 ± 209</td>
<td>2002 ± 346</td>
<td>1817 ± 186</td>
<td>1942 ± 204</td>
<td>1279 ± 382&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.32</td>
<td>1.19</td>
<td>1.28</td>
<td>0.85</td>
<td>1.16</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the appropriate control, *p* < 0.02,
- b " " " " " " , *p* < 0.005,
- c " " " " " " , *p* < 0.001.
are shown in table 4.16. Only the 3 and 9 h incubations are presented as the 6 h results are intermediate between them. Initially, in the 3 h incubation, RFX has caused increases in propionate, acetate and succinate production. After 9 h incubation with RFX the amount of propionate excreted is less than in the control, the amount of acetate excreted is the same as in the control, and there is still more succinate excreted than in the control.

The total end products excreted in the incubations (table 4.16) are the sum of the end products shown for each group and so no standard deviation is given. It seems that there is an increase in the total amount of end products excreted by the RFX treated group after 3 h. However, after 9 h incubation there is probably no difference, or perhaps a slight decrease by the RFX treated group, in the total amount excreted.

The P/A ratio for both control groups is 1. In the 3 and 9 h RFX treated groups it is less than 1 due to more acetate production by the 3 h group and less propionate production by the 9 h group.

(b) NSC

The amount of end products excreted during incubation with and without NSC is shown in table 4.17. The total end products are the sum of the means of each product for each incubation time so no standard deviation is given. After 3 h incubation with NSC there is significantly more lactate excreted compared with the controls and overall there seems to be a greater excretion of total end products by the drug treated group.

Following 12 h incubation there is an increase in the amount
TABLE 4.16

The effect of RFX on the excretion of end products by *F. hepatica* maintained *in vitro*.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>3 h Control</th>
<th>3 h RFX treated</th>
<th>9 h Control</th>
<th>9 h RFX treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>1.2 ± 0.3 (3)</td>
<td>4.3 ± 1.3 (3)(^a)</td>
<td>1.5 ± 1.0 (3)</td>
<td>7.2 ± 5.2 (3)(^a)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.4 ± 0.8 (6)</td>
<td>1.7 ± 0.9 (6)</td>
<td>3.5 ± 1.9 (3)</td>
<td>8.5 ± 3.4 (3)</td>
</tr>
<tr>
<td>Acetate</td>
<td>37.8 ± 14.4 (9)</td>
<td>54.2 ± 11.1 (9)(^b)</td>
<td>113.8 ± 45.1 (7)</td>
<td>111.6 ± 36.3 (7)</td>
</tr>
<tr>
<td>Propionate</td>
<td>35.3 ± 8.5 (8)</td>
<td>41.7 ± 9.2 (8)(^b)</td>
<td>111.9 ± 19.3 (7)</td>
<td>82.7 ± 14.5 (7)(^a)</td>
</tr>
<tr>
<td>Total end products</td>
<td>75</td>
<td>101</td>
<td>229</td>
<td>209</td>
</tr>
<tr>
<td>P/A</td>
<td>0.95</td>
<td>0.76</td>
<td>0.98</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Tests for significance by paired 't' show that:

\(^a\) significantly different from the appropriate control, p < 0.05,

\(^b\) " " " "", p < 0.005.
TABLE 4.17

The effect of NSC on the excretion of end products by *F. hepatica* maintained *in vitro*. The results are expressed as µmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>3 h Control</th>
<th>3 h NSC</th>
<th>12 h Control</th>
<th>12 h NSC</th>
<th>20 h Control</th>
<th>20 h NSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>3 ± 1 (4)</td>
<td>3 ± 1 (4)</td>
<td>4 ± 2 (4)</td>
<td>6 ± 3 (4)</td>
<td>6 ± 4 (4)</td>
<td>7 ± 4 (4)</td>
</tr>
<tr>
<td>Lactate</td>
<td>5 ± 1 (4)</td>
<td>16 ± 5 (4)b</td>
<td>19 ± 4 (4)</td>
<td>27 ± 6 (4)</td>
<td>40 ± 12 (4)</td>
<td>49 ± 7 (4)</td>
</tr>
<tr>
<td>Acetate</td>
<td>32 ± 6 (4)</td>
<td>36 ± 6 (4)</td>
<td>78 ± 17 (6)</td>
<td>91 ± 20 (6)a</td>
<td>136 ± 23 (5)</td>
<td>164 ± 38 (5)</td>
</tr>
<tr>
<td>Propionate</td>
<td>42 ± 17 (4)</td>
<td>48 ± 9 (4)</td>
<td>222 ± 66 (6)</td>
<td>214 ± 55 (6)</td>
<td>245 ± 45 (7)</td>
<td>231 ± 54 (7)</td>
</tr>
<tr>
<td>Total end products</td>
<td>82</td>
<td>103</td>
<td>323</td>
<td>338</td>
<td>427</td>
<td>451</td>
</tr>
<tr>
<td>P/A</td>
<td>1.3</td>
<td>1.3</td>
<td>2.9</td>
<td>2.4</td>
<td>1.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the appropriate control, *p* < 0.05,
- b " " " " " " " " " , *p* < 0.001.
of acetate excreted by the NSC treated flukes. The amount of lactate excreted by the drug treated flukes is no longer significantly higher than that by the control group.

After 20 h incubation with NSC there are no significant differences between the amounts of any of the end products excreted compared with the control group.

The P/A ratios after 3 h incubation are the same for the control and NSC treated groups. After 12 h incubation there is an increase in the control P/A ratio, as found in the in vitro maintenance study (see chapter 3). The P/A ratio is also increased in the 12 h NSC treated group but it is not as high as the control value, due to the increase in acetate excretion by the drug treated group. Following 20 h incubation the P/A ratio of the controls is around 2, which is similar to the findings in chapter 3. The P/A ratio of the 20 h NSC treated flukes is not as high as the corresponding control value.

(c) MBZ

The end products excreted during the incubations are given in table 4.18. The total end products excreted are the sum of the means of each product for each time so no standard deviation is given. The most significant feature is the increase in the amount of succinate excreted when MBZ is present, at all three sampling times. After 12 h incubation there is also a decrease in the amount of propionate excreted. Following 36 h incubation with MBZ there is a decrease in the amount of lactate excreted, which is also evident after 48 h. There are no significant effects on the amounts of acetate and propionate excreted after 36 and 48 h incubation with MBZ. At all times the P/A ratio is lower in the MBZ treated groups.
TABLE 4.18

The effect of MBZ on the excretion of end products by *F. hepatica* maintained *in vitro*.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>12 h Control</th>
<th>12 h MBZ</th>
<th>36 h Control</th>
<th>36 h MBZ</th>
<th>48 h Control</th>
<th>48 h MBZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>4 ± 2 (4)</td>
<td>60 ± 9 (4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15 ± 4 (4)</td>
<td>76 ± 27 (4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13 ± 3 (4)</td>
<td>114 ± 53 (4)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate</td>
<td>19 ± 4 (4)</td>
<td>20 ± 5 (4)</td>
<td>85 ± 21 (4)</td>
<td>52 ± 12 (4)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113 ± 4 (3)</td>
<td>77 ± 25 (4)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetate</td>
<td>102 ± 11 (3)</td>
<td>102 ± 11 (3)</td>
<td>162 ± 80 (5)</td>
<td>190 ± 110 (4)</td>
<td>207 ± 25 (4)</td>
<td>233 ± 60 (6)</td>
</tr>
<tr>
<td>Propionate</td>
<td>182 ± 4 (3)</td>
<td>122 ± 42 (3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>305 ± 120 (5)</td>
<td>298 ± 125 (4)</td>
<td>425 ± 95 (4)</td>
<td>332 ± 117 (6)</td>
</tr>
<tr>
<td>Total end products</td>
<td>307</td>
<td>304</td>
<td>567</td>
<td>616</td>
<td>758</td>
<td>756</td>
</tr>
<tr>
<td>P/A</td>
<td>1.6</td>
<td>1.2</td>
<td>1.9</td>
<td>1.6</td>
<td>2.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the appropriate control, p < 0.025,
- b , p < 0.001.
4.4 DISCUSSION

The results of this study show that three drugs (RFX, NSC and MBZ) which, according to field trial data, are effective fasciolicides, have marked effects on the energy metabolism of *F. hepatica* during *in vitro* incubation. The most profound effects are on the adenine nucleotide concentrations. All three drugs disturb the ability of the parasite to phosphorylate ADP; RFX within 9 h, NSC by 20 h and MBZ after 36 h incubation.

The effects of RFX on the adenine nucleotide levels are consistent with the view that RFX acts as an uncoupler and so impairs the efficiency of the ATP synthesising system. Thus, the results of Yorke and Turton (1974) and Van den Bossche (1972a) are complemented by the present findings and together they show that RFX is capable of traversing the body or intestinal wall of the fluke and affecting ATP synthesis in the whole animal as well as in isolated mitochondria.

The disturbance in ADP phosphorylation caused by MBZ after 36 h incubation is in agreement with the finding of Van den Bossche (1972a) that MBZ inhibits the incorporation of $^{32}\text{Pi}$ into ATP in isolated *Ascaris* mitochondria. Following 48 h incubation with MBZ there is a further effect on the adenine nucleotides. The ATP concentration has dropped to only 28% of the total adenine nucleotide concentration and the ADP and total adenine nucleotide levels have decreased significantly. This implies that there is an effect on adenine nucleotide synthesis or turnover. It is possible that this effect is due to a diminished synthesis of precursors resulting from insufficient ATP. For example, the formation of adenylic acid, a precursor of ATP, requires energy in the form of GTP (McGilvery, 1970).
Rahman (1976) also found a decrease in ATP and total adenine nucleotide levels in the cestode *M. expansa* following treatment with MBZ *in vitro* and *in vivo*. The effect *in vitro* occurs within 30 min. For a similar effect in *F. hepatica* incubation for at least 36 h is required. The large difference in time of effect between *F. hepatica* and *M. expansa* may be due to different permeability barriers in the two worms. *M. expansa* has a large surface area due to the presence of microtriches, which allow greater absorption of nutrients from the host's intestinal contents. *F. hepatica* has an oral sucker and a gut but also absorbs low molecular weight nutrients through the tegument (see 1.5.2). It is not known whether MBZ is absorbed by flukes through the tegument or whether it can get into the caeca of the gut and penetrate the intestinal wall. Perhaps the permeability barriers in flukes are greater than in *M. expansa*.

The decrease in total adenine nucleotides found in the flukes after 48 h incubation with MBZ is an important consideration if the adenylate energy charge is used as an index of 'condition' (see 1.6.8). Implicit in the formula for the energy charge is that the total adenine nucleotide concentration remain constant. If there is a change in the totals, a misleading value is obviously obtained. The adenylate energy charges have not been given in the present study because of the decrease in total adenine nucleotide concentration in the 48 h MBZ treated flukes. Instead, the ATP/ADP ratios are considered to be better indicators as, though the ADP concentration does fall in the 48 h treated group, the ATP concentration decreases to a greater extent.

The effects on the adenine nucleotide concentrations by the three drugs must have certain consequences for the intact animal.
Carbon flow along respiratory metabolic pathways is regulated by elaborate mechanisms to ensure an optimum energy status. Regulation is mediated by the relative concentrations of adenine nucleotides. Changes in ATP/ADP ratios will have secondary effects on the pathway which must not be confused with those that occur as a direct result of the drug. Decreased ATP/ADP ratios lead to an increase in carbon flow through the pathways of energy metabolism (Newsholme & Start, 1973). If this is the only effect of the drugs, the formation of end products should also increase, i.e. there is an increase in carbon flow.

Tables 4.16 and 4.17 show that the presence of RFX and NSC in the incubations cause initial increases in the excretion of end products by the flukes. After 3 h incubation with RFX there are increases in the amounts of propionate, acetate and succinate formation, of about 25% in total. 3 h incubation with NSC results in an increase in the amount of lactate excreted and also an overall increase of about 25%. Thus, the effects on the adenine nucleotides and increases in end product excretion of flukes incubated with RFX and NSC suggest that the two drugs act as uncouplers.

There does not seem to be a marked increase in the amount of end products excreted by any of the MBZ treated group despite the decrease in the ATP/ADP ratios of each sample. It is possible that the pathways of energy metabolism are already operating at maximal activity as a result of adaptation to the in vitro conditions. Alternatively, there may have been an early increase in end product formation in the MBZ treated flukes, before the first media samples were taken. The control flukes may have 'caught up' by this time in response to in vitro incubation.
There are similar effects of the three drugs on some of the concentrations of intermediates of the energy producing pathway. There are decreases in the internal glucose pools in the RFX treated group, in the 12 and 20 h NSC treated groups and in the 36 and 48 h MBZ treated groups. This could reflect either an increase in glucose utilisation by the flukes or a decrease in uptake from the medium.

Isseroff and Read (1974) measured the uptake of 3-O-methyl glucose by *F. hepatica*. Their studies showed that it is not accumulated against a concentration difference. The concentration of the sugar in the worm reaches equilibrium with the level in the medium in about 90 min and remains at a constant level for up to 180 min. Chromatographic analysis of worm extract following incubation with $^{14}$C-3-O-methyl glucose shows that the compound is not metabolised by *F. hepatica*. The presence of glucose in the medium inhibits 3-O-methyl glucose uptake. Isseroff and Read (1974) conclude from the study that glucose and 3-O-methyl glucose are transported through the same mechanism, which is referred to as the glucose site (see 1.5.2).

In the present study, incubations were carried out with individual flukes to measure $^{14}$C-3-O-methyl glucose uptake over a period of 180 min. The simple technique employed (see 1.4.2.(c)) provides an accurate measurement of 3-O-methyl glucose uptake and avoids the complication of the loss of radiocarbon due to metabolism of the compound by the fluke. Thus, the results can be equated with glucose uptake. Tables 4.4, 4.5 and 4.6 show that within the first 3 h none of the drugs affects methyl glucose uptake by the flukes. Due to the length of time required for NSC and MBZ to disturb the energy metabolism of *F. hepatica* some flukes were preincubated with NSC and
with MBZ to ensure that an effect does not become apparent at a later time. The results show that methyl glucose uptake is not affected by preincubation with these drugs. Thus, the decreases in the internal glucose concentrations, caused by the three drugs, are not due to inhibition of glucose uptake from the medium. There must be increases in glucose utilisation.

The finding that there is no effect of any of the drugs on glucose uptake by *F. hepatica* or on the glycogen reserves supports the hypothesis, discussed in 2.6.1 for MBZ, that in cestodes and nematodes, the effect of MBZ on the energy levels may secondarily cause inhibition of glucose uptake and depletion of glycogen reserves. This is not in agreement with the conclusion of Van den Bossche (1972b) and De Nollin and Van den Bossche (1973), who state that MBZ inhibits glucose uptake primarily, therefore glycogen reserves are utilised and eventually ATP levels fall. The reason for the different effect in *F. hepatica* is probably because of the different mode of glucose uptake. In cestodes and nematodes the uptake of glucose is an active process, i.e. energy requiring (e.g. Pappas, Uglem & Read, 1974; Read, Stewart & Pappas, 1974), whereas in *F. hepatica* glucose uptake is not energy dependent but occurs by a mediated process (Isseroff & Read, 1974). Thus, it seems that the effect of MBZ on the energy levels of the parasite is a more direct effect and the inhibition of glucose uptake is due to the low ATP levels in those parasites which employ active transport, and therefore, is a secondary effect.

There are decreases in the G6P and F6P concentrations of the RFX treated group, the 20 h NSC treated group and the 48 h MBZ treated group. There are also decreases in the level of FDP in the RFX treated
group and the G6P concentration of the 36 h MBZ treated group. These effects probably reflect the lower glucose pools resulting from increased glucose utilisation in the drug treated groups.

The malate concentrations of the RFX treated group and the 36 h MBZ treated group are lower than those of the controls. It has been found by Van Vugt et al. (1976) that the addition of an uncoupler to a preparation of isolated F. hepatica mitochondria results in an increase in malate utilisation. The results of this and other studies suggest that RFX is an uncoupler. The effect of MBZ on the adenine nucleotide concentrations and the slight increase in total end product excretion after 36 h incubation are characteristic of an uncoupling action. Thus, the decreases in malate concentration may reflect an increase in utilisation.

The flukes incubated with NSC and RFX have higher pyruvate concentrations than the corresponding control groups. With NSC present there is an increase in the amount of lactate excreted after 3 h incubation and after 20 h incubation there is an increase in the internal lactate concentration. After 12 h, more acetate is excreted by the NSC treated group. Thus, the increased pyruvate concentration seems to be contributing to both the lactate and acetate pools of the flukes. In the RFX treated group there is no corresponding increase in the lactate pool but there is an increase in acetate production. It has been suggested in chapter 3 that there are probably two pyruvate pools; a cytosolic and a mitochondrial one. It appears that in the RFX treated group the increase in the pyruvate level may be due to an increase in the mitochondrial pool. There may be a greater conversion of malate to pyruvate, via malic enzyme, in the mitochondria. The
increased flow of carbon into the acetate branch of the pathway may be greater than the rate of conversion of pyruvate to acetate, i.e. the pyruvate dehydrogenase step is rate-limiting, which would explain the increase in the pyruvate pool in the mitochondria. When NSC is present both pyruvate pools seem to increase, thus contributing to both the acetate and lactate pools. Pyruvate accumulation may be due to an increase in synthesis and/or inability of the worm to metabolise it at a faster rate, i.e. the enzymes LDH and pyruvate dehydrogenase may be rate-limiting. Alternatively, there may be increases in amino acid metabolism in both the RFX and NSC treated flukes which would contribute to the increase in pyruvate concentration.

In the RFX treated flukes there are increases in the internal concentrations of succinate and the amount of succinate excreted. A similar effect was observed by Bryant, Smith and Williams (1963) in a study on the effect of mepacrine on *F. hepatica*. They found that the drug interferes with the phosphorylation of glucose and also increases the formation of radioactive succinate from $^{14}$C-glucose. There is also a decrease in the amount of propionate formed after 9 h incubation with RFX. This suggests that the further metabolism of succinate to propionate is inhibited in some way. The disturbance in stoichiometry between acetate and propionate formation may be due to the inhibition of succinate metabolism.

In the 3 h NSC treated group the P/A ratio is similar to the control indicating that the relationship between propionate and acetate formation (discussed in chapter 3) is maintained. However, after 20 h incubation the P/A ratio is lower in the NSC treated groups, in both cases due to less propionate and more acetate being excreted compared
with the controls. Thus, the relationship between acetate and propionate production is changed.

After 12 h incubation with MBZ there is a drop in the amount of propionate produced and a corresponding increase in the amount of succinate excreted. 36 and 48 h incubation with MBZ also result in marked increases in succinate excretion. If the amount of succinate excreted is added to the amount of propionate excreted at each time of sampling, the totals obtained are similar to the total propionate produced by the controls. Hence, there appears to be an inhibition of the further metabolism of succinate in the MBZ treated flukes. Similar amounts of carbon flow through the fumarate reductase step. The inhibition of the conversion of succinate to propionate in the RFX and MBZ treated flukes will result in a decrease in energy production, as one molecule of ATP is produced per molecule of propionate formed from succinate.

4.5 GENERAL DISCUSSION

The studies described in this chapter show that a simple in vitro system can be used to examine the effects of anthelmintics on the energy metabolism of *F. hepatica*. The system is easy to manipulate, sample and assay. These are important considerations when evaluating the efficacy of new anthelmintics. It has the added advantage that flukes may be maintained for more than 48 h without adverse effects on their energy metabolism, which should be sufficient time to determine whether a drug is going to have an effect on energy production in vitro.

The results have shown that the energy producing pathway is essential for the maintenance of the integrity of the parasite and that
it has considerable capacity to adjust to adverse conditions. The most important indicators of anthelmintic effect appear to be the concentrations of adenine nucleotides. All three drugs disturb the ability of the parasite to phosphorylate ADP. MBZ has a further, later effect of reduction of the total adenine nucleotide concentration, which may be due to disruption of adenine nucleotide synthesis or turnover.

The effects of NSC and RFX on the adenine nucleotide levels and the initial increases in end product excretion suggest that the two drugs act as uncouplers of phosphorylation. The other in vitro studies carried out with RFX (see 2.6.3) also suggest that it is an uncoupler of electron transport and phosphorylation.

There are changes in the concentrations of some of the intermediates of the pathway which may be direct effects of the drugs or result from changes in the adenine nucleotide levels and carbon flow through the pathway, e.g. decreases in glucose, G6P, F6P and FDP levels.

None of the drugs affect glucose uptake, as measured by 3-O-methyl glucose uptake, or glycogen utilisation. Thus, the flukes do not die from starvation; there are sufficient carbohydrate reserves available. That MBZ does not inhibit glucose uptake or stimulate glycogen utilisation supports the hypothesis proposed in 2.6.1 that a major effect of the drug is a decrease in ATP concentration which causes inhibition of active transport of nutrients in some parasites. *F. hepatica* does not take up glucose by an energy dependent mechanism.

There are some drug-specific effects on various components of the pathway. MBZ causes a slight inhibition of lactate excretion and a large increase in succinate excretion. These effects will have
consequences for energy production and the redox balance of the parasite. NSC causes a marked increase in pyruvate concentration which contributes to an increase in lactate and acetate excretion. RFX appears to inhibit the further metabolism of succinate to propionate, which disrupts the relationship between propionate and acetate formation and will affect energy production.

This study has shown that three structurally unrelated fasciolicides have several similar effects on the energy metabolism of F. hepatica. It shows that the parasite cannot maintain normal energy production in the presence of the drugs. The effects of MBZ, NSC and RFX on the adenine nucleotide concentrations may not be the primary action but they ultimately contribute to the death of the parasite.
CHAPTER 5

THE EFFECTS OF MBZ, NSC AND RFX ON F. HEPATICA
FOLLOWING IN VIVO TREATMENT

5.1 INTRODUCTION

Once an anthelmintic is shown to have an effect in vitro it is necessary to establish that it has a similar effect on the parasite in vivo. There may be permeability barriers in vivo which stop the drug reaching the parasite or the site of action within the parasite. In the case of blood feeders, the concentration of the drug available to the parasite in vivo may be too low to cause an effect. It is also possible that the drug is metabolised by the host and the product may not have anthelmintic properties. The reverse situations are also possible. That is, a drug which is effective in vivo may not affect the parasite in vitro. This may be because host metabolism of the drug is necessary to produce the active metabolite, or the host may accumulate levels of the drug which are much higher than the level experienced by the parasite in vitro. Another possibility is that in vitro some parasites may not feed in the same manner as they do in vivo and so do not take up the drug.

Rahman (1976) has examined the effects of two benzimidazoles, cambendazole and MBZ, on M. expansa in vivo, following the demonstration of their activity in vitro. He found that the major effects of the drugs on the adenine nucleotide levels, glucose uptake,
glycogen utilisation and succinate production found in vitro are similar to what occurs in vivo and so concluded that they are associated with the anthelmintic efficacy of the compounds.

The drugs used in the present study have efficacy against F. hepatica in vivo as shown by field trial data (see 2.6.1, 2.6.2, 2.6.3). Experiments with the drugs were carried out to determine whether they have effects on the energy producing pathway in vivo similar to those observed in vitro and thus contribute to the death and/or expulsion of the parasite from the host.

5.2 METHODS

(a) MBZ

The in vivo MBZ experiment was carried out at Ethnor Pty Ltd, The Oaks, N.S.W. and the infected sheep, slaughtering facilities and assistance were also provided.

Three groups of five sheep were infected with 100 metacercariae (obtained from Dr J.D. Kelly, University of Sydney) 15 weeks prior to the experiment. One group of five sheep was left as the control group. The other two groups were treated orally with a 5% w/v suspension of MBZ (Telmin RLT Sheep drench) at a dose rate of 100 mg/kg and were killed 18 h and 30 h after treatment.

The flukes were removed from the livers and washed in warm Hédon-Fleig salt solution (see appendix 1 (d)). The total number of flukes recovered, together with the sites in which they were found were recorded, i.e. liver, gall bladder or intestine. Some flukes were immediately blotted and frozen in liquid nitrogen ('0' time) for intermediate assays and some were also taken for glycogen
determinations. The rest of the flukes were incubated in Hédon-Fleig containing glucose, streptomycin and penicillin (see appendix 1 (d)), for 24 h. The media were changed after 8-12 h and samples of flukes were taken for intermediate assays and glycogen determination at the completion of the incubation.

(b) NSC

The in vivo NSC experiment was carried out at CIBA-GEIGY Australia Ltd, Research Centre, Kemps Creek, N.S.W. Infected sheep, slaughtering facilities and assistance were provided.

Fourteen sheep were infected with 200 metacercariae obtained from CIBA-GEIGY's culture system (Boray, 1969), 13 weeks prior to the experiment. Four sheep were kept as controls. Ten sheep were drenched with NSC (a 25% w/v suspension of the active ingredient with a particle size of 2-3 µ (95% < 5 µm) at a dose rate of 150 mg/kg. Five were killed 12 h after treatment and five 24 h after treatment.

The flukes were removed from the livers and prepared as described in 5.2.(a). Samples were taken immediately after washing ('0' time) and following 2h incubation with glucose in the medium.

(c) RFX

The in vivo RFX experiment was carried out at Merck Sharp and Dohme (Australia) Pty Ltd, Ingleburn, N.S.W. The infected sheep, slaughtering facilities and assistance were provided.

Twenty seven sheep were infected with 200 metacercariae obtained from the Merck Sharp and Dohme culture system 20 weeks prior to the experiment. Eight sheep were left as controls. The rest were drenched with a 2.27% w/v suspension of RFX (RANIDE™) intraruminally
at a dose rate of 7.5 mg/kg four days, 24 h and 12 h prior to killing.

The flukes were removed from the hosts and prepared as described in 5.2.(a). Samples were taken immediately after washing ('0' time) and following 24 h incubation. Some glycogen samples were also taken 6 h after incubation.

(d) ASSAYS

The samples taken for intermediate assays were treated as described in 3.2.(c) except that the perchloric acid extracts were centrifuged for 4 min at 10,000 g in a Quickfit microcentrifuge. The final extracts were frozen immediately and assayed as described in 3.2.(d).

As the experiments were carried out at the research farms of the three drug companies it was not possible to perform the intermediate assays on the day of sample preparation. The samples had to be frozen immediately after preparation. Some of the intermediates do not withstand freezing - DHAP and G3P - so could not be determined in these experiments. Some of the other intermediates - PEP, 3PGA and 2PGA were not assayed because they did not change in the *in vitro* experiments and it was considered of greater importance to carry out the adenine nucleotide assays and related coupled assays as soon as the samples were transported back to the laboratory. The time from the preparation of a sample to the assays was less than three days.

Glycogen samples were prepared and assayed as described in 3.2.(e) except that the glucose was assayed using a Boehringer blood sugar assay kit (GOD method, Boehringer Mannheim catalogue number 15982, 1973/4). 5 μl samples were used and the concentration of
glucose was determined from a standard curve carried out at each time.

(e) ASSAYS OF NECROTIC FLUKES

One sheep infected with *F. hepatica* was supplied by Merck Sharp and Dohme (Australia) Pty Ltd and was drenched with RFX (RANIDE™) at a dose rate of 7.5 mg/kg. It was killed 30 h after treatment. Most of the flukes had necrotic posterior portions and were stained with bile. Samples of normal anterior and necrotic posterior portions were taken by cutting the flukes with a scalpel at the junction of the two regions. They were immediately frozen in liquid nitrogen, separately. Extracts were made as described in 3.2.(c) and 5.2.(d) and assays were carried out as described in 3.2.(d) and 5.2.(d).

(f) HISTOLOGY

Samples of flukes were taken from a sheep drenched with NSC (at 150 mg/kg) and a sheep drenched with RFX (RANIDE™, Merck Sharp and Dohme (Australia) Pty Ltd at 7.5 mg/kg), killed 30 h after treatment. The flukes had necrotic posterior portions which were stained with bile posteriorly and laterally; the anterior ends appeared normal and active. Flukes were also taken from a control sheep. The flukes were rinsed in Hédon-Fleig salt solution, placed gently between two glass slides to keep them flat and were immersed in 70% ethanol or Bouin's fluid.

(i) CATECHOL REACTION

The catechol technique described by Smyth (1954) for the demonstration of polyphenol-oxidase activity in whole mounts of *F. hepatica* was carried out to determine whether the necrotic portions of the flukes contained the enzyme.
The flukes in 70% ethanol were washed in distilled water for about 30 min. They were transferred to a freshly prepared 0.2% catechol solution and incubated for about 60 min at 40°. They were then washed in distilled water for about 30 min and whole mounts were prepared using the procedures described by Smyth (1954). Before clearing with xylene the flukes were examined under a binocular microscope.

(ii) SECTIONS

The flukes, fixed in Bouin's fluid, were embedded in paraffin wax using standard procedures. 5-7 μm sections were cut and stained with Mayer's haemotoxylin and eosin.

(g) SCANNING ELECTRON MICROSCOPY

The flukes, collected in 70% ethanol, as described in 5.2 (f), were cut with a scalpel to produce pieces approximately 25 mm². They were freeze-dried at -40° in a Dynavac cold stage freeze drier for 24 h. The specimens were then coated with gold palladium (80%/20%) in a Dynavac Special Coating Unit by vacuum deposition on a rotating planetary head. The thickness of the coating was estimated, by a measurement made with a digital thin film thickness monitor (Sloan), to be approximately 300Å. The specimens were examined by a Cambridge Stereoscope 180 at 30 KV accelerating voltage.

5.3 RESULTS

5.3.1 MBZ

(a) OBSERVATIONS MADE DURING COLLECTION OF THE FLUKES

The total fluke recoveries from the control and MBZ treated groups of sheep were similar, but the sites of recovery were different
TABLE 5.1

Fluke recovery from the liver, gall bladder and intestine of the host following *in vivo* treatment with MBZ.

The results are expressed as a percentage of the total number of flukes recovered from each group. There were five sheep in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Gall bladder</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>97.8%</td>
<td>1.1%</td>
<td>1.1%</td>
<td>0%</td>
</tr>
<tr>
<td>18 h MBZ treated</td>
<td>90.0%</td>
<td>3.7%</td>
<td>6.3%</td>
<td>0%</td>
</tr>
<tr>
<td>30 h MBZ treated</td>
<td>71.0%</td>
<td>7.8%</td>
<td>9.5%</td>
<td>11.7%</td>
</tr>
</tbody>
</table>
following drug treatment. This is shown in table 5.1. In the control sheep no flukes were found in the large intestine and in one sheep two flukes were found in the small intestine. In another, two were found in the gall bladder. However, in the 18 h treated group there was an increase in the number of flukes recovered in the gall bladder and small intestine. 30 h after treatment some flukes were found in the large intestine and a greater proportion were found in the gall bladder and small intestine. Those recovered from the small intestine were alive and active but those recovered from the large intestine appeared moribund when placed in warm Hédon-Fleig solution. Most of the control flukes were located in the main bile duct, whereas in the treated groups they were in the peripheral regions of the liver.

In the control group, the majority of the flukes were firmly attached by their oral suckers, which were embedded in the bile duct walls. They had to be removed very carefully to avoid damaging them. The majority of control flukes also had full caeca, shown by the dark pigment inside them. In contrast, many of the flukes recovered from the 18 h treated sheep were not attached and had empty caeca. The flukes recovered 30 h after treatment were all detached and had empty caeca. Also, some of them appeared to have necrotic posterior regions which were stained with bile, but their anterior regions were normal in colour and moving actively. None of the necrotic flukes were included in the samples taken at '0' time or for incubation.

(b) GLYCOGEN LEVELS

The glycogen levels of the flukes removed immediately from the livers ('0' time) and following 24 h incubation in media containing glucose are given in table 5.2. The control level at '0' time is
TABLE 5.2

The effect of MBZ on the glycogen levels of *F. hepatica* treated *in vivo* and after further incubation for 24 h *in vitro*.

The results are expressed as µmoles of glucosyl units/g wet weight and they are means ± standard deviation. (n) is the number of samples.

<table>
<thead>
<tr>
<th></th>
<th>'0' time</th>
<th>24 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>163 ± 18 (5)</td>
<td>257 ± 43 (5)a</td>
</tr>
<tr>
<td>18 h MBZ treated</td>
<td>139 ± 18 (5)</td>
<td>224 ± 23 (5)b</td>
</tr>
<tr>
<td>30 h MBZ treated</td>
<td>90 ± 7 (5)a</td>
<td>192 ± 48 (2)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from '0' time control, p < 0.001,

b significantly different from '0' time, p < 0.001.

and 18 h MBZ treated '0' time, p < 0.001.
comparable with the level found in flukes collected from the abattoir (table 3.1). After incubation there is a significant increase in the glycogen level of the controls.

18 h after treatment the glycogen level at '0' time is not significantly different from the control value and there is a similar increase in the level following 24 h incubation. 30 h after treatment there is a marked decrease in the glycogen level of the flukes at '0' time. These flukes seem able to restore their glycogen levels when incubated for 24 h with glucose in the medium, but this has not been tested statistically due to the low numbers of non-necrotic flukes available for incubation.

(c) ADENINE NUCLEOTIDE LEVELS

Table 5.3 presents the effects of MBZ on the adenine nucleotide concentrations of flukes removed immediately from the livers. The control values are similar to the values found in flukes collected from the abattoirs (table 3.4). There is no effect of the drug on the adenine nucleotide levels of the 18 h treated flukes. There are marked drops in the ATP level and the total adenine nucleotide concentration of the flukes removed 30 h after treatment with MBZ.

The effects on the adenine nucleotide levels following 24 h incubation are given in table 5.4. There is a decrease in the ATP level of the 18 h MBZ treated group. There are decreases in the ADP, AMP and total adenine nucleotide concentrations in the 30 h treated group.

(d) METABOLIC INTERMEDIATE POOL SIZES

Table 5.5 shows the effects of MBZ on the concentrations of
TABLE 5.3

The effect of MBZ on the adenine nucleotide levels of *F. hepatica* treated *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. The number of determinations in each group is five.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>18 h MBZ treated</th>
<th>30 h MBZ treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>890 ± 212</td>
<td>1001 ± 81</td>
<td>627 ± 82</td>
</tr>
<tr>
<td>ADP</td>
<td>826 ± 106</td>
<td>947 ± 75</td>
<td>757 ± 169</td>
</tr>
<tr>
<td>AMP</td>
<td>342 ± 91</td>
<td>327 ± 58</td>
<td>290 ± 23</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>2058 ± 305</td>
<td>2274 ± 168</td>
<td>1674 ± 228</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.25</td>
<td>1.37</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the control and 18 h MBZ treated, p < 0.05,
- b significantly different from 30 h MBZ treated, p < 0.005,
- c 18 h MBZ treated, p < 0.05.
The effect of MBZ on the adenine nucleotide levels of *F. hepatica* following *in vitro* incubation for 24 h after treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>18 h MBZ treated (n = 5)</th>
<th>30 h MBZ treated (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1016 ± 114</td>
<td>794 ± 160^a</td>
<td>823 ± 178</td>
</tr>
<tr>
<td>ADP</td>
<td>1025 ± 186</td>
<td>973 ± 162</td>
<td>723 ± 140^a</td>
</tr>
<tr>
<td>AMP</td>
<td>241 ± 25</td>
<td>253 ± 45</td>
<td>147 ± 47^c</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>2281 ± 165</td>
<td>2021 ± 353</td>
<td>1692 ± 359^b</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>0.99</td>
<td>0.82</td>
<td>1.14</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from control, *p* < 0.05,
- b " " " " , *p* < 0.01,
- c " " " " , *p* < 0.005.
TABLE 5.5

The effect of MBZ on some of the metabolic intermediate levels of *F. hepatica* following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. n is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>18 h MBZ treated (n = 10)</th>
<th>30 h MBZ treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>257 ± 113</td>
<td>253 ± 113</td>
<td>224 ± 89</td>
</tr>
<tr>
<td>G6P</td>
<td>895 ± 233</td>
<td>1173 ± 234&lt;sup&gt;a&lt;/sup&gt;</td>
<td>594 ± 120&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>F6P</td>
<td>168 ± 57</td>
<td>189 ± 69</td>
<td>142 ± 32</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>302 ± 72</td>
<td>201 ± 66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96 ± 49&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate</td>
<td>773 ± 148</td>
<td>966 ± 413</td>
<td>1260 ± 249&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malate</td>
<td>768 ± 172</td>
<td>1071 ± 132&lt;sup&gt;b&lt;/sup&gt;</td>
<td>623 ± 177&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Succinate</td>
<td>3266 ± 1196</td>
<td>2783 ± 1136</td>
<td>2338 ± 1030</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- <sup>a</sup> significantly different from the control, p < 0.01,
- <sup>b</sup> significantly different from the control, p < 0.001,
- <sup>c</sup> 18 h MBZ treated, p < 0.01,
- <sup>d</sup> 18 h MBZ treated, p < 0.001.
The effect of MBZ on some of the metabolic intermediate levels of *F. hepatica* after incubation *in vitro* for 24 h following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>18 h MBZ treated (n = 5)</th>
<th>30 h MBZ treated (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>6155 ± 859</td>
<td>3396 ± 864^c^</td>
<td>5332 ± 1218</td>
</tr>
<tr>
<td>G6P</td>
<td>1769 ± 123</td>
<td>1634 ± 290</td>
<td>1833 ± 181</td>
</tr>
<tr>
<td>F6P</td>
<td>420 ± 43</td>
<td>355 ± 70</td>
<td>288 ± 58^b^</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>151 ± 87</td>
<td>156 ± 57</td>
<td>199 ± 53</td>
</tr>
<tr>
<td>Lactate</td>
<td>1286 ± 622</td>
<td>598 ± 113^b^</td>
<td>364 ± 240^a^</td>
</tr>
<tr>
<td>Malate</td>
<td>1192 ± 259</td>
<td>1372 ± 325</td>
<td>1124 ± 542</td>
</tr>
<tr>
<td>Succinate</td>
<td>3068 ± 796</td>
<td>2572 ± 983</td>
<td>4125 ± 557</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- ^a^ significantly different from the control, *p* < 0.01,
- ^b^ " " " " " , *p* < 0.005,
- ^c^ " " " " " , *p* < 0.001.
some of the intermediates of the energy producing pathway. There is a marked difference between the internal glucose pool of the flukes collected from the abattoirs (table 3.2) and those in this experiment. After the death of the host there may be an increase in the blood glucose level in the liver due to increased glycogen breakdown. If the flukes continue to feed after the host's death this may result in an increase in the internal glucose concentration of the flukes.

The other pool sizes of the flukes obtained from the abattoir are comparable with those of the flukes in this study.

There are increases in the G6P and malate concentrations and a decrease in the pyruvate pool in the 18 h treated flukes compared with the control group. 30 h after treatment the G6P pool is much lower than in the control group, the pyruvate concentration is lower than the control and 18 h treated group and there is an increase in the lactate internal concentration.

Table 5.6 shows the effects of MBZ treatment on the intermediate levels following 24 h incubation. There are increases in the internal glucose concentrations of all groups due to uptake of the glucose in the medium. The increase in the 18 h treated group is not as great as for the control. There is a decrease in the lactate concentration in both drug treated groups and there is also a decrease in the F6P level in the 30 h MBZ treated group.

(e) GLUCOSE UPTAKE AND END PRODUCT EXCRETION

Table 5.7 shows the amount of glucose taken up and end products excreted by the control and MBZ treated flukes following 24 h incubation. There are no differences between the groups in the amounts of propionate, acetate and succinate excreted. However, there is a
The effects of MBZ on glucose uptake and end products excreted by *F. hepatica* during 24 h incubation *in vitro* following treatment *in vivo*.

The results are expressed as μmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>18 h MBZ treated</th>
<th>30 h MBZ treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose uptake</td>
<td>1616 ± 435 (10)</td>
<td>1340 ± 175 (10)</td>
<td>2433 ± 892 (10)</td>
</tr>
<tr>
<td>Succinate excreted</td>
<td>24 ± 12 (7)</td>
<td>30 ± 19 (8)</td>
<td>38 ± 19 (6)</td>
</tr>
<tr>
<td>Lactate</td>
<td>42 ± 9 (5)</td>
<td>16 ± 12 (5)a</td>
<td>23 ± 9 (6)b</td>
</tr>
<tr>
<td>Acetate</td>
<td>453 ± 149 (10)</td>
<td>372 ± 61 (10)</td>
<td>464 ± 102 (6)</td>
</tr>
<tr>
<td>Propionate</td>
<td>623 ± 209 (10)</td>
<td>563 ± 111 (10)</td>
<td>523 ± 146 (6)</td>
</tr>
<tr>
<td>Total end products excreted</td>
<td>1059 ± 285 (5)</td>
<td>967 ± 183 (5)</td>
<td>1049 ± 244 (6)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from control, p < 0.05,
b significantly different from control, p < 0.01.
decrease in the amount of lactate excreted by the 18 and 30 h treated groups. There are no significant differences between the amounts of glucose taken up by the groups.

5.3.2 NSC

(a) OBSERVATIONS MADE DURING COLLECTION OF THE FLUKES

Fluke recovery from sites within the host is shown in table 5.8. The total recovery of flukes in all groups was similar. All the control flukes were recovered from the liver. In the 12 h NSC treated group some flukes were found in the gall bladder and small intestine and they were all alive. After 24 h there was an increase in the number of flukes recovered from the gall bladder and small intestine. As was found in the in vivo MBZ experiment most of the control flukes were found in the main bile duct whereas flukes from the treated sheep were recovered from the peripheral regions of the liver.

A similar situation to that described following MBZ treatment (5.3.1.(a)) was found with regard to the attachment of the flukes and caecal contents. The control flukes were firmly attached and had full caeca; the 12 h treated flukes were mostly detached and had empty caeca; the 24 h treated flukes were all detached and had empty caeca. Some of the flukes recovered from the 24 h treated sheep were necrotic and stained with bile at their posterior ends, as was found after MBZ treatment. Some of these necrotic flukes were included in the samples taken for '0' time determinations and for incubation as there were not enough non-necrotic flukes available.

(b) GLYCOGEN LEVELS

The effect of NSC on the glycogen levels of flukes taken at '0' time and following 24 h incubation are shown in table 5.9. There
Fluke recovery from the liver, gall bladder and intestine of the host following *in vivo* treatment with NSC.

The results are expressed as a percentage of the total number of flukes recovered from each group. *n* is the number of sheep in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>n</em></th>
<th>Liver</th>
<th>Gall bladder</th>
<th>Small intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>12 h NSC treated</td>
<td>5</td>
<td>91.4%</td>
<td>3.8%</td>
<td>4.9%</td>
</tr>
<tr>
<td>24 h NSC treated</td>
<td>5</td>
<td>71.0%</td>
<td>23.7%</td>
<td>5.3%</td>
</tr>
</tbody>
</table>
TABLE 5.9

The effect of NSC on the glycogen levels of *F. hepatica* treated *in vivo* and after further incubation for 24 h *in vitro*.

The results are expressed as µmoles of glucosyl units/g wet weight and they are means ± standard deviation. *n* = 5 for each determination.

<table>
<thead>
<tr>
<th></th>
<th>'0' time</th>
<th>24 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>359 ± 86</td>
<td>463 ± 45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>12 h NSC treated</td>
<td>269 ± 21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>221 ± 28&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 h NSC treated</td>
<td>119 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

<sup>a</sup> significantly different from '0' time control, *p* < 0.05,

<sup>b</sup> significantly different from '0' time 12 h NSC treated, *p* < 0.001,

<sup>c</sup> '0' time 12 h NSC treated, *p* < 0.02 and from incubated control, *p* < 0.001.
were insufficient flukes recovered from the 24 h treated sheep to incubate for 24 h. Some of the necrotic flukes were incubated but they did not survive for 24 h.

There is an increase in the control glycogen level after incubation in media containing glucose, as was found in the MBZ in vivo experiment. The flukes recovered at '0' time from the 12 h NSC treated sheep have significantly lower glycogen levels compared with the control group and following 24 h incubation with glucose in the medium, the levels have dropped further. The 24 h NSC treated group at '0' time have lower glycogen levels than both the control and 12 h treated groups.

(c) ADENINE NUCLEOTIDE LEVELS

The adenine nucleotide concentrations of flukes removed at '0' time are given in table 5.10. The only significant differences are the drops in ATP and total adenine nucleotide concentration in the 24 h treated group compared with the 12 h treated group.

Table 5.11 presents the adenine nucleotide levels after 24 h incubation. There are decreases in the ATP and AMP concentrations and also in the total adenine nucleotides in the 12 h treated group. Only one incubation of 24 h treated flukes was carried out due to the low numbers recovered. The results of this group have been included to show that there seem to be further drops in the ATP, ADP and total adenine nucleotide concentrations.

(d) METABOLIC INTERMEDIATE POOL SIZES

Table 5.12 shows the effects of NSC on some of the intermediate concentrations in the energy producing pathway of
TABLE 5.10

The effect of NSC on the adenine nucleotide levels of F. hepatica treated in vivo.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. n is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control 12 h NSC treated</th>
<th>24 h NSC treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>ATP</td>
<td>969 ± 268</td>
<td>1186 ± 233</td>
</tr>
<tr>
<td>ADP</td>
<td>811 ± 131</td>
<td>924 ± 160</td>
</tr>
<tr>
<td>AMP</td>
<td>289 ± 70</td>
<td>281 ± 49</td>
</tr>
<tr>
<td>Total adenine</td>
<td>2069 ± 423</td>
<td>2390 ± 376</td>
</tr>
<tr>
<td>nucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.20</td>
<td>1.28</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

<sup>a</sup> significantly different from 12 h NSC treated, p < 0.02.
TABLE 5.11

The effect of NSC on the adenine nucleotide levels of *F. hepatica* following *in vitro* incubation for 24 h after treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th>Control (n = 8)</th>
<th>12 h NSC treated (n = 8)</th>
<th>24 h NSC treated (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1027 ± 145</td>
<td>873 ± 130&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>695 ± 121</td>
<td>586 ± 134</td>
</tr>
<tr>
<td>AMP</td>
<td>233 ± 48</td>
<td>172 ± 41&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>1955 ± 253</td>
<td>1631 ± 246&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.48</td>
<td>1.49</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- <sup>a</sup> significantly different from the control, *p* < 0.05.
- <sup>b</sup> significantly different from the control, *p* < 0.025.
TABLE 5.12

The effect of NSC on some of the metabolic intermediate levels of *F. hepatica* following treatment *in vivo*. The results are expressed as nmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>12 h NSC treated</th>
<th>24 h NSC treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>206 ± 85 (8)</td>
<td>304 ± 104 (8)</td>
<td>333 ± 231 (7)</td>
</tr>
<tr>
<td>G6P</td>
<td>680 ± 248 (8)</td>
<td>1354 ± 285 (8)b</td>
<td>1036 ± 362 (7)b</td>
</tr>
<tr>
<td>F6P</td>
<td>206 ± 134 (8)</td>
<td>337 ± 108 (8)a</td>
<td>195 ± 58 (7)c</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>416 ± 131 (8)</td>
<td>176 ± 56 (8)b</td>
<td>163 ± 142 (7)b</td>
</tr>
<tr>
<td>Lactate</td>
<td>1835 ± 897 (5)</td>
<td>1102 ± 222 (5)</td>
<td>1183 ± 407 (5)</td>
</tr>
<tr>
<td>Malate</td>
<td>1055 ± 193 (5)</td>
<td>1441 ± 688 (5)</td>
<td>863 ± 311 (5)</td>
</tr>
<tr>
<td>Succinate</td>
<td>1221 ± 274 (5)</td>
<td>1715 ± 384 (5)</td>
<td>1186 ± 261 (5)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from the control, p < 0.05,

b " " " " " " , p < 0.001,

c " " " " 12 h NSC treated, p < 0.005.
TABLE 5.13

The effect of NSC on some of the metabolic intermediate levels of *F. hepatica* after incubation *in vitro* for 24 h following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>12 h NSC treated</th>
<th>24 h NSC treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>7374 ± 1826 (8)</td>
<td>6465 ± 1455 (8)</td>
<td>5223 (1)</td>
</tr>
<tr>
<td>G6P</td>
<td>1866 ± 293 (8)</td>
<td>1613 ± 217 (8)</td>
<td>1282 (1)</td>
</tr>
<tr>
<td>F6P</td>
<td>437 ± 87 (8)</td>
<td>376 ± 56 (8)</td>
<td>306 (1)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>253 ± 189 (8)</td>
<td>277 ± 99 (8)</td>
<td>230 (1)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1906 ± 930 (5)</td>
<td>1505 ± 763 (5)</td>
<td>not measured</td>
</tr>
<tr>
<td>Malate</td>
<td>788 ± 275 (5)</td>
<td>888 ± 74 (5)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Succinate</td>
<td>535 ± 198 (5)</td>
<td>417 ± 220 (5)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that there are no significant differences between the control and 12 h NSC treated groups.
F. hepatica recovered at '0' time. There are marked increases in the G6P and F6P concentrations in the 12 h treated group. The G6P level in the 24 h treated group is lower than in the 12 h treated group, however, it is still significantly higher than the control level. The F6P concentration in the 24 h treated group is lower than in the 12 h group. In both NSC treated groups there is a marked decrease in the pyruvate concentration.

Following 24 h incubation with glucose present in the medium (table 5.13) there are no significant differences between the control and 12 h NSC treated flukes. The results of the single sample of the 24 h NSC treated group suggest that there may be a decrease in the G6P concentration following incubation.

(e) GLUCOSE UPTAKE AND END PRODUCT EXCRETION

Table 5.14 shows the amount of glucose taken up and end products excreted during 24 h incubation of the control and NSC treated flukes. There are no significant differences between any of the measurements of the control and 12 h treated group.

5.3.3 RFX

(a) OBSERVATIONS MADE DURING COLLECTION OF THE FLUKES

The total fluke recoveries from the control and RFX treated sheep were similar but, as with MBZ and NSC treatment in vivo, there were changes in the sites of recovery. All the control flukes were found in the liver but 12 and 24 h after treatment some were found in the gall bladder and small intestine, alive. Four days after treatment no flukes were found in any of the sites. Table 5.15 shows the proportions of flukes found in the various sites after RFX treatment. The plasma levels of RFX in the sheep at the time of killing were
TABLE 5.14

The effects of NSC on glucose uptake and end products excreted by *F. hepatica* during 24 h incubation in vitro following treatment in vivo.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>12 h NSC treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose uptake</td>
<td>1022 ± 215 (5)</td>
<td>1075 ± 275 (5)</td>
</tr>
<tr>
<td>Succinate excreted</td>
<td>11 ± 2 (5)</td>
<td>12 ± 5 (5)</td>
</tr>
<tr>
<td>Lactate</td>
<td>77 ± 27 (5)</td>
<td>90 ± 29 (5)</td>
</tr>
<tr>
<td>Acetate</td>
<td>306 ± 55 (4)</td>
<td>273 ± 61 (4)</td>
</tr>
<tr>
<td>Propionate</td>
<td>445 ± 46 (4)</td>
<td>554 ± 264 (4)</td>
</tr>
<tr>
<td>Total end products excreted</td>
<td>838 ± 104 (4)</td>
<td>929 ± 277 (4)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that there are no significant differences between any of the control and NSC treated measurements.
TABLE 5.15

Fluke recovery from the liver, gall bladder and intestine of the host, and plasma levels of RFX following *in vivo* treatment.

The results are expressed as a percentage of the total number of flukes recovered from each group. 
n is the number of sheep in each group and (n) is the number of plasma level determinations.

<table>
<thead>
<tr>
<th>Site of recovery</th>
<th>Treatment</th>
<th>n</th>
<th>Liver</th>
<th>Gall bladder</th>
<th>Small intestine</th>
<th>RFX level µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>8</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0 (4)</td>
</tr>
<tr>
<td></td>
<td>12 h RFX treated</td>
<td>5</td>
<td>99.6%</td>
<td>0.2%</td>
<td>0.2%</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>24 h RFX treated</td>
<td>9</td>
<td>93.6%</td>
<td>4.3%</td>
<td>2.1%</td>
<td>16.4 ± 3.4 (5)</td>
</tr>
<tr>
<td></td>
<td>4 day RFX treated</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18.6 ± 2.8 (5)</td>
</tr>
</tbody>
</table>

* no measurement was taken 12 h after treatment, however, 6 h after treatment the drug plasma level of RFX was 6.4 ± 1.5 µg/ml.
determined by Merck Sharp and Dohme (Australia) Pty Ltd, and are also shown in table 5.15. The plasma drug level rises within 6 h of treatment and remains high for up to four days.

The observations made on the attachment and caecal contents of the flukes were similar to those made after MBZ and NSC treatment. In the control sheep, most of the flukes were firmly attached and had full caeca. In the 12 h treated group the flukes were mostly detached and had empty caeca while the flukes from the 24 h treated group were all detached and had empty caeca. It was also noted that most of the control flukes were located in the main bile duct whereas in the treated groups they were mainly in the peripheral regions. Some of the 24 h treated flukes were stained with bile posteriorly and laterally and appeared necrotic; however, their anterior ends were still normal in appearance and were active. Some flukes were completely stained with bile and were dead but had not started to degenerate. In the four day treated group no whole live flukes were found but some apparent 'ghosts' were recovered from the peripheral regions of the livers. Thus, some flukes die in the bile ducts and then degenerate.

(b) GLYCOGEN LEVELS

The glycogen levels of flukes removed immediately from the liver and following 6 and 24 h incubation are shown in table 5.16. There is an increase in the control group level within 6 h incubation in medium containing glucose. By 24 h incubation it has increased further. The 12 h RFX treated group glycogen level is not significantly different from the control level. It also increases on incubation in vitro. The glycogen level of the 24 h RFX treated flukes at '0' time is significantly lower than the control level at that time. On
TABLE 5.16
The effect of RFX on the glycogen levels of *F. hepatica* treated *in vivo* and after further incubation for 24 h *in vitro*.

The results are expressed as µmoles of glucosyl units/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>'0' time</th>
<th>6 h incubation</th>
<th>24 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>153 ± 60 (9)</td>
<td>228 ± 42 (7)</td>
<td>256 ± 28 (5)</td>
</tr>
<tr>
<td>12 h RFX treated</td>
<td>201 ± 30 (5)</td>
<td>220 ± 42 (5)</td>
<td>-</td>
</tr>
<tr>
<td>24 h RFX treated</td>
<td>71 ± 34 (10)</td>
<td>134 ± 32 (5)</td>
<td>134 ± 45 (10)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- **a** significantly different from the appropriate control, p < 0.005,
- **b** significantly different from the '0' time group, p < 0.01.
incubation the concentration increases slightly but remains lower than the control incubated level.

(c) ADENINE NUCLEOTIDE LEVELS

Table 5.17 shows the effect of RFX on the adenine nucleotide concentrations of flukes at '0' time. There is no effect of the drug on the nucleotides of the 12 h treated group, but in the 24 h treated group the ATP concentration is very low. Also, the AMP level is higher than that of the 12 h treated group.

The adenine nucleotide levels measured in the flukes after incubation for 24 h are given in table 5.18. The 12 h RFX treated flukes have not maintained their nucleotide levels during incubation. There are significant drops in the ATP, AMP and total adenine nucleotide levels. The 24 h treated group has even lower levels of ATP, the ADP level is lower than the control and the AMP concentration is higher than the 12 h treated group. There is also a decrease in the total adenine nucleotide concentration of the incubated 24 h treated group compared with the control.

(d) METABOLIC INTERMEDIATE POOL SIZES

Table 5.19 presents the effects of RFX on the concentrations of some of the intermediates of the energy producing pathway in F. hepatica. There are increases in the G6P, F6P and lactate concentrations in the 12 h treated group compared with the control. The G6P and F6P levels of the 24 h treated group are lower than both the control and 12 h treated group levels. The internal lactate concentration of the 24 h RFX treated group is higher than the control and the malate concentration is lower than the control. There is an increase in the succinate concentration of the 24 h treated group
TABLE 5.17

The effect of RFX on the adenine nucleotide levels of *F. hepatica* treated *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>12 h RFX treated (n = 5)</th>
<th>24 h RFX treated (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>812 ± 102</td>
<td>867 ± 24</td>
<td>598 ± 157&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>841 ± 80</td>
<td>869 ± 61</td>
<td>793 ± 134</td>
</tr>
<tr>
<td>AMP</td>
<td>395 ± 80</td>
<td>338 ± 43</td>
<td>465 ± 68&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total adenine</td>
<td>2047 ± 144</td>
<td>2074 ± 94</td>
<td>1856 ± 298</td>
</tr>
<tr>
<td>nucleotides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>0.97</td>
<td>1.00</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from the control, *p* < 0.005,

b 12 h RFX treated, *p* < 0.001.
TABLE 5.18

The effect of RFX on the adenine nucleotide levels of *F. hepatica* following *in vitro* incubation for 24 h after treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>12 h RFX treated (n = 5)</th>
<th>24 h RFX treated (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1002 ± 99</td>
<td>782 ± 41b</td>
<td>656 ± 253b</td>
</tr>
<tr>
<td>ADP</td>
<td>833 ± 82</td>
<td>739 ± 77</td>
<td>657 ± 158a</td>
</tr>
<tr>
<td>AMP</td>
<td>280 ± 34</td>
<td>206 ± 33a</td>
<td>302 ± 54c</td>
</tr>
<tr>
<td>Total adenine</td>
<td>2115 ± 183</td>
<td>1726 ± 90b</td>
<td>1615 ± 400a</td>
</tr>
<tr>
<td>nucleotides AT/ADP</td>
<td>1.20</td>
<td>1.06</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the control, *p* < 0.005,
- b " " " " " " " " , *p* < 0.001,
- c " " " " 12 h RFX treated, *p* < 0.005.
TABLE 5.19

The effect of RFX on some of the metabolic intermediate levels of *F. hepatica* following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>12 h RFX treated</th>
<th>24 h RFX treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>242 ± 156 (8)</td>
<td>303 ± 167 (5)</td>
<td>161 ± 93 (10)</td>
</tr>
<tr>
<td>G6P</td>
<td>885 ± 355 (8)</td>
<td>1151 ± 79 (5)</td>
<td>632 ± 184 (10)</td>
</tr>
<tr>
<td>F6P</td>
<td>146 ± 70 (8)</td>
<td>269 ± 11 (5)</td>
<td>128 ± 30 (8)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>371 ± 157 (8)</td>
<td>168 ± 83 (5)</td>
<td>139 ± 59 (10)</td>
</tr>
<tr>
<td>Lactate</td>
<td>288 ± 189 (6)</td>
<td>2356 ± 1383 (5)</td>
<td>1895 ± 1989 (5)</td>
</tr>
<tr>
<td>Malate</td>
<td>850 ± 77 (5)</td>
<td>643 ± 197 (5)</td>
<td>385 ± 160 (5)</td>
</tr>
<tr>
<td>Succinate</td>
<td>1547 ± 118 (5)</td>
<td>1604 ± 379 (5)</td>
<td>3483 ± 275 (5)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the control, p < 0.025,
- b , p < 0.01,
- c , p < 0.001,
- d 12 h RFX treated, p < 0.001.
TABLE 5.20

The effect of RFX on some of the metabolic intermediate levels of *F. hepatica* after incubation *in vitro* for 24 h following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>12 h RFX treated</th>
<th>24 h RFX treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3700 ± 673 (9)</td>
<td>2334 ± 435 (5)(^b)</td>
<td>3056 ± 1568 (10)</td>
</tr>
<tr>
<td>G6P</td>
<td>2063 ± 175 (9)</td>
<td>1676 ± 207 (5)(^b)</td>
<td>1238 ± 630 (10)(^b)</td>
</tr>
<tr>
<td>F6P</td>
<td>474 ± 48 (9)</td>
<td>412 ± 62 (5)</td>
<td>280 ± 133 (10)(^b)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>94 ± 32 (9)</td>
<td>86 ± 47 (5)</td>
<td>221 ± 51 (9)(^b)</td>
</tr>
<tr>
<td>Lactate</td>
<td>785 ± 365 (4)</td>
<td>483 ± 201 (4)</td>
<td>2050 ± 788 (5)(^a)</td>
</tr>
<tr>
<td>Malate</td>
<td>1021 ± 168 (4)</td>
<td>897 ± 207 (5)</td>
<td>512 ± 249 (5)(^a)</td>
</tr>
<tr>
<td>Succinate</td>
<td>916 ± 132 (5)</td>
<td>1390 ± 443 (5)</td>
<td>3393 ± 910 (5)(^b)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- \(^a\) significantly different from the control, \(p < 0.01\),
- \(^b\) significantly different, \(p < 0.005\).
compared with the control and 12 h treated group. The pyruvate levels of both treated groups are lower than the control value.

Following incubation for 24 h in medium containing glucose, there are increases in all the glucose internal concentrations (table 5.20); however, the increase in the 12 h treated group is not as great as in the control group. The G6P concentrations of the 12 and 24 h treated groups are lower than the control group level. The F6P and malate concentrations of the 24 h treated group are lower than the control group. The pyruvate, lactate and succinate concentrations are higher in the 24 h treated group compared with the control group.

(e) GLUCOSE UPTAKE AND END PRODUCT EXCRETION

The amount of glucose taken up from the medium and the end products excreted by the control and 24 h RFX treated groups during a 24 h incubation are shown in table 5.21. There is a significant increase in the amount of succinate excreted by the 24 h treated group compared with the control group. There are no significant differences between the total amount of end products excreted by the two groups or in the amount of glucose taken up.

5.3.4 HISTOLOGY AND SCANNING ELECTRON MICROSCOPY

(a) CATECHOL REACTION ON WHOLE FLUKES

The whole flukes were examined following the completion of the catechol reaction. The control flukes were stained dark purple around their perimeter in the area of the vitelline glands, showing the characteristic distribution of the polyphenol-oxidase. They also had purple staining regions mainly in the centre of their bodies due to the presence of some caecal contents. An example of a control fluke is shown in plate 1. The drug treated flukes (plates 3 and 5) have no
**TABLE 5.21**

The effect of RFX on glucose uptake and end products excreted by *F. hepatica* during 24 h incubation *in vitro* following treatment *in vivo*.

The results are expressed as µmoles/g wet weight and they are means + standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>24 h RFX treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose uptake</td>
<td>703 ± 137 (5)</td>
<td>741 ± 262 (5)</td>
</tr>
<tr>
<td>Succinate excreted</td>
<td>8 ± 2 (5)</td>
<td>12 ± 2 (5)(^a)</td>
</tr>
<tr>
<td>Lactate</td>
<td>10 ± 5 (5)</td>
<td>7 ± 2 (5)</td>
</tr>
<tr>
<td>Acetate</td>
<td>218 ± 40 (4)</td>
<td>261 ± 73 (5)</td>
</tr>
<tr>
<td>Propionate</td>
<td>450 ± 27 (4)</td>
<td>484 ± 123 (5)</td>
</tr>
<tr>
<td>Total end products</td>
<td>676 ± 65 (4)</td>
<td>764 ± 195 (5)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

\(^a\) significantly different from the control, p < 0.02.
PLATE 1

A control fluke stained by the catechol reaction. The vitelline areas around the perimeter are characteristically stained purple. Caecal contents, also stained purple, are apparent in the white inner region.

Magnification:  $\times 4$

v = vitelline area
cc = caecal contents

PLATE 2

The posterior region of the control fluke from plate 1 showing surface texture.

Magnification:  $\times 8$
PLATE 3

A drug treated fluke stained by the catechol reaction. The vitelline areas around the perimeter are characteristically stained purple except for a small posterior, lateral region which is necrotic. The white inner region shows that the caeca are empty.

Magnification: $x \ 4$
$v =$ vitelline area
$ec =$ empty caecal area
$n =$ necrotic portion

PLATE 4

The posterior region of the drug treated fluke from plate 3. The surface texture is apparent on the non-necrotic region but is absent from the necrotic portion.

Magnification: $x \ 8$
$s =$ normal surface texture
$ns =$ necrotic surface
PLATE 5

A drug treated fluke stained by the catechol reaction. The anterior vitelline areas are characteristically stained purple. The posterior necrotic portion shows little staining. The caeca are empty.

Magnification: $\times 4$

$av =$ normal anterior vitelline area

$ec =$ empty caecal area

$n =$ necrotic portion

PLATE 6

The mid-region of the drug treated fluke from plate 5. The surface texture is apparent on the anterior non-necrotic area but is absent from the necrotic posterior portion.

Magnification: $\times 8$

$s =$ normal surface texture

$ns =$ necrotic surface
caecal contents, as shown by their white interior regions. Plate 2 shows part of the control fluke shown in plate 1 at a higher magnification. It is possible to see surface texture. The necrotic portions of the flukes from the *in vivo* experiment did not stain with the catechol reaction. Plates 3 and 5 show this. A closer examination of the necrotic portions shows that the texture found on the surface of the controls (e.g. plate 2) is no longer present (plates 4 and 6).

The necrotic flukes from each drug treatment all appeared similar; the necrotic portions did not stain with the catechol reaction and the texture of their body surface was absent.

(b) SECTIONS

Transverse sections of control and necrotic flukes were examined under the light microscope. Plate 7 is an example of a section of a control fluke. The outer layer of the body has some structures which have been termed spines (Threadgold, 1963) and the caeca contain debris. Plate 8 shows a section through a necrotic portion of a fluke. The outer body layer seen in the control fluke is absent. There is less contrast in the photograph due to the absence of normal dark purple-staining nuclei. In some places there are dark bodies (labelled 'granules') but these are brown and when viewed at higher magnification appear to be granular and characteristic of autotanning (plate 10). Plate 9 is also taken from a necrotic portion of a fluke and probably shows a slightly later effect. There is marked vacuolation and tissue degeneration within the body of the fluke; however, the body is still intact.

Plate 11 is also a transverse section of a necrotic fluke showing the edge of the necrotic zone. The outer body wall with spines
PLATE 7

A photomicrograph of a transverse section of part of a control fluke.

Magnification: x 380

c = caeca and caecal contents
t = tegument
v = vitellaria

PLATE 8

A photomicrograph of a transverse section of part of a necrotic portion of a drug treated fluke. The outer tegument is absent and there are very few darkly staining nuclei.

Magnification: x 380

c = caeca and caecal contents
v = vitellaria
PLATE 9

A photomicrograph of a transverse section of a necrotic portion of a drug treated fluke. The outer tegument is absent and there is marked vacuolation and tissue degeneration within the body. The caeca are empty and there are no darkly staining nuclei. The dark areas labelled 'granules' are brown and are not nuclei.

Magnification:   x 380

ec = empty caeca

g = granules

PLATE 10

A photomicrograph of a transverse section of part of a necrotic portion from a drug treated fluke showing the 'granules' in more detail.

Magnification:   x 950


g = granules
PLATE 11

A photomicrograph of part of a transverse section of the border between the necrotic and non-necrotic regions of a drug treated fluke. The region on the right is non-necrotic and has a tegument and darkly staining nuclei in the underlying region. The necrotic area on the left is vacuolated and has no tegument or darkly staining nuclei.

Magnification: x 380

\( t \) = tegument
\( s \) = spine
\( n \) = necrotic area
\( ec \) = empty caeca

Plate 12

A photomicrograph of part of a transverse section of the border between the necrotic and non-necrotic regions of a drug treated fluke. The region on the right is not necrotic; it has a tegument with spines and normal staining tissue. The area on the left is necrotic; the tegument is absent from the dorsal and ventral surfaces of the fluke.

Magnification: x 380

\( t \) = tegument
\( s \) = spine
\( n \) = necrotic area
PLATE 13

A photomicrograph of part of a transverse section of a drug treated fluke showing the degeneration of the tegument. The region on the right is non-necrotic.

Magnification: $\times$ 950

t = tegument
is also on the cirri. The process of the cell of the "dying" layer is absent, there is atrophy and degeneration and the cell wall is very thin and indicates that the inner cell walls are almost completely covered from the NCC and becomes very thin. The outer body walls are broken down, and the contents are pushed out. No NCC or NCC-particles were observed. POMNNT ND

Some fluke from the area is shown in the light microscope. Plate 18 of the light microscope in plate 7 is due to this area. There is also a folded appearance of the outer body wall at the base of the fluke. The particles around the fluke are probably along. Plate 18 shows the normal surface at a higher magnification.

The necrotic portion of the fluke viewed by scanning electron microscopy is shown in plates 16 and 17. There is a complete absence of spines and there are what appear to be indentations which may indicate where some of the spines were. There are no folds apparent in any of the normal portion or in the necrotic portion, which shows that the whole outer layer of the fluke is absent. Plate 19 shows the necrotic region at higher magnification. It appears to be flaky and the indentations are not very deep.

Plates 18 and 19 show the area between the normal and
is seen on the right and there is a lot of contrast due to the darkly staining nuclear material. On the left hand side the outer layer is absent, there is a lack of contrast and there are signs of tissue degeneration and vacuolation. Plate 12 shows a similar situation and indicates that the body wall is removed from the dorsal and ventral surfaces of the fluke. Plate 13 gives a more detailed picture of the body wall degenerating. The necrotic areas of the flukes recovered from the NSC and RFX treated sheep appear very similar. The outer body walls are absent from the necrotic portions and there is marked vacuolation and tissue degeneration within the bodies of the flukes. No NSC- or RFX-specific effects are apparent.

(c) SCANNING ELECTRON MICROSCOPY

Some flukes were observed under the scanning electron microscope. Plate 14 shows that the texture observed under the light microscope in plate 2 is due to the spines. There is also a folded appearance of the outer body wall, at the base of the spines. The particles around the spines are probably mucus. Plate 15 shows the normal surface at a higher magnification.

The necrotic portion of the fluke viewed by scanning electron microscopy is shown in plates 16 and 17. There is a complete absence of spines and there are what appear to be indentations which may indicate where some of the spines were. There are no folds apparent as seen in the normal portion, so it seems that the whole outer layer of the fluke is absent. Plate 17 shows the necrotic region at higher magnification. It appears to be flaky and the indentations are not very deep.

Plates 18 and 19 show the area between the normal and
PLATE 14

A scanning electron micrograph of the non-necrotic surface of a fluke showing the spines and underlying folded area.

Magnification: $ \times 750$

$s =$ spines

$f =$ folds

$m =$ mucus

PLATE 15

A scanning electron micrograph of part of plate 14 showing the spines and folds in greater detail.

Magnification: $ \times 3000$

$s =$ spines

$f =$ folds

$m =$ mucus
PLATE 16

A scanning electron micrograph of the surface of a necrotic portion of a drug treated fluke. No spines are present but there appear to be indentations in the surface.

Magnification: x 750

i = indentations

PLATE 17

A scanning electron micrograph of part of plate 16 showing the surface in greater detail. The surface appears to be flaky and the marks are indentations.

Magnification: x 3000

i = indentations
PLATE 18

A scanning electron micrograph of the region between the necrotic and non-necrotic areas of a drug treated fluke. The top area is not necrotic and has spines and underlying folds. The mid-region is made up of flaky debris and the bottom area is necrotic and has no spines or folds.

Magnification: x 750
d = debris
s = spines
f = folded area

PLATE 19

A scanning electron micrograph of the region between the necrotic and non-necrotic areas of a drug treated fluke. The necrotic area is at the top.

Magnification: x 750
d = debris
n = necrotic area
PLATE 20

A scanning electron micrograph of the oral sucker of a fluke showing the presence of spines.

Magnification:  x 1000
s = spines

PLATE 21

A scanning electron micrograph of the ventral sucker of a fluke showing the flattened spines around it.

Magnification:  x 750
vs = ventral sucker
fs = flattened spines
necrotic portions of the fluke. There is a lot of flaky debris present and it is obvious from plate 18 that the folded layer containing the spines is completely absent from the necrotic portion.

All areas of the fluke were viewed under the scanning electron microscope to ensure that the spines normally cover the whole body and that the features found in the necrotic areas are not normally present elsewhere. Plate 20 shows that the spines are even on the oral sucker. Plate 21 shows that the area around the ventral sucker appears at first to be devoid of spines but on closer examination they can be seen, flattened against the body of the fluke.

(d) ASSAYS OF NECROTIC FLUKES

Table 22 shows the adenine nucleotide levels in the anterior and posterior ends of F. hepatica from a control sheep and from a sheep treated with RFX to produce necrotic posterior portions. There are no differences between the anterior and posterior portions of control flukes. There are significant decreases in the ATP, ADP and total adenine nucleotide concentrations of the RFX treated anterior portions compared with the anterior portions of control flukes. The adenine nucleotide levels of the RFX treated posterior portions are very low compared with both the control portions and the RFX treated anterior portions.

Table 5.23 shows the concentrations of some of the intermediate levels of anterior and posterior portions of the control and necrotic flukes. All the intermediates measured, except for lactate and succinate, are very low in the RFX treated anterior and posterior portions of the flukes. The lactate concentrations are the same in both the RFX anterior and posterior portions compared with the controls. The succinate concentration in the RFX anterior portion is
TABLE 5.22

The adenine nucleotide levels in anterior and posterior portions of *F. hepatica* following *in vivo* treatment with RFX to produce necrotic posterior portions.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* = 5 for all determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control anterior</th>
<th>RFX anterior</th>
<th>Control posterior</th>
<th>RFX posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1006 ± 85</td>
<td>377 ± 52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1051 ± 139</td>
<td>25 ± 15&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>955 ± 53</td>
<td>645 ± 88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>955 ± 168</td>
<td>57 ± 27&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMP</td>
<td>371 ± 18</td>
<td>433 ± 56</td>
<td>358 ± 89</td>
<td>61 ± 17&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>2332 ± 100</td>
<td>1453 ± 139&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2343 ± 376</td>
<td>143 ± 24&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.05</td>
<td>0.59</td>
<td>1.10</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

<sup>a</sup> significantly different from appropriate control, *p* < 0.001,

<sup>b</sup> " " " " RFX anterior, *p* < 0.001.
TABLE 5.23

Some of the intermediate levels in the anterior and posterior portions of *F. hepatica* following *in vivo* treatment with RFX to produce necrotic portions.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. n = 5 for all determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control anterior</th>
<th>RFX anterior</th>
<th>Control posterior</th>
<th>RFX posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>900 ± 133</td>
<td>252 ± 143</td>
<td>914 ± 258</td>
<td>183 ± 191</td>
</tr>
<tr>
<td>G6P</td>
<td>1893 ± 129</td>
<td>599 ± 39</td>
<td>1514 ± 251</td>
<td>17 ± 27</td>
</tr>
<tr>
<td>F6P</td>
<td>429 ± 46</td>
<td>146 ± 11</td>
<td>353 ± 61</td>
<td>13 ± 11</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>205 ± 52</td>
<td>146 ± 41</td>
<td>236 ± 54</td>
<td>49 ± 69</td>
</tr>
<tr>
<td>Lactate</td>
<td>950 ± 122</td>
<td>1107 ± 543</td>
<td>1060 ± 298</td>
<td>657 ± 346</td>
</tr>
<tr>
<td>Malate</td>
<td>734 ± 129</td>
<td>560 ± 147</td>
<td>862 ± 194</td>
<td>114 ± 47</td>
</tr>
<tr>
<td>Succinate</td>
<td>1264 ± 325</td>
<td>10210 ± 1299</td>
<td>1701 ± 450</td>
<td>1643 ± 1368</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from appropriate control, p < 0.05,
- b " " " " " " , p < 0.001,
- c " " " " RFX anterior, p < 0.001.

There are no significant differences between the anterior and posterior control values.
very high, but in the RFX treated posterior portions it is similar to
the controls.

5.4 DISCUSSION

The observations made during the removal of the flukes from
the hosts are similar for all three \textit{in vivo} drug treatments. The
majority of flukes are detached and most have empty caeca. It was
suggested in 1.5.1 that flukes may feed intermittently \textit{in vivo}. It is
possible that treatment of the host with MBZ, NSC and RFX causes the
flukes to detach and/or those that are already detached, following
feeding, do not reattach. Thus, the caecal contents are regurgitated
after the fluke's last meal is processed and the caeca remain empty
because the fluke does not reattach for the next meal.

Some of the detached flukes, probably those in the main bile
duct are washed into the gall bladder by the flow of bile. Some of
these ultimately appear in the intestine. The flukes which were still
present in the livers of the 24 h treated RFX and NSC groups and the
30 h MBZ treated group were located in the peripheral portions of the
liver where there is a greater mechanical resistance to their
elimination with bile flow. In the four day RFX treated group, the
'ghosts' are probably flukes which die \textit{in situ} in the peripheral
portions of the liver.

In field trials with these three drugs, a few flukes are
often found alive and apparently normal following treatment of the
host. This is especially so after MBZ treatment as it only causes a
94\% reduction in fluke numbers at a dose rate of 100 mg/kg (Kelly
\textit{et al.}, 1975). It is possible that some of the flukes which are not
washed out of their sites by bile flow are able to reestablish their position once the concentration of the drug drops in the host's tissues and providing they did not receive a large concentration of the drug with the last meal of host tissue. The data in table 5.15 show that the level of RFX in the host's blood remains high for at least four days after treatment, which may explain why it is such an effective fasciolicide.

There are marked differences in the pyruvate concentrations of the drug treated flukes immediately after removal from the liver (tables 5.5, 5.12 and 5.19). When this was observed in chapter 3, following incubation of the flukes it was suggested that, as pyruvate is a component of whole blood, the concentration probably decreases when the parasites disgorge their caecal contents. A similar explanation can be given for the in vivo experiments as the drug treated flukes mainly had empty caeca. In support of this explanation is the finding that the control flukes disgorge their caecal contents on incubation and their pyruvate levels also drop (tables 5.6, 5.13 and 5.20).

Once the drug treated flukes detach and stop feeding on host tissue they must rely on the utilisation of storage products to maintain sufficient energy production. As soon as the flukes utilise the nutrients from their last meal they will start to metabolise their glycogen reserves as a result of the regulatory role of the adenine nucleotide concentrations. The flow of carbon through the energy producing pathway is regulated by the concentrations of the adenine nucleotides (Newsholme & Start, 1973).

Following in vivo treatment with the three drugs there are decreases in the glycogen levels of the flukes removed immediately
from the livers (tables 5.2, 5.9 and 5.16). These results reflect the post-mortem observations that the flukes are detached and have empty caeca. They suggest that the glycogen reserves are utilised to compensate for the cessation of feeding. In the \textit{in vitro} studies with these drugs there were no effects on the glycogen levels, probably because of the large external concentration of glucose available to the incubated flukes, either for direct metabolism or for maintaining the glycogen levels.

Incubation of the control flukes from the \textit{in vivo} experiments results in an increase in their glycogen levels (tables 5.2, 5.9 and 5.16). Tables 5.7, 5.14 and 5.21 show that glucose is taken up from the medium and that none of the drug treatments have any effect on this uptake when the treated flukes are incubated. This indicates that the glucose present in the medium is taken up and utilised by the flukes to build up their glycogen reserves. Such an effect was not observed in the \textit{in vitro} studies with flukes obtained from the abattoir. An explanation could be that the flukes removed immediately from livers and then incubated (i.e. the \textit{'in vivo'} flukes) are in better 'condition' than the flukes collected from the abattoir. The flukes used in the \textit{in vitro} studies were often in the livers of the dead hosts for a considerable period and the time from death of the host to incubation of the flukes was up to 3 h. Although the control flukes used in the \textit{in vitro} studies were capable of maintaining their energy metabolism it is possible that other pathways and/or functions may not have been operating normally. Thus, energy reserves may have been utilised rather than built up in the abattoir flukes. For example, it was suggested in chapter 3 that the initial drop in the glycogen level
following incubation is due to the loss of eggs and the lower glycogen level is maintained during incubation. The control flukes used in the *in vivo* experiments may not have excreted all their eggs and may have continued to produce eggs during their *in vitro* incubation.

The glycogen levels of the 18 h MBZ treated flukes (table 5.2) increase on incubation. Although there are only two determinations for the incubated 30 h MBZ treated flukes it appears that the depleted glycogen reserves found at '0' time are restored on incubation with glucose. Thus, glucose in the medium is taken up and utilised by the treated flukes.

In the 12 h NSC treated group (table 5.9) the glycogen levels do not increase on incubation. It is possible that the glucose which is taken up from the medium is completely utilised for maintaining the energy levels of the flukes and is not available for building up glycogen levels.

The low glycogen levels found 24 h after RFX treatment (table 5.16) increase after incubation with glucose in the medium; however, the increase is not as great as in the control group. This may indicate that most of the glucose taken up from the medium is utilised to maintain the flukes' energy metabolism but some is available to restore part of the glycogen reserves.

The effects of the *in vivo* drug treatments on the glycogen levels of the flukes at '0' time may be explained by their detachment from the bile ducts and subsequent starvation. The utilisation of glycogen reserves also has effects on the concentrations of some of the intermediates of the energy producing pathway. An increase in glycogen
utilisation will result in an increase in G6P formation (see the pathway of glycogen metabolism, 1.6.2). There are increases in the G6P concentrations of flukes recovered 12 h after NSC and RFX treatment \textit{in vivo} (tables 5.12 and 5.19) and after 18 h MBZ treatment (table 5.5). There are also increases in the F6P pools of the NSC and RFX groups 12 h after treatment, which probably reflect the increased G6P concentrations. Following prolonged starvation, i.e. 24 h after NSC and RFX treatment and 30 h after MBZ treatment \textit{in vivo} the G6P concentrations are lower than the corresponding earlier treated groups and, in the case of MBZ and RFZ treatment, are lower than the control values. Thus, as the glycogen levels become markedly depleted there is a decrease in G6P formation. The F6P concentrations in the 24 h NSC and RFX treated groups are also lower and probably reflect the drop in G6P concentrations.

When the control flukes are incubated for 24 h with glucose in the medium there are increases in the G6P and F6P concentrations, as was found in the \textit{in vitro} study (table 3.3). The explanation given in 3.4 can also be given in this case, that is, glucose is taken up due to the high concentration available in the medium and the internal intermediate pools also fill up.

When the treated flukes are incubated for 24 h in media containing glucose, the internal glucose pools increase (tables 5.6, 5.13 and 5.20), as was found in the \textit{in vitro} study (table 3.3). However, the increase is not as great in the 18 h MBZ treated and 12 h RFX treated groups when compared with the controls. This may be due to greater utilisation of the glucose by the treated flukes to maintain energy production and to restore glycogen reserves.
There are decreases in the G6P concentrations of the incubated 12 and 24 h RFX treated groups compared with the control. In the 12 h treated group it may reflect the lower internal glucose concentration and in the 24 h treated group it may be due to the low glycogen level which is not restored during incubation with glucose present in the medium. The decreases in the F6P concentrations of the 30 h MBZ treated group and the 24 h RFX treated group may reflect the low glycogen levels.

Some of the changes in the intermediate concentrations may also be due to the changes observed in the adenine nucleotide levels as these are the ultimate regulators of carbohydrate metabolism. Fluctuations in the concentrations of adenine nucleotides affect the entry of carbon into the energy producing pathway since they act as regulators of, and substrates for, the hexokinase and PFK catalysed reactions. All three in vivo drug treatments have a marked effect on the adenine nucleotide concentrations of the flukes.

The adenine nucleotide levels of the control flukes at '0' time (tables 5.3, 5.10 and 5.17) are comparable with those of the '0' time flukes in the in vitro study (table 3.4). Thus, it is likely that these values correspond to those found in flukes in vivo. There are increases in the ATP concentrations of abattoir flukes (table 3.4) and those obtained from the in vivo control sheep (tables 5.4, 5.11 and 5.18) following 24 h incubation in vitro. This may reflect an increase in carbohydrate metabolism due to the availability of glucose from the medium and thus, increased ATP formation. It is also possible that some ATP requiring processes cease to function, e.g. egg production, so allowing a build up of ATP.
There are no apparent effects of MBZ 18 h after treatment, or NSC and RFX 12 h after treatment, on the adenine nucleotide concentrations of flukes removed immediately from the liver (tables 5.3, 5.10 and 5.17). However, when these flukes are incubated for 24 h some effects are observed (tables 5.4, 5.11 and 5.18). In all cases there are decreases in the ATP concentrations and in the incubated 12 h treated NSC and RFX groups there are also decreases in the AMP and total adenine nucleotide concentrations. So, there are disturbances of some kind in the energy metabolism of the flukes at the early times after treatment, which do not become apparent until they are incubated.

In the flukes removed immediately from the host 30 h after MBZ treatment and 24 h after NSC treatment there are decreases in the ATP and total adenine nucleotide concentrations (tables 5.3 and 5.10). In the MBZ treated group there is also a decrease in the ADP concentration. In the 24 h RFX treated group there is a decrease in the ATP level and an increase in AMP concentration (table 5.17) which is characteristic of the effect of an uncoupler and was also found in the in vitro study (chapter 4). When the flukes from the later drug treated times are incubated, there are decreases in the total adenine nucleotide concentrations with each treatment. The effect on the MBZ treated flukes is similar to the in vitro observations when the flukes are incubated for 48 h with MBZ in the medium (table 4.10). However, NSC was found to have an apparent uncoupling action in vitro (table 4.14). The reason for the decrease in the total adenine nucleotide levels in the 24 h NSC treated flukes at '0' time and after incubation may be due to the inclusion of some necrotic flukes in the samples. Table 5.22 shows that there are very low levels of adenine nucleotides
in the necrotic portions of the flukes, thus inclusion of necrotic flukes will result in an overall decrease in total adenine nucleotide levels. A similar explanation can be given for the decrease in total adenine nucleotide levels in the incubated 24 h RFX treated flukes, as some necrotic flukes were included in the samples.

The consequence of disturbances in the adenine nucleotide concentrations is an increase in carbon flow through the energy producing pathway to maintain the ATP levels. Its confirmation, in the treated flukes, by direct determination of end product excretion is not possible in vivo. Neither is it possible to detect such changes in flukes treated in vivo and subsequently incubated in vitro. In the in vitro studies described earlier (chapter 4), increased flux through the pathway was detected early in some incubations; the controls 'caught up' as the drug treated flukes were unable to sustain their initial rate of carbohydrate metabolism. In the in vivo experiments such adjustments will already have been made during exposure to the drug. Tables 5.7, 5.14 and 5.21 show that there is no significant difference between the total amount of end products excreted during 24 h incubation after in vivo treatment with any of the drugs. The treated and untreated flukes are probably processing maximum amounts of carbohydrate; the former group in an attempt to overcome the effects of the drugs and the latter group in adaptation to the new conditions.

There are some effects of drug treatment on the excretion of end products. There is a decrease in the amount of lactate excreted by the MBZ treated flukes (table 5.7). Table 5.6 also shows that there is a marked decrease in the internal lactate concentration of the
incubated 18 and 30 h MBZ treated flukes. A similar decrease in lactate excretion was observed in the \textit{in vitro} MBZ study (table 4.18). These observations may reflect a decrease in carbon flow through the lactate producing branch of the pathway.

There is an increase in the amount of succinate excreted by the 24 h RFX treated group compared with the control (table 5.21). There is also an increase in the internal succinate concentration of the incubated 24 h RFX treated group (table 5.20). It was found that in the RFX treated anterior portions there is a high succinate concentration compared with the control anteriors, however the necrotic posterior succinate concentration is similar to the control level (table 5.23). Therefore, in the 24 h RFX treated group, which included some necrotic flukes, there must be a very marked increase in the succinate concentration of the anterior portions to produce a high overall succinate concentration. A similar increase in succinate concentration was found in the \textit{in vitro} RFX study. It seems that the further metabolism of succinate may be inhibited or else there is an increase in succinate production from another pathway, e.g. amino acid metabolism.

There is an increase in the pyruvate concentration of the incubated 24 h RFX treated group (table 5.20) and also an increase in the internal lactate concentration. This may be due to an increase in carbon flow to the pyruvate/lactate pathway which in turn may result from an inhibition of the further metabolism of succinate.

The malate concentration of the 24 h RFX treated flukes at '0' time and following 24 h incubation is low compared with the controls (tables 5.19 and 5.20). A similar result was found in the
in vitro RFX study (table 4.8). It was suggested in chapter 4 that the decrease in the malate pool is due to the uncoupling of electron transport and phosphorylation by RFX. Van Vugt et al. (1976) have shown that uncouplers cause increased utilisation of malate in isolated F. hepatica mitochondria. The observations from the in vivo RFX experiment are consistent with the hypothesis that RFX acts as an uncoupler.

The results of the biochemical measurements made on the anterior and posterior portions of RFX treated flukes with necrotic posterior regions show that there are marked effects on the apparently normal anterior ends and the necrotic posterior regions (tables 5.22 and 5.23). In the anterior portions the only metabolite which does not change in concentration is lactate. Apart from succinate and AMP all other changes in the metabolite concentrations are decreases, as found in the in vivo drug experiments on whole adult flukes. The increase in the internal succinate concentration also occurred in the RFX in vivo experiment (table 5.19). The levels of metabolites measured in the necrotic posterior regions are all very low compared with the controls, except for the concentration of lactate. Lactate is not as labile as most of the other compounds so will remain unchanged for a longer period.

Some of the decreases in the metabolite concentrations found in the 24 h NSC and RFX treated flukes may be due to the very low levels present in the necrotic portions. Insufficient non-necrotic flukes were available for sampling at these times so some necrotic flukes were included.

The biochemical results from the in vivo MBZ, NSC and RFX
experiments suggest that there are two major effects of drug treatment which can explain most of the differences found between the control and drug treated flukes. One effect is that most of the treated flukes are detached and no longer feeding; thus, to maintain sufficient energy production, the carbohydrate reserves are utilised and changes in some of the concentrations of the intermediates of the pathway occur. The other major effect is on the adenine nucleotide concentrations. The drugs have an effect on these within 12 h, for NSC and RFX, and 18 h, for MBZ, which becomes apparent when the flukes are incubated. The effects on the adenine nucleotides 30 h after MBZ treatment and 24 h after RFX treatment are similar to the effects found in the in vitro studies. The results with NSC are complicated because of the inclusion of necrotic flukes in the 24 h treated group.

Death of the flukes remaining in the livers after treatment with these three drugs probably occurs because of the very low adenine nucleotide levels; they are probably insufficient to act as substrates in the various reactions of glycolysis, i.e. there is a lack of 'kinetic efficiency' (Newsholme, personal communication).

The results of the histological examination and the scanning electron microscopy of the necrotic portions of drug treated flukes indicate that the outer tegumental layer is removed. There is marked tissue degeneration in the underlying regions, as shown in plates 8, 9, 11 and 12. In some cases brown granular vesicles are found (plate 10) which are characteristic of auto-tanning of the egg shell proteins. This is due to early oxidation of the phenolic groups of the proteins which may occur spontaneously due to an increase in oxygen concentration of the necrotic tissue (Howell, personal communication).
Alternatively, it may occur because tissue degeneration may allow the combination of the polyphenol-oxidase with the egg shell proteins within the vitellaria. During normal egg production in flukes, tanning of the egg shell proteins does not occur until the eggs are passed through the uterus, then oxidation of the phenolic material takes place to produce the hard, brown shell. The normal reactions in a quinone-tanning system can be summarised as follows:

\[
\text{protein} + \text{diphenyl polyphenol oxidase} \rightarrow \text{protein} + \text{quinone} = \text{tanned protein}
\]


The results of the catechol reaction on the necrotic portions of the flukes show that there is no active polyphenol-oxidase present (plates 3, 4, 5 and 6). This is not surprising in view of the amount of tissue degeneration found.

Several studies have been carried out on the tegument of *F. hepatica* to determine its structure. Bennett (1975a,b) has examined the surface of newly excysted juvenile flukes by scanning electron microscopy and described the spines and various sensory structures. Threadgold (1963) carried out an ultrastructural study of the cuticle and showed that it is cellular, i.e. a tegument not a cuticle. The tegument is composed of a syncytial, anucleate surface-layer which is joined by protoplasmic tubes to individual, nucleated areas of cytoplasm - the tegumental cells - which lie within the parenchyma. The surface syncytium contains many mitochondria aligned at right angles to the surface (Threadgold, 1963; Björkman & Thorsell, 1964).
The tegumental cells also contain many dense mitochondria, some granular endoplasmic reticulum and vesicular Golgi complexes.

A more recent study by Threadgold (1967) has shown that there are two types of tegumental cells and both types are connected to the same surface syncytium. They are designated types 1 and 2. Type 1 is more common; there are usually two or three type 1 cells to one type 2 cell. They are clearly differentiated at low magnification because the type 2 cell has a lower electron density overall and there are only a few mitochondria, concentrated at the pole of the cell which faces the surface syncytium.

The type 1 cells contain many small, dense, membrane-bound vesicles which arise from the Golgi complex of the cells and these appear in the surface syncytium. There is an intimate relationship between the granular endoplasmic reticulum and the Golgi complex. The type 2 cells, in contrast, have large numbers of small biconcave disc-like secretion bodies lying throughout the cytoplasm. The discs consist of a membrane enclosing a granular material. They are scattered randomly throughout the cell and also occur in small numbers in the protoplasmic extensions of the cells which project upwards into the muscle layers (Threadgold, 1963, 1967; Björkman & Thorsell, 1964).

The function of the two cell types is not known. Threadgold (1967) states that both types contribute cytoplasm, cell organelles and secretory products to the surface syncytium. Also, they both have a characteristic secretory product, originating from the Golgi complexes. Thus, the tegument functions as both a secretory epithelium and a protective covering. The presence of the secretory granules in both types of tegumental cells and in the surface syncytium is evidence for
an outward flow of protoplasm and cellular fluid which carries the secretory products to the surface epithelium. However, once there, it is not known what role they play. Neither type of secretion has been seen in the process of release onto the apical surface.

In view of these observations, Threadgold (1967) suggests two possible roles for the secretory products:

1. their function is to combine with absorbed substances. This could serve as an extension of the general protective function of the tegument or as a preliminary part of the process of assimilation and digestion; and,

2. they are excretory substances, although this is unlikely in view of their mode of origin and the presence of a complex excretory system in the fluke.

The finding that the necrotic regions produced by in vivo treatment with MBZ, NSC and RFX are without a tegument presents several possibilities. All three drugs have one major biochemical effect in common in both the in vitro and in vivo studies: marked disturbance of the adenine nucleotide concentrations, in particular, ATP. It is possible that the drugs are taken up by the fluke either when feeding or by absorption through the tegument from the bile, or both. Once inside the fluke the drugs eventually disrupt the synthesis of ATP either by an uncoupling action or by affecting adenine nucleotide turnover, or by some other indirect way. Mitochondria are the major sites of ATP production and there are large numbers of these in the surface syncytium (Threadgold, 1963, 1967; Björkman & Thorsell, 1964). Presumably they provide ATP for the secretory activity of the tegumental cells and the overall maintenance of the tegument. If the
production of ATP is inhibited these functions will cease. The secretory products of the two types of cells may have lytic action and on prolonged storage in the tegumental cells, may burst and digest them, thus causing necrosis.

Another possibility is that the low levels of ATP may inhibit the production of the secretory products. If these are responsible for maintaining the 'barrier' between the internal areas of the fluke and the external bile constituents, breakdown of this function may result in adverse effects on the tegument. Bile contains many enzymes (e.g. lipases and proteases) which may contribute to the removal of the tegument.

The effect of MBZ on the intracellular structure of nematodes and cestodes was discussed in 2.6.1. It is possible that similar effects occur in *F. hepatica* and ultimately result in the removal of the tegument. The finding that all three drugs used in this study have a similar effect on the tegument supports the hypothesis suggested in 2.6.1 for MBZ that the ATP levels are too low to maintain structural integrity.

It is also possible that all three drugs have a direct effect on the tegument causing degeneration of it and the underlying tissue and thus a decrease in the adenine nucleotide levels. However, the effects on the nucleotides occur well before any necrotic areas appear and the changes in them and other labile intermediates are not always reductions. Also, the *in vitro* effects correspond with most of the *in vivo* observations but the appearance of necrotic areas never occurred *in vitro*. Flukes that died during *in vitro* incubation were uniformly white and still had the texture shown in plate 1 when viewed
under the light microscope. It is possible that if the flukes were left long enough in contact with a very low level of drug \textit{in vitro}, necrotic regions may develop. However, the \textit{in vitro} MBZ study was carried out for 48 h and necrosis was not found. It seems therefore, that removal of the tegument and necrosis only occurs in the \textit{in vivo} situation after drug treatment, so it may result from the action of bile and mechanical effects caused by bile flow, spines of other flukes, eggs and the confines of the bile ducts.

The reason for necrosis occurring in the posterior region is not clear. It is possible that the drug is ingested with host tissue and is moved into the diverticula where it remains until the caecal contents are disgorged. Thus, the drug is present mainly in the posterior portion of the fluke and is probably absorbed with the soluble nutrients during the digestion/absorption process described in 1.5.2. The drug would therefore affect the energy metabolism of the posterior fluke tissues and eventually result in necrosis. This explanation implies that flukes do not feed in the same manner as they do \textit{in vivo} when they are incubated \textit{in vitro}. Necrotic regions were never found in flukes incubated with MBZ, NSC or RFX \textit{in vitro} where glucose is taken up through the tegument. It seems likely that \textit{in vivo} the main source of the drug is from the host tissues which are ingested by the flukes and not from the concentration present in the bile. The effect on the energy metabolism of the flukes is similar whether the drug is ingested or absorbed through the tegument but different physical features are apparent in the \textit{in vivo} situation.

All three \textit{in vivo} drug treatments result in detachment of the flukes from the bile duct walls. The actual process of attachment
of flukes has not been studied fully. It is not known whether they are
attached by the ventral sucker all the time, while feeding
intermittently with the oral sucker, or whether they detach and lie
free in the bile ducts after a meal is taken. The ventral sucker has
muscle layers around it. It is not known whether they are in a state
of contraction when the fluke is attached to the bile duct wall or
whether they are in a relaxed state. There may be circular and
longitudinal muscle layers which oppose one another in action in such a
way that contraction of the longitudinal layer opens the ventral sucker
and contraction of the circular layer closes it, or vice versa. A
detailed study of the orientation of the muscle layers is required to
determine which of these occurs. No matter which occurs, energy is
required for muscular contraction. Once the effects of a drug cause a
reduction in ATP concentration there may not be sufficient to allow
contraction of the sucker. Thus, the flukes are forced to lie free in
the bile ducts.

Low levels of ATP may also affect the feeding processes of
the fluke. Howell (1970) has examined the process of ingestion via the
oral sucker by the metacercariae of two species of digenetic trematodes,
*Philophthalmus burrili* and *Echinoparyphium serratum*. It is generally
considered that ingestion in digenetic trematodes is a suctorial
process mediated by the action of the pharynx. Howell (1970) has shown
that in these species ingestion does not occur by a suctorial mechanism.
Instead, the pharynx is used as a transfer organ which appears to
dilate passively as fluid is forced into the lumen by contraction of
the oral sucker. In turn, the pharynx contracts, expelling the fluid
into the oesophagus and caeca. Presumably this process requires the
expenditure of energy. If a similar process occurs in adult *F. hepatica* there may be insufficient ATP available for muscle contraction in the drug treated flukes.

The studies carried out on flukes treated with MBZ, NSC and RFX *in vivo* complement and extend the results obtained from *in vitro* treatment of flukes with these drugs. Most of the effects on the metabolites are comparable in both studies except where necrotic flukes were included in the *in vivo* treated samples. The major effects are the disturbances in the adenine nucleotide concentrations, in particular the decrease in ATP. It is suggested that the lack of ATP may be responsible for other effects which are observed in the *in vivo* situation; detachment and cessation of feeding, decreased glycogen levels and necrosis of the posterior portions. Various possibilities have been presented to explain these effects and it is apparent that a lot more information on the habits of *F. hepatica* is required before the effect of an anthelmintic on the parasite *in vivo* can be fully understood.
CHAPTER 6

THE EFFECTS OF A POLYMORPH OF MBZ, BROMOPHENOPHOS AND A DISULPHONAMIDE
ON F. HEPATICA FOLLOWING IN VIVO TREATMENT

6.1 INTRODUCTION

The preceding chapters have shown that three drugs, with known fasciolicidal activity, cause disturbances in the energy producing pathway of F. hepatica when they are incubated with the drugs in vitro and when the drugs are administered to the host. At the same time as the in vivo experiments described in chapter 5 were carried out, two different drugs and a different form of MBZ were tested in a similar manner. In vitro studies with these drugs have not been carried out. It is known that the three drugs are effective against F. hepatica in field trials but there is no information on their mode of action. The three drugs are: MBZ-Polymorph C, bromphenophos and 4-amino-6-trichloroethenyl-1,3-benzenedisulphonamide.

(a) MBZ-POLYMORPH C (MBZ-PCLY C)

Three crystalline forms of MBZ have been found and these have been designated polymorph A, B and C. Polymorph A has negligible activity against nematodes in sheep whereas polymorphs B and C are extremely active (Chevis, personal communication). A dose of 100 mg/kg of a formulation containing an undefined mixture of the polymorphs is required to remove 94% of adult F. hepatica. However, only 15 mg/kg of pure polymorph C is required to remove the same fluke burden (Chevis,
personal communication).

(b) BROMOPHENOPHOS

Bromophenophos is an active fasciolicide according to field trial data. The dose required to remove adult flukes is given in table 2.2 and its formula and structure are presented in tables 2.1. Laboratory tests with mice and rats (Kruyt & van der Steen, 1969) and field trials with cattle indicate that it is active against immature flukes (Reinders, 1969, cited by van der Meer & Pouwels, 1969).

The study carried out by van Miert and Groeneveld (1969) (see 2.5) with bromophenophos suggests that it is an uncoupler of oxidative phosphorylation in rodents. It causes retarded and abnormal egg development of fluke eggs and also a reduction in egg production (Kruyt & van der Steen, 1969; Burrows, 1973).

(c) DISULPHONAMIDE

4-amino-6-trichloroethenyl-1,3-benzenedisulphonamide is a new anthelmintic developed for effect against adult and juvenile F. hepatica. It is more potent against adult flukes but is over 90% effective at 15 mg/kg against six week old flukes and 98% effective at 30 mg/kg against four week old flukes in sheep (Ostlind & Mrozik, 1976). The drug is well tolerated by sheep and showed no gross toxic symptoms in one sheep dosed at 200 mg/kg and another at 400 mg/kg (Ostlind & Mrozik, 1976). No information is available on its mode of action.

6.2 METHODS

(a) MBZ-POLY C

The in vivo experiment with MBZ-Poly C was carried out at
Ethnor Pty Ltd, The Oaks, N.S.W. at the same time as the *in vivo* MBZ experiment (chapter 5).

The sheep were infected as described in 5.2.(a). The control sheep were used for both the MBZ and MBZ-Poly C *in vivo* experiments. Five sheep were treated with MBZ-Poly C at a dose rate of 15 mg/kg and were killed 17 h after treatment. The flukes were removed and prepared as described in 5.2.(a). Samples were taken at '0' time and extracts were prepared and assayed as described in 5.2.(d). Insufficient flukes were recovered to carry out incubations for 24 h.

(b) BROMOPHENOPHOS

The bromophenophos *in vivo* experiment was carried out at CIBA-GEIGY Australia Ltd, Kemps Creek, N.S.W. at the same time as the *in vivo* NSC experiment (chapter 5).

The sheep were infected as described in 5.2.(b). One group of five sheep was the control group for both the NSC and bromophenophos *in vivo* experiments. Six sheep were drenched with bromophenophos at a dose rate of 15 mg/kg. Three were killed 12 and 24 h after treatment. The flukes were removed and prepared as described in 5.2.(a).

Samples were taken from each sheep at '0' time for assays and incubations. When sufficient flukes were recovered the remainder were pooled and further samples were taken to increase the number of determinations. Hence, in the 12 h bromophenophos treated group there were only three sheep but one more sample was taken from the remaining pooled flukes to give four determinations of adenine nucleotide levels and intermediate concentrations. The assays were carried out as described in 5.2.(d).
One sheep infected with *F. hepatica* and drenched with bromophenophos as described above was killed 30 h after treatment. The flukes were prepared as described in 5.2.(f) for histological examination.

(c) **DISULPHONAMIDE**

The *in vivo* experiment with the disulphonamide was carried out at Merck Sharp and Dohme (Australia) Pty Ltd, Ingleburn, N.S.W. at the same time as the RFX *in vivo* experiment (chapter 5).

The sheep were infected as described in 5.2.(c). The control sheep were used for both the RFX and disulphonamide *in vivo* experiments. Nine sheep were treated with the disulphonamide at a dose rate of 10 mg/kg. The weighed dose was dissolved in 7.5 ml of DMSO and was injected intraruminally. Four sheep were killed 12 h after treatment and five sheep 24 h after treatment.

The flukes were removed and prepared as described in 5.2.(a). Samples were taken at '0' time and following incubation for 24 h. The assays were carried out as described in 5.2.(d). A pooled sample of flukes was taken from the 12 h disulphonamide treated sheep to increase the number of determinations and incubations to five.

6.3 RESULTS

6.3.1 **OBSERVATIONS MADE DURING COLLECTION OF THE FLUKES**

Table 6.1 shows the various proportions of flukes recovered from the liver, gall bladder and intestine of the hosts following treatment with MBZ-Poly C, bromophenophos and the disulphonamide. The total numbers of flukes recovered from the controls and drug treated hosts from each experiment were similar. The majority of flukes from
Fluke recovery from the liver, gall bladder and intestine of the host following *in vivo* treatment with MBZ-Poly C, bromophenophos and the disulphonamide.

The results are expressed as a percentage of the total number of flukes recovered from each group. *n* is the number of sheep in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Liver</th>
<th>Gall bladder</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>97.8%</td>
<td>1.1%</td>
<td>1.1%</td>
</tr>
<tr>
<td>17 h MBZ-Poly C</td>
<td>5</td>
<td>87.3%</td>
<td>0</td>
<td>12.7%</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>100.0%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12 h bromophenophos</td>
<td>3</td>
<td>89.0%</td>
<td>5.5%</td>
<td>5.5%</td>
</tr>
<tr>
<td>24 h bromophenophos</td>
<td>3</td>
<td>83.2%</td>
<td>5.6%</td>
<td>11.2%</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>100.0%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12 h disulphonamide</td>
<td>4</td>
<td>90.5%</td>
<td>9.0%</td>
<td>0.5%</td>
</tr>
<tr>
<td>24 h disulphonamide</td>
<td>5</td>
<td>93.4%</td>
<td>5.2%</td>
<td>3.1%</td>
</tr>
</tbody>
</table>
the control sheep were firmly attached and had full caeca, as described in chapter 5. Treatment with the three drugs increases the numbers of flukes recovered from the gall bladder and intestine (table 6.1).

Some of the flukes from the MBZ-Poly C and the 12 h bromophenophos and disulphonamide treated sheep were detached and had empty caeca as was a greater proportion of flukes from the 24 h bromophenophos and disulphonamide treated sheep. Many also had necrotic posterior areas similar to those described in chapter 5 from RFX, NSC and MBZ treated sheep. Some of the necrotic flukes were included in the 24 h bromophenophos samples but none from the disulphonamide treated sheep was included.

6.3.2 GLYCOGEN LEVELS

The effects of MBZ-Poly C, bromophenophos and the disulphonamide on the glycogen levels of *F. hepatica* at '0' time and following 24 h incubation *in vitro* are given in table 6.2. Insufficient flukes were recovered from the MBZ-Poly C and the 24 h bromophenophos treated sheep to carry out incubations. Only two groups of flukes were available from the 12 h bromophenophos treated sheep for incubation.

The glycogen levels of the MBZ-Poly C and disulphonamide treated flukes at '0' time are similar to the appropriate control levels. There is a marked decrease in the glycogen level of the 24 h bromophenophos treated group at '0' time compared with the control.

The control glycogen levels of the incubated flukes have increased on incubation, as already discussed in chapter 5. Although there are only two samples in the incubated 12 h bromophenophos treated
TABLE 6.2

The effect of MBZ-Poly C, bromophenophos and the disulphonamide on the glycogen levels of *F. hepatica* after *in vivo* treatment ('0' time) and following 24 h incubation *in vitro*.

The results are expressed as µmoles of glucosyl units/g wet weight and they are means ± standard deviation.

(n) is the number of determinations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>'0' time</th>
<th>24 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>163 ± 18 (5)</td>
<td>not carried</td>
</tr>
<tr>
<td>17 h MBZ-Poly C</td>
<td>174 ± 24 (5)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>359 ± 86 (5)</td>
<td>463 ± 45b (5)</td>
</tr>
<tr>
<td>12 h bromophenophos</td>
<td>272 ± 110 (4)</td>
<td>203 ± 19 (2)</td>
</tr>
<tr>
<td>24 h bromophenophos</td>
<td>22 ± 11a(5)</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>126 ± 36 (5)</td>
<td>256 ± 30c (5)</td>
</tr>
<tr>
<td>12 h disulphonamide</td>
<td>127 ± 24 (5)</td>
<td>239 ± 11c (5)</td>
</tr>
<tr>
<td>24 h disulphonamide</td>
<td>114 ± 14 (5)</td>
<td>214 ± 35c (5)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from the appropriate control, p < 0.001,
b '0' time, p < 0.05,
c '0' time, p < 0.001.
group there does not appear to be an increase in the glycogen level on incubation. Both disulphonamide treated groups increase their glycogen reserves on incubation.

6.3.3 ADENINE NUCLEOTIDE LEVELS

Table 6.3 shows the effect of MBZ-Poly C on the adenine nucleotide concentrations of *F. hepatica* following treatment *in vivo*. There are marked increases in the ATP, ADP and total adenine nucleotide levels.

12 h after bromophenophos treatment there are increases in the ADP and AMP concentrations at '0' time (table 6.4). However, 24 h after treatment there are marked decreases in the ATP, ADP and total adenine nucleotide levels compared with the control values. There is also a decrease in the AMP level compared with the 12 h treated group at '0' time.

There were only sufficient numbers of flukes recovered to incubate two groups from the 12 h bromophenophos treated sheep and one group from the 24 h treated sheep. The adenine nucleotide levels of these groups (table 6.5) suggest that there are marked decreases of all the adenine nucleotide levels following incubation.

Table 6.6 presents the effects of the disulphonamide on the adenine nucleotide concentrations of *F. hepatica*. There are no differences between the adenine nucleotide levels of the 12 h treated flukes and the controls, but 24 h after treatment there are increases in the ADP and total adenine nucleotide levels compared with the control and 12 h treated groups.

When the 12 h disulphonamide treated flukes are incubated for
TABLE 6.3

The effect of MBZ-Poly C on the adenine nucleotide levels of *F. hepatica* treated *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>17 h MBZ-Poly C treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>869 ± 104</td>
<td>1397 ± 419&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>697 ± 98</td>
<td>1240 ± 196&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMP</td>
<td>345 ± 94</td>
<td>385 ± 65</td>
</tr>
<tr>
<td>Total adenine</td>
<td>1911 ± 159</td>
<td>3022 ± 649&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>nucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.25</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

<sup>a</sup> significantly different from the control, *p* < 0.005,

<sup>b</sup> " " " " " " , *p* < 0.001.
TABLE 6.4

The effect of bromo phenophos on the adenine nucleotide levels of *F. hepatica* treated *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>12 h bromophenophos treated (n = 4)</th>
<th>24 h bromophenophos treated (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1225 ± 198</td>
<td>1126 ± 258</td>
<td>183 ± 43d</td>
</tr>
<tr>
<td>ADP</td>
<td>845 ± 103</td>
<td>996 ± 69a</td>
<td>401 ± 79d</td>
</tr>
<tr>
<td>AMP</td>
<td>289 ± 51</td>
<td>414 ± 77b</td>
<td>240 ± 21c</td>
</tr>
<tr>
<td>Total adenine</td>
<td>2259 ± 341</td>
<td>2536 ± 312</td>
<td>823 ± 34d</td>
</tr>
<tr>
<td>nucleotides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.33</td>
<td>1.13</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Tests for significance by Student's *t* show that:

- a significantly different from the control, *p* < 0.05,
- *b* " " " " " " , *p* < 0.025,
- *c* " " " 12 h bromophenophos treated, *p* < 0.001,
- *d* " " " " " " and the control, *p* < 0.001.
TABLE 6.5

The effect of bromophenophos on the adenine nucleotide levels of *F. hepatica* after 24 h incubation *in vitro* following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>12 h bromophenophos treated (n = 2)</th>
<th>24 h bromophenophos treated (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1118 ± 83</td>
<td>580 ± 1</td>
<td>108</td>
</tr>
<tr>
<td>ADP</td>
<td>740 ± 110</td>
<td>406 ± 89</td>
<td>113</td>
</tr>
<tr>
<td>AMP</td>
<td>257 ± 39</td>
<td>123 ± 1</td>
<td>57</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>2113 ± 125</td>
<td>1109 ± 89</td>
<td>278</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.51</td>
<td>1.43</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Tests for significance have not been carried out due to the low sample sizes.
### TABLE 6.6

The effect of the disulphonamide on the adenine nucleotide levels of *F. hepatica* treated *in vivo*.  

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 4)</th>
<th>12 h disulphonamide treated (n = 5)</th>
<th>24 h disulphonamide treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>751 ± 68</td>
<td>618 ± 119</td>
<td>698 ± 134</td>
</tr>
<tr>
<td>ADP</td>
<td>777 ± 36</td>
<td>744 ± 47</td>
<td>936 ± 65b,c</td>
</tr>
<tr>
<td>AMP</td>
<td>428 ± 69</td>
<td>509 ± 135</td>
<td>494 ± 157</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>1956 ± 105</td>
<td>1872 ± 54</td>
<td>2128 ± 58a,c</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>0.97</td>
<td>0.83</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- **a** significantly different from the control, *p* < 0.02,
- **b** " " " " " , *p* < 0.005,
- **c** " " " 12 h disulphonamide treated, *p* < 0.001.
TABLE 6.7

The effect of the disulphonamide on the adenine nucleotide levels of *F. hepatica* after 24 h in vitro incubation following in vivo treatment.

The results are expressed as nmols/g wet weight and they are means ± standard deviation. n is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 4)</th>
<th>12 h disulphonamide treated (n = 5)</th>
<th>24 h disulphonamide treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>995 ± 50</td>
<td>830 ± 26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>927 ± 45&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADP</td>
<td>780 ± 53</td>
<td>870 ± 60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1069 ± 54&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMP</td>
<td>250 ± 8</td>
<td>316 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>481 ± 105&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total adenine nucleotides</td>
<td>2014 ± 91</td>
<td>2015 ± 83</td>
<td>2508 ± 114&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.28</td>
<td>0.95</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the control, p < 0.05,
- b " " " " " " " "  " p < 0.001,
- c " " " 12 h disulphonamide treated, p < 0.005,
- d " " " " " " " and control, p < 0.001.
24 h, effects on the adenine nucleotide concentrations become apparent (table 6.7); there is a decrease in the ATP concentration and increases in the ADP and AMP levels. The ATP concentration of the incubated 24 h treated flukes is greater than the incubated 12 h treated flukes and the ADP, AMP and total adenine nucleotide concentrations are greater than both the incubated control and 12 h treated values (table 6.7).

6.3.4 METABOLIC INTERMEDIATE POOL SIZES

Table 6.8 shows the effect of MBZ-Poly C on some of the intermediates of the energy producing pathway of *F. hepatica*. There are increases in the glucose, G6P, F6P, lactate, malate and succinate concentrations compared with the control group.

The effects of bromophenophos on some of the intermediates at '0' time are given in table 6.9. In the 12 h treated group there are increases in the glucose, G6P and succinate concentrations and a decrease in the pyruvate levels compared with the control. In the 24 h treated group the glucose, G6P, F6P, lactate and malate levels are lower than the 12 h treated group levels and the G6P, F6P, pyruvate, lactate and malate concentrations are lower than the controls.

Only small numbers of flukes were available for incubation following bromophenophos treatment thus the sample sizes are very low. Also, the incubated 24 h treated group includes some necrotic flukes. Despite the low numbers, it appears that on incubation the 12 h treated group has lower glucose, G6P, F6P, lactate and succinate concentrations compared with the control. The single incubation of the 24 h treated group shows decreases in all the intermediates except succinate, compared with the control (table 6.10).
TABLE 6.8

The effect of MBZ-Poly C on some of the metabolic intermediate levels of *F. hepatica* treated *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. The number of determinations is 5 in each case.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>MBZ-Poly C treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>208 ± 146</td>
<td>640 ± 455&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G6P</td>
<td>949 ± 290</td>
<td>1797 ± 212&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>F6P</td>
<td>170 ± 49</td>
<td>347 ± 20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>306 ± 64</td>
<td>331 ± 120</td>
</tr>
<tr>
<td>Lactate</td>
<td>892 ± 109</td>
<td>1561 ± 482&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malate</td>
<td>792 ± 180</td>
<td>1232 ± 206&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Succinate</td>
<td>3011 ± 1161</td>
<td>7518 ± 2205&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- <sup>a</sup> significantly different from the control, *p* < 0.01,
- <sup>b</sup> " " " " " *, *p* < 0.005,
- <sup>c</sup> " " " " " *, *p* < 0.001.
TABLE 6.9

The effect of bromophenophos on some of the metabolic intermediate levels of *F. hepatica* treated *in vivo.*

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>12 h bromophenophos treated (n = 4)</th>
<th>24 h bromophenophos treated (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>231 ± 101</td>
<td>654 ± 556a</td>
<td>162 ± 21d</td>
</tr>
<tr>
<td>G6P</td>
<td>819 ± 199</td>
<td>1505 ± 275b</td>
<td>204 ± 58c,d</td>
</tr>
<tr>
<td>F6P</td>
<td>282 ± 109</td>
<td>380 ± 50</td>
<td>72 ± 42c,d</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>488 ± 80</td>
<td>263 ± 142a</td>
<td>191 ± 150b</td>
</tr>
<tr>
<td>Lactate</td>
<td>1835 ± 897</td>
<td>1781 ± 778</td>
<td>331 ± 254c,d</td>
</tr>
<tr>
<td>Malate</td>
<td>1055 ± 193</td>
<td>1272 ± 262</td>
<td>439 ± 43c,d</td>
</tr>
<tr>
<td>Succinate</td>
<td>1221 ± 274</td>
<td>1710 ± 298a</td>
<td>1367 ± 400</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

a significantly different from the control, *p* < 0.05,
b " " " " " " , *p* < 0.005,
c " " " " " , *p* < 0.001,
d " " " " 12 h bromophenophos treated, *p* < 0.005.
TABLE 6.10

The effect of bromophenophos on some of the metabolic intermediate levels of *F. hepatica* after 24 h in *vitro* incubation following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>12 h bromophenophos treated (n = 2)</th>
<th>24 h bromophenophos treated (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>8536 ± 1095</td>
<td>3431 ± 1233</td>
<td>1694</td>
</tr>
<tr>
<td>G6P</td>
<td>2052 ± 170</td>
<td>1100 ± 132</td>
<td>132</td>
</tr>
<tr>
<td>F6P</td>
<td>493 ± 51</td>
<td>250 ± 40</td>
<td>47</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>147 ± 90</td>
<td>175 ± 131</td>
<td>14</td>
</tr>
<tr>
<td>Lactate</td>
<td>1906 ± 930</td>
<td>677 ± 129</td>
<td>176</td>
</tr>
<tr>
<td>Malate</td>
<td>788 ± 275</td>
<td>590 ± 111</td>
<td>282</td>
</tr>
<tr>
<td>Succinate</td>
<td>535 ± 198</td>
<td>265 ± 50</td>
<td>798</td>
</tr>
</tbody>
</table>

Tests for significance have not been carried out due to the low sample sizes.
Table 6.11 shows the effects of the disulphonamide on some of the metabolic intermediates of *F. hepatica*. 12 h after treatment there are decreases in the glucose, G6P and F6P levels and an increase in the succinate concentration compared with the control. 24 h after treatment there are increases in the pyruvate and malate levels compared with the control and increases in glucose, G6P, F6P, pyruvate and succinate compared with the 12 h treated group.

When the disulphonamide treated groups are incubated for 24 h there are also effects on some of the intermediate levels. In the incubated 12 h treated group there are increases in the pyruvate, lactate, malate and succinate levels compared with the control. In the 24 h treated group there are increases in the G6P, pyruvate, lactate, malate and succinate levels compared with the control and increases in the glucose, G6P, F6P and succinate pools and a decrease in the lactate level compared with the incubated 12 h treated group (table 6.12).

6.3.5 GLUCOSE UPTAKE AND END PRODUCT EXCRETION

Table 6.13 shows the amount of glucose taken up and end products excreted by two groups of 12 h bromophenophos treated flukes during a 24 h incubation. It is possible that there is an increase in the amount of glucose taken up by the treated flukes, although the low sample size precludes statistical analysis. There do not appear to be any differences between the amounts of end products excreted by the two groups.

The effects of the disulphonamide on glucose uptake and end product excretion are shown in table 6.14. There are decreases in glucose uptake and excretion of total end products by the 24 h treated group. There are marked increases in succinate excretion by both drug
TABLE 6.11

The effect of the disulphonamide on some of the metabolic intermediate levels of *F. hepatica* treated *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. n is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 4)</th>
<th>12 h disulphonamide treated (n = 5)</th>
<th>24 h disulphonamide treated (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>334 ± 165</td>
<td>171 ± 26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>259 ± 39&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G6P</td>
<td>999 ± 505</td>
<td>674 ± 56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>954 ± 58&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>F6P</td>
<td>166 ± 92</td>
<td>97 ± 18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>207 ± 30&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>345 ± 100</td>
<td>248 ± 84</td>
<td>650 ± 82&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate</td>
<td>259 ± 189</td>
<td>266 ± 215</td>
<td>188 ± 134</td>
</tr>
<tr>
<td>Malate</td>
<td>824 ± 197</td>
<td>1101 ± 494</td>
<td>1420 ± 146&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Succinate</td>
<td>1547 ± 118</td>
<td>2504 ± 580&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2913 ± 503&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:
- <sup>a</sup> significantly different from the control, **p** < 0.05,
- <sup>b</sup> significantly different from 12 h disulphonamide treated, **p** < 0.05,
- <sup>c</sup> significantly different from 12 h disulphonamide treated, **p** < 0.005.
The effect of the disulphonamide on some of the metabolic intermediates of *F. hepatica* after 24 h *in vitro* incubation following treatment *in vivo*.

The results are expressed as nmoles/g wet weight and they are means ± standard deviation. The number of determinations in each case is 5.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>12 h disulphonamide treated</th>
<th>24 h disulphonamide treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3717 ± 826</td>
<td>3552 ± 799</td>
<td>4282 ± 289^c</td>
</tr>
<tr>
<td>G6P</td>
<td>2000 ± 122</td>
<td>1843 ± 157</td>
<td>2472 ± 238^b,d</td>
</tr>
<tr>
<td>F6P</td>
<td>468 ± 62</td>
<td>390 ± 30</td>
<td>522 ± 49^d</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>93 ± 21</td>
<td>314 ± 108^b</td>
<td>351 ± 31^b</td>
</tr>
<tr>
<td>Lactate</td>
<td>666 ± 274</td>
<td>1548 ± 222^b</td>
<td>1068 ± 245^a,c</td>
</tr>
<tr>
<td>Malate</td>
<td>1067 ± 152</td>
<td>2765 ± 279^b</td>
<td>3113 ± 298^b</td>
</tr>
<tr>
<td>Succinate</td>
<td>916 ± 132</td>
<td>2361 ± 454^b</td>
<td>2966 ± 251^b,c</td>
</tr>
</tbody>
</table>

Tests for significance by Student’s 't' show that:

- ^a^ significantly different from the control, *p* < 0.02,
- ^b^ " " " " " " " , *p* < 0.005,
- ^c^ " " " " 12 h disulphonamide treated, *p* < 0.05,
- ^d^ " " " " " " " " " , *p* < 0.001.
TABLE 6.13

The effect of bromophenophos on glucose uptake and end product excretion by *F. hepatica* during 24 h incubation *in vitro* following treatment *in vivo*.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. *n* is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>12 h bromophenophos treated (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose taken up</td>
<td>1022 ± 215</td>
<td>1736 ± 76</td>
</tr>
<tr>
<td>Succinate excreted</td>
<td>11 ± 2</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>Lactate</td>
<td>77 ± 27</td>
<td>107 ± 44</td>
</tr>
<tr>
<td>Acetate</td>
<td>306 ± 55</td>
<td>262 ± 102</td>
</tr>
<tr>
<td>Propionate</td>
<td>445 ± 46</td>
<td>405 ± 177</td>
</tr>
<tr>
<td>Total end products</td>
<td>838 ± 104</td>
<td>779 ± 328</td>
</tr>
</tbody>
</table>

Tests for significance have not been carried out due to the low sample size of the drug treated group.
TABLE 6.14

The effect of the disulphonamide on glucose uptake and end product excretion by *F. hepatica* during 24 h incubation *in vitro* following treatment *in vivo*.

The results are expressed as µmoles/g wet weight and they are means ± standard deviation. (n) is the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>12 h disulphonamide treated</th>
<th>24 h disulphonamide treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose taken up</td>
<td>703 ± 137 (5)</td>
<td>617 ± 128 (5)</td>
<td>585 ± 45 (5)</td>
</tr>
<tr>
<td>Succinate excreted</td>
<td>8 ± 2 (5)</td>
<td>30 ± 9 (5)</td>
<td>75 ± 12 (5)</td>
</tr>
<tr>
<td>Lactate</td>
<td>10 ± 5 (5)</td>
<td>20 ± 4 (5)</td>
<td>17 ± 7 (5)</td>
</tr>
<tr>
<td>Acetate</td>
<td>218 ± 40 (4)</td>
<td>149 ± 36 (4)</td>
<td>130 ± 18 (4)</td>
</tr>
<tr>
<td>Propionate</td>
<td>450 ± 27 (4)</td>
<td>348 ± 51 (4)</td>
<td>272 ± 30 (4)</td>
</tr>
<tr>
<td>Total end products</td>
<td>676 ± 65 (4)</td>
<td>551 ± 84 (4)</td>
<td>488 ± 33 (4)</td>
</tr>
</tbody>
</table>

Tests for significance by Student's 't' show that:

- a significantly different from the control, *p* < 0.05,
- b , *p* < 0.005,
- c , *p* < 0.001.
treated groups and corresponding decreases in propionate excretion. There is also a decrease in the amount of acetate excreted by the 24 h treated group.

6.3.6 HISTOLOGY

The flukes removed from the sheep 30 h after treatment with bromophenophos had necrotic portions similar to those described in chapter 5 following RFX, NSC and MBZ treatment. Examination of whole flukes after treatment with the catechol reaction gave similar results to those described in 5.3.4.(a) for the drug treated flukes. The sections of necrotic flukes were also similar to the other sections taken from drug treated flukes, described in 5.3.4.(b); i.e. their outer tegumental layer was absent and the underlying tissue was degenerating.

6.4 DISCUSSION

Many of the effects of \textit{in vivo} treatment of \textit{F. hepatica} with MBZ-Poly C, bromophenophos and the disulphonamide are similar to those observed following \textit{in vivo} treatment with RFX, NSC and MBZ, discussed in chapter 5. All six drugs cause an increase in fluke recovery from the gall bladder and intestine, detachment of the flukes from the bile duct walls and cessation of feeding, as shown by their empty caeca. Five of the drug treatments result in the formation of necrotic posterior regions. MBZ-Poly C does not produce necrosis but this may be because the flukes were removed only 17 h after treatment. It was suggested in chapter 5 that necrosis occurs as a result of insufficient ATP to maintain structural integrity. The mechanical effects (e.g. abrasion by other flukes and eggs and the bile flow) experienced by the flukes in
the bile ducts probably aid in the removal of the tegument. It is possible that 17 h is not long enough to produce necrotic areas.

The suggestions put forward in chapter 5 to explain the effects of RFX, NSC and MBZ on detachment, cessation of feeding and necrosis could also apply to the effects produced by \textit{in vivo} treatment of \textit{F. hepatica} with MBZ-Poly C, bromophenophos and the disulphonamide. All six drugs have marked effects on the adenine nucleotide levels of the parasites but some are increases rather than decreases in concentration. Presumably any significant change in the adenine nucleotide levels indicates that the parasite has been stressed beyond its capacity to adapt.

MBZ-Poly C causes marked increases in the ATP, ADP and total adenine nucleotide concentration of flukes 17 h after \textit{in vivo} treatment (table 6.3). Such increases have not previously been found following drug treatment. It was found in chapters 3 and 5 that there is a significant increase in the ATP concentration of flukes incubated for 24 h in media containing glucose (tables 3.4, 5.4, 5.11 and 5.18). It was suggested that this may reflect an increase in carbohydrate metabolism due to the availability of glucose from the medium, and thus increased ATP formation. Alternatively, it is possible that some ATP requiring processes cease to function and so allow a build up of ATP. Similar explanations can be considered in this case.

It is possible that the drug has stimulated an increase in metabolism. The changes in some of the metabolic intermediate levels (tables 6.8) after MBZ-Poly C treatment suggest that there is an increase in carbon flow through the energy producing pathway. There are increases in the glucose, G6P, F6P, lactate, malate and succinate
pools in the 17 h MBZ-Poly C treated flukes. Increases in the glucose, G6P, F6P concentrations and in some cases increases in the lactate, malate and succinate levels occur when flukes are incubated with glucose (tables 3.2, 3.3, 5.5, 5.6, 5.12, 5.13, 5.19 and 5.20) and it was suggested that this occurs as a result of an increase in carbohydrate metabolism.

There is no effect of MBZ-Poly C on the glycogen levels of the flukes 17 h after treatment. It is possible that the increase in carbohydrate metabolism, suggested above, is due to an increase in the metabolism of the caecal contents and has not yet required the depletion of the flukes' glycogen reserves. If the flukes had remained in the treated sheep for longer than 17 h decreases in the glycogen reserves may have become apparent, as has been found for all the drugs so far tested in vivo in this study.

It is possible that the increases in the adenine nucleotide levels are due to decreased utilisation, i.e. some ATP requiring processes may cease following MBZ-Poly C treatment, but this study gives no indication as to which processes could be affected. As MBZ-Poly C treatment ultimately results in the elimination of *F. hepatica* from infected sheep (Chevis, personal communication) it is likely that the effects on the adenine nucleotide levels contribute to their death.

Treatment with bromophenophos also has marked effects on the adenine nucleotide levels of *F. hepatica*. 12 h after treatment there are increases in the ADP and AMP concentrations (table 6.4). Also at this time there are increases in the glucose, G6P and succinate concentrations (table 6.9) which may reflect an increase in
carbohydrate metabolism. The decrease in the pyruvate concentration of
the 12 h bromophenophos treated flukes probably reflects the absence of
caecal contents, as discussed in chapters 3 and 5.

There is no significant decrease in the glycogen levels of
the 12 h bromophenophos treated flukes. Following incubation of only
two groups of these flukes for 24 h the results suggest that there is
no increase in the glycogen levels whereas there are increases in the
glycogen levels of the incubated controls. Although the results cannot
be tested statistically due to the low number of samples, they suggest
that there is an increase in carbohydrate metabolism in the 12 h
treated flukes and that the glucose taken up from the medium is
utilised in the energy producing pathway rather than for building up
the glycogen reserves. The intermediate levels measured in the two
groups of incubated flukes support this suggestion. The internal
glucose, G6P, F6P, lactate and succinate pools are low compared with
the control. The increase in carbohydrate metabolism of the 12 h
treated flukes is not sufficient to maintain the energy levels of the
parasites. Table 6.5 shows that there are marked decreases in all the
adenine nucleotide concentrations following 24 h incubation and,
although there are only two groups of flukes in the sample, the results
are very similar and markedly lower than the control values.

Table 6.13 shows the amount of glucose taken up and end
products excreted after 24 h incubation by the control flukes and the
two groups of 12 h bromophenophos treated flukes. These results cannot
be tested statistically; however, it seems that more glucose is taken
up by the bromophenophos treated group. This may indicate that there
is a greater rate of glucose transport which may result because of a
greater rate of glucose utilisation by the treated flukes.

The glycogen levels of the 24 h bromophenophos treated flukes are markedly lower than the control values. As suggested in chapter 5, this is probably a result of the detachment of the flukes and cessation of feeding. The glycogen reserves are metabolised to maintain the flukes' energy levels.

Most of the flukes recovered 24 h after bromophenophos treatment had necrotic posterior areas and some were included in the samples taken for intermediate and nucleotide assays. The adenine nucleotide and intermediate concentrations reflect this. Table 6.4 shows that the ATP, ADP and total adenine nucleotide levels of the 24 h treated flukes are very low compared with the control values. All the metabolic intermediates measured in the 24 h treated flukes are low, except for succinate, compared with the control values. These findings agree with the results discussed in chapter 5 where the metabolite levels of necrotic portions of flukes produced by RFX treatment were measured. There did not appear to be any histological differences between the necrotic areas of the bromophenophos treated flukes and those described in chapter 5.

The metabolic effects of the disulphonamide on F. hepatica are different from the other drugs so far studied. Table 6.2 shows that there are no effects of the disulphonamide on the glycogen levels of flukes removed at '0' time or following 24 h incubation in media containing glucose, despite the detachment of the flukes from the bile duct walls and the consequent cessation of feeding. Therefore, there must either be sufficient nutrients available for
energy production or an inhibition of carbohydrate metabolism.

There is no effect of the disulphonamide on the adenine nucleotide levels of the 12 h treated flukes at '0' time but on incubation for 24 h there are increases in the ADP and AMP concentrations and a decrease in the ATP level compared with the control. 24 h after treatment there are increases in the ADP and total adenine nucleotide concentrations at '0' time and following incubation the ATP level increases to the same extent as the control and the ADP, AMP and total adenine nucleotide levels are higher than the control values. Thus, the adenine nucleotide concentrations are disturbed compared with the controls but the effects are increases rather than the decreases which have been found following *in vivo* treatment with other fasciolicides (RFX, NSC, MBZ and bromophenophos). It is possible that the turnover of adenine nucleotides is affected in some way. Alternatively, the explanations suggested for the increases in adenine nucleotide concentrations following MBZ-Poly C treatment may be applicable in this case.

Disulphonamide treatment results in the formation of necrotic areas of flukes recovered 24 h after treatment. Necrotic flukes were not included in the samples of disulphonamide treated flukes. Presumably the adenine nucleotide concentrations of the necrotic portions are very low, as was found in the necrotic areas of flukes recovered from RFX treated sheep (table 5.22).

There are also effects of the disulphonamide treatment on some of the metabolic intermediates of the energy producing pathway of *F. hepatica*. In the 12 h disulphonamide treated flukes at '0' time there are decreases in the glucose, G6P and F6P
concentrations compared with the control. This could indicate that the rate of carbohydrate metabolism is lower in the 12 h treated flukes, perhaps due to inhibition of glycogen utilisation or because the activity of another pathway has increased and hence decreases glycogen metabolism (e.g. amino acid metabolism). Alternatively there may be an increase in carbon flow which causes the depletion of the intermediate pools.

24 h after disulphonamide treatment the glucose, G6P and F6P levels are no longer lower than the control values. There are also increases in the pyruvate, malate and succinate concentrations compared with the control which suggest that there is a disturbance in the metabolism of PEP. The increases in the earlier intermediates of the pathway may result from the increases in the later ones.

Table 6.14 shows that when the 24 h disulphonamide treated flukes are incubated for 24 h with glucose present in the medium, less glucose is taken up compared with the control. Also, smaller amounts of end products are excreted. Glucose uptake by *F. hepatica* occurs by a mediated process and depends upon the concentration gradient between the worm and its environment. Table 6.12 shows that the glucose concentration within the worm is greater than the control. Thus, glucose has been taken up from the medium but may not have been utilised as quickly as by the control flukes. This is supported by the decrease in end product excretion by the treated flukes. The rate of glucose uptake will therefore be slower than for the control. These results suggest that the rate of carbohydrate metabolism is lower in the 24 h drug treated group.

Following incubation of both disulphonamide treated groups
there are large increases in the pyruvate concentrations. It has been suggested in chapter 3 that there are two pools of pyruvate, one generated by PK and retained in the cytosol, the other generated by malic enzyme and retained in the mitochondria. There are slight increases in the internal lactate concentrations (table 6.12) but no significant increases in lactate excretion (table 6.14), which suggests that the increased pyruvate concentration contributes to the lactate pool within the worm but not sufficiently to cause an increase in lactate excretion.

The increases in the pyruvate concentration may be due to increased production or decreased utilisation. If it is due to decreased utilisation, and lactate excretion is not greatly affected, there must be a decrease in acetate production. This occurs in the 24 h treated group on incubation (table 6.14). Thus, it seems that the utilisation of the mitochondrial pyruvate pool is affected. The increases in the malate concentrations of the incubated treated flukes also support this explanation. The mitochondrial pyruvate pool derives from malate via malic enzyme. If the further metabolism of pyruvate is inhibited there will be a build up of malate.

Succinate production increases dramatically in the disulphonamide treated flukes. At '0' time there are increases in the internal succinate concentrations of both drug treated groups (table 6.11) and following 24 h incubation the internal pools remain high (table 6.12) and there are increases in the amounts of succinate excreted (table 6.14). The increase in succinate concentration may be due to either an increase in formation or a decrease in utilisation. Table 6.14 shows there is a decrease in the amount of propionate
excreted by the incubated 24 h treated flukes which suggests that there is an inhibition in the further metabolism of succinate.

A possible explanation for a decrease in both acetate production from pyruvate and propionate production from succinate could be that there is an effect on CoA metabolism. The conversion of pyruvate to acetate in *F. hepatica* has not been studied in detail. It is likely that pyruvate is metabolised to acetyl CoA by the pyruvate dehydrogenase complex (Prichard & Schofield, 1968c). The conversion of acetyl CoA to acetate may occur in several ways. In bacterial systems acetyl CoA is converted into acetyl phosphate, catalysed by phosphotransacetylase, from which the phosphate group is transferred to ADP in the acetate thiokinase reaction. Alternatively, acetyl CoA synthetase may catalyse acetate formation from acetyl CoA.

\[
\text{pyruvate} + \text{NAD} + \text{CoA} \xrightarrow{\text{pyruvate dehydrogenase complex}} \text{acetyl CoA} + \text{CO}_2 + \text{NADH}
\]

The initial reaction involved in the formation of propionate from succinate is the conversion of succinate to succinyl CoA, catalysed by succinyl CoA synthetase.

\[
\text{succinate} + \text{GTP} + \text{CoA} \xrightarrow{\text{succinyl CoA synthetase}} \text{succinyl CoA} + \text{GDP} + \text{Pi}
\]

The drug may inhibit CoA synthesis or may increase its rate of degradation. In either case there would be a reduction in acetate and propionate formation. One consequence of inhibiting the conversion of succinate to succinyl CoA may be that GTP utilisation is spared. The synthesis of adenylates requires GTP in the reaction catalysed by
adenylosuccinate synthetase. Thus, more GTP may be available for adenylate synthesis which may explain the increases found in adenine nucleotide concentrations in the disulphonamide treated groups.

It is likely that the build up of various intermediates such as pyruvate, malate, lactate and succinate, caused by disulphonamide treatment, may result in a decrease in the rate of carbohydrate metabolism, as has already been suggested. Ultimately there would be an effect on ATP production due to the lack of carbon flow through the energy producing pathway, which presumably results in the inability of the parasite to maintain its structural integrity. As a result the effects found following other in vivo drug treatments also occur; detachment, cessation of feeding and necrosis.

It is possible that the increase in succinate formation in F. hepatica caused by the disulphonamide treatment is due to an increase in amino acid metabolism. However, an explanation is then required for the inhibition of pyruvate metabolism and the further metabolism of succinate as both acetate and propionate excretion are inhibited.

6.5 GENERAL DISCUSSION

The results of the in vivo experiments with MBZ-Poly C bromophenophos and the disulphonamide show that they disturb the energy metabolism of F. hepatica following in vivo treatment. However, the effects are not, in all cases, similar to those observed after RFX, NSC and MBZ treatment.

MBZ-Poly C, the most active polymorph of MBZ against
F. hepatica, causes changes in the adenine nucleotide levels and some of the metabolic intermediates 17 h after treatment. The changes are all marked increases. This was not found following MBZ treatment of flukes either in vitro or in vivo. In chapter 4 the effects of MBZ on the adenine nucleotide concentrations after 36 h were characteristic of an uncoupling action. Also there were indications of an increase in carbohydrate metabolism. The increases in adenine nucleotide levels and the intermediates found 17 h after MBZ-Poly C treatment may indicate an early effect on the flukes of increased metabolism to maintain energy production. At this time they are being maintained at higher levels than normal. Later, the ATP concentration probably decreases, as found after MBZ treatment. Further work is required with MBZ-Poly C to determine whether the adenine nucleotide levels decrease on prolonged treatment and whether the effects are similar to MBZ treatment.

Most of the effects of bromophenophos treatment are similar to those found following RFX, NSC and MBZ treatment in vivo. There are marked effects on the adenine nucleotide levels. The changes in some of the intermediates of the energy producing pathway and the decrease in glycogen concentration suggest an increase in carbohydrate metabolism to maintain the energy levels. These effects are consistent with the suggestion by van Miert and Groeneveld (1969) that the drug acts as an uncoupler.

The results of the disulphonamide treatment are different from those found following in vivo treatment with the other drugs (RFX, NSC, MBZ and bromophenophos). There are no reductions in any of the adenine nucleotide levels, no glycogen depletion and no
indication of increased carbohydrate metabolism. Therefore, the drug does not appear to have an uncoupling action. There are changes in some of the intermediate concentrations and inhibition of acetate and propionate excretion which may be explained by an effect of the drug on CoA metabolism. Presumably, the inhibition of end product excretion by the disulphonamide would result in a decrease in carbohydrate metabolism and energy production.

The observation that necrotic posterior portions occur in flukes after disulphonamide treatment, despite the apparently different mode of action of the drug, suggests that lack of ATP and mechanical effects encountered in their habitat are responsible for the appearance of necrotic areas, as discussed in chapter 5.

The disturbances in the adenine nucleotide concentrations of *F. hepatica* treated *in vivo* with MBZ-Poly C, bromophenophos and the disulphonamide indicate that the drugs have affected energy production in some way but appear to have some different effects on the intermediate concentrations and the excretion of end products. This supports the suggestion made earlier that adenine nucleotide levels may act as indicators of anthelmintic effect.
CHAPTER 7

GENERAL DISCUSSION AND FUTURE WORK

*F. hepatica* can be maintained *in vitro*, in a simple medium containing glucose, for at least 48 h, without adverse effects on the respiratory metabolic pathway (chapter 3). There are indications that initial changes occur, in response to the new conditions, in carbohydrate metabolism of the flukes. Thus, the *in vitro* incubations do not resemble the *in vivo* situation. It was not the purpose of this study to determine the optimal culture technique for *F. hepatica*, but to observe the effects of several anthelmintics on the energy producing pathway. Hence, the medium used in earlier, successful studies was adopted for the *in vitro* maintenance study.

Other *in vitro* studies carried out on the biochemical effects of anthelmintics on parasites fall into two groups. In the first, enzymes or organelles are isolated from the parasite, or tissue slices are cut, and the drug is tested on them (e.g. Van den Bossche, 1972b; Prichard, 1973; Yorke & Turton, 1974; Hanna & Threadgold, 1976). In the second group the effects of the anthelmintic on storage products or on the incorporation of isotopes are determined in the whole parasite (e.g. Van den Bossche & De Nollin, 1973; Van den Bossche, 1972a). By itself, each approach is unsatisfactory. In the first case there may be little relevance to the whole animal, as permeability barriers may preclude access of the drug to the enzyme being tested. In the second
case, the studies have not been taken far enough as, ideally, the behaviour of the whole energy producing pathway of the parasite should be monitored. The work of Metzger and D'Awel (1973) and Rahman (1976) are exceptions. Both studies show some correlation with effects of the drugs on the respiratory metabolic pathway and isolated enzymes. However, Metzger and D'Awel (1973) did not measure end product excretion or storage product concentrations, and used only a single time interval, whereas different effects may occur at different times. The present study and that of Rahman (1976) examine in detail the effects of anthelmintics on the energy producing pathway by measuring a range of parameters in whole parasites.

The large amounts of exogenous glucose are taken up \textit{in vitro} and utilised by the flukes, thus causing increases in the internal glucose concentration and several other intermediate pool sizes. It is likely that this leads to a greater rate of carbohydrate metabolism and reduces the contribution of amino acid metabolism to energy production. In turn, this may explain the decrease in the internal succinate concentration, following \textit{in vitro} incubation; there may be a decrease in the contribution to the succinate pool from sources other than carbohydrate metabolism.

The stoichiometrical relationship between acetate and propionate excretion is similar to that observed by de Zoeten \textit{et al.} (1969). Prichard (1976) and Van Vugt \textit{et al.} (1976) suggest that acetate formation is necessary to drive the fumarate reductase reaction. Reduced nucleotides produced in other parts of the pathway are reoxidised either by LDH or MDH. The present findings are in agreement with this hypothesis.
An area of further research which would benefit this type of study is an examination of the host-parasite relationship to determine the *in vivo* conditions experienced by a parasite. This should lead to the design of a better culture technique which would allow a complete study of the regulatory mechanisms used by the parasite under adverse conditions, e.g. in the presence of anthelmintics. It was mentioned earlier (see 1.4) that work of this kind on the rat tapeworm, *H. diminuta*, has shown that the presence of the parasite alters several parameters in its habitat, e.g. pH, oxygen tension and CO\textsubscript{2} concentration, thus showing the necessity of studying the parasite within its normal environment.

Until more detailed information is available about the habits and environment of adult *F. hepatica*, it may be possible to improve the *in vitro* incubations by including a physical substrate, containing nutrients, to which the flukes can attach and feed as they do *in vivo*. It may also be advantageous to use a defined growth medium, rather than a balanced salt solution, in the incubations. A suitable parameter to monitor when experimenting with different media is egg production, as this seems in flukes, to be a process which is adversely affected by *in vitro* culture. If a medium could be found in which flukes continue to produce normal eggs for several days and maintain energy production and carbohydrate reserves, the flukes would probably be close to their normal *in vivo* condition and thus even more suited for a complete study of anthelmintic action. The *in vitro* study described in chapter 3 shows that the pathways of carbohydrate metabolism are maintained and energy production is not adversely affected during 48 h incubation in Hédon-Fleig solution containing glucose. It was concluded therefore, that
the culture method used is suitable for examining the effects of several anthelmintics on the pathways.

The effects of the fasciolicides RFX, NSC and MBZ, on the carbohydrate metabolism of *F. hepatica* show that it is an essential pathway for the maintenance of the integrity of the parasite during *in vitro* incubation. It is also evident that the energy producing pathway has considerable capacity to adjust to adverse conditions. The most important indicators of anthelmintic effect appear to be the concentrations of adenine nucleotides. The three drugs disturb the ability of the parasite to phosphorylate ADP. This is in agreement with the findings of Van den Bossche (1972b) that RFX and MBZ cause an inhibition of $^{32}$Pi incorporation into ATP in *Ascaris* mitochondria.

The changes in adenine nucleotide concentration following incubation of flukes with RFX and NSC, and MBZ after 36 h, are characteristic of an uncoupling action. There are also increases in carbon flow through the energy producing pathway, as measured by increased end product formation and inferred from decreases in the internal glucose and hexose phosphate pool sizes. Several other studies with RFX also suggest that it is an uncoupler of electron transport and phosphorylation (see 2.6.3).

Little is known of the effects of uncouplers on parasites. Van Vugt *et al.* (1976) have examined the effect of pentachlorophenol on substrate consumption and phosphate esterification by *F. hepatica* mitochondria. It was found that malate consumption is increased in the presence of the uncoupler, indicating that the phosphorylation process is rate-limiting for the overall dismutation of malate. A similar study could be carried out, using the same methods, to determine
whether RFX, NSC and MBZ stimulate malate utilisation.

Van den Bossche (1972a) has examined the effects of some known uncouplers on \(^{32}\text{Pi}\) incorporation into ATP in isolated \(A.\text{caris}\) mitochondria. They inhibit radioactive ATP production; RFX and MBZ have a similar effect. Fluke mitochondria could be prepared by the methods of Van Vugt et al. (1976) and similar experiments could be carried out to determine the effects of RFX, NSC and MBZ on \(^{32}\text{Pi}\) incorporation. A known uncoupler of fluke mitochondria, such as pentachlorophenol, could be included as a comparison.

As \(F.\text{hepatica}\) has an electron transport system using oxygen as a terminal electron acceptor, it should be possible to isolate intact fluke mitochondria and carry out a polarographic study, measuring oxygen uptake and ADP utilisation under different conditions. The effects of known uncouplers of mammalian mitochondria, such as 2,4-dinitrophenol, and RFX, NSC and MBZ could be examined in fluke and mammalian mitochondria to determine whether they have an uncoupling action and if they have similar effects on both types of mitochondria. Preliminary studies with RFX and NSC show that they act as uncouplers of mammalian mitochondria (A.J. Wilkes, personal communication). It is possible that there are less permeability barriers for the drugs to reach fluke mitochondria, or mammalian mitochondria may be less susceptible, which may explain the lack of effect of the drugs on the host.

It would also be of interest to determine whether the drugs have an effect on energy production from the fumarate reductase system. As electron transport is involved in this reaction (see 1.6.7) uncoupling agents should destroy the link between fumarate reduction to succinate and ATP production. The measurement of \(^{14}\text{C}-\text{fumarate}\)
conversion to radioactive succinate together with $^{32}$Pi incorporation into ATP in intact fluke mitochondria, isolated from control and drug treated flukes, or, in control mitochondria with added drug, should determine whether the drugs uncouple the reactions.

The action of uncouplers on whole flukes has not been studied. It would be of interest to carry out an in vitro study on an uncoupler known to have an effect on F. hepatica, e.g. pentachlorophenol, and to compare its effects with the present findings for RFX, NSC and MBZ.

The experiments suggested will provide a lot of information on the effects of known uncoupling agents on whole flukes and isolated mitochondria and should indicate whether the three fasciolicides used in the in vitro study have an uncoupling action.

MBZ appears to have a further effect on the adenine nucleotide levels; a decrease in the total adenine nucleotide concentration after 48 h incubation. This may be due to an effect of the drug on adenine nucleotide turnover or it may be due to insufficient energy available for adenylic acid synthesis. It may be possible to use the techniques of Pfaff and Klingenberg (1968) and Klingenberg (1970) to examine the exchange reactions of ADP and ATP in mitochondria isolated from control and MBZ treated flukes, or, control mitochondria with MBZ added. The results should indicate whether the drug affects the transport of the nucleotides in and out of the mitochondria and also whether there is a decrease in adenine nucleotide turnover.

The other major finding from the in vitro study with RFX, NSC and MBZ, is that none of the drugs has an effect on glucose uptake
from the medium or on glycogen utilisation. This is an important
observation in the case of MBZ, as previous work on nematodes and
cestodes has shown that MBZ inhibits glucose uptake, stimulates
glycogen utilisation and decreases ATP production (Van den Bossche,
1972b; De Nollin and Van den Bossche, 1973). More recent work with
MBZ has shown that it has an effect on the microtubular system which
results in the degeneration of the tegumental cells in some nematodes
and cestodes (Borgers et al., 1975a,b; Verheyen et al., 1976). It has
been suggested that this is the primary site of action of MBZ and
results in the inhibition of glucose uptake which in turn causes the
reduction of glycogen and ATP levels. However, the results of Rahman
(1976) and the present study offer a different explanation. Rahman
(1976) has shown an effect of MBZ on the ATP and total adenine
nucleotide levels in M. expansa within 30 min contact in vitro and
within 3 h in vivo. The intracellular effects observed on nematodes
and cestodes take many hours to become apparent (Borgers & De Nollin,
1975; Borgers et al., 1975a,b; Verheyen et al., 1976). The present
study on F. hepatica, a trematode, shows no effect of MBZ on glucose
uptake. Isseroff and Read (1974) have studied the mechanism of glucose
uptake in flukes and have shown that it is an active process (see
1.5.2). In the nematodes and cestodes so far studied, glucose uptake
is energy requiring. If MBZ causes a decrease in ATP concentration by
increasing its rate of breakdown or decreasing its rate of synthesis,
active transport of glucose in cestodes and nematodes will be inhibited
and glycogen reserves will be utilised. The decrease in ATP levels in
F. hepatica will not inhibit glucose uptake or stimulate glycogen
utilisation. It is suggested by Rahman (1976) that as ATP is necessary
for the maintenance of intracellular structure, degeneration of microtubules in nematodes and cestodes is probably due to the lack of ATP. Thus, the primary effect of MBZ could be on energy production.

Several effects of the three drugs on the intermediate levels are similar and probably reflect an increase in glucose metabolism to maintain ATP levels. There also appear to be some drug-specific effects. For example, there are marked increases in the pyruvate concentrations of flukes incubated with RFX and NSC. It is not possible to decide from the present study whether pyruvate accumulation is due to an increase in synthesis from glucose or amino acids, and/or, inability of the parasite to metabolise it at a faster rate.

Incubation of flukes with radioactive precursors and RFX or NSC in the medium, followed by analysis of the distribution and specific activity of the radiocarbon within the flukes and excreted into the medium should indicate whether there is an inhibition of the further metabolism of pyruvate.

MBZ and RFX appear to increase the amount of succinate produced and excreted by *F. hepatica* and decrease the amount of propionate excreted. Again, this may be due to an increase in synthesis from glucose or other sources, and/or due to an inhibition in part of the succinate - propionate pathway. It is possible that the low levels of adenine nucleotides present in the flukes after incubation with the drugs are insufficient to act as substrates in the reactions of the pathway catalysed by propionyl CoA carboxylase and propionate thiokinase; that is, there is a lack of kinetic efficiency. The use of \(^{14} \text{C}-\text{glucose}\) in the medium and analysis of the distribution of the radiocarbon as suggested above, should also determine whether
the increased succinate is due to increased utilisation of glucose and/or due to inhibition of its metabolism to propionate. A detailed study of the enzymes involved in the metabolism of succinate to propionate from control and drug treated flukes may also provide an answer to the problem.

The decrease in lactate excretion in flukes incubated in vitro with MBZ could be examined with the use of radioactive precursors in the medium. The enzymes involved in lactate production from PEP, PK and LDH, could also be tested in the presence of MBZ or their activity could be examined from MBZ treated flukes to determine whether MBZ has a direct effect on either of them. Rahman (1976) studied the effects of MBZ on PK and LDH from *M. expansa* and on the commercial enzyme preparations obtained from Boehringer, and found that it has no effect.

The *in vivo* studies with RFX, NSC and MBZ were carried out to determine whether the effects of the drugs measured *in vitro* also occur in flukes treated *in vivo*, following administration of the drugs to the host. The results of the *in vivo* study complement and extend the observations made in the *in vitro* study. The major effects appear to be the disturbances in adenine nucleotide concentrations after removal of the flukes from the host and/or following 24 h incubation *in vitro*. Most of the changes in the intermediate concentrations found *in vitro* also occur *in vivo*.

The observations made during the *in vivo* study emphasise the need to reproduce the conditions experienced by the parasite within the host as closely as possible during an *in vitro* study. Surprisingly little is known about the habits of adult *F. hepatica* and the
environment in which it lives. The detachment and/or lack of reattachment of the flukes following in vivo treatment complicates the results when comparing them with the in vitro study. The consequent cessation of feeding leads to the utilisation of glycogen reserves which was not observed in vitro presumably because of the large supply of glucose available in the medium.

Another feature observed following in vivo drug treatment but not in the in vitro study is the appearance of necrotic areas in the posterior regions of the flukes, from which the tegument is absent. It was suggested in 5.4 that several factors probably operate to produce these regions: a lack of ATP to maintain structural integrity, the digestion/absorption processes of the flukes and the mechanical effects caused by bile flow, spines of other flukes, eggs and the confines of the bile ducts. The absence of adenine nucleotides and other labile intermediates from the necrotic regions are probably responsible for masking some of the changes caused by the drugs in vitro but not shown in vivo, e.g. the reduction of the total adenine nucleotide concentration in flukes following in vivo treatment with NSC compared with the apparent uncoupling effect of the drug in vitro.

The finding that three known fasciolicides, of unrelated structure, cause marked changes in the adenine nucleotide concentrations of flukes maintained in vitro and treated in vivo suggests that adenine nucleotides may be used as indicators of anthelmintic efficacy. Two parameters, using adenine nucleotide concentrations, are often used as indicators of the energy status of a tissue or organism; ATP/ADP ratios and adenylate energy charges (see 1.6.8). Implicit in the definitions of these measurements is that the
total adenine nucleotide concentration remains constant. In the *in vitro* study MBZ causes a reduction in total adenine nucleotide concentration and in the *in vivo* study all three drugs produce a similar reduction, although in some cases this is due to the inclusion of necrotic flukes in the samples. If ATP/ADP ratios or adenylate energy charges are used in these cases a misleading value is obviously obtained. Therefore, it is necessary to measure all three adenine nucleotide concentrations and to compare the absolute values instead of false ratios.

As mentioned earlier (see 2.7), current methods used for testing the efficiency of anthelmintics and the screening of new compounds, are expensive, lengthy and labour-intensive. Simple *in vitro* and *in vivo* tests of the kind described in this thesis may provide a cheap, additional, or even alternative, method for screening anthelmintics. The study of Rahman (1976) on the *in vitro* and *in vivo* effects of MBZ and cambendazole on *M. expansa* shows that adenine nucleotide levels are affected by the two drugs. Preliminary *in vivo* studies with the nematode, *H. contortus*, also show that some anthelmintics effective against it in field trials cause disturbances in the adenine nucleotide concentrations (Rahman, Cornish, Chevis & Bryant, 1977; Cornish, unpublished).

It is necessary to carry out both *in vitro* and *in vivo* studies, as some anthelmintics, although effective *in vitro*, are ineffective *in vivo*, and *vice versa* (see 2.7 and 5.1). However, the numbers of parasites and hosts required for the experiments described in this thesis are far less than are required for present methods of screening. In addition, by measuring other parameters (as done in this
study) it is possible to acquire information about the mode of action of the drug.

The effects of MBZ-Poly C, bromophenophos and trichloroethenyl-disulphonamide on *F. hepatica* in vivo also support the suggestion that adenine nucleotide levels can be used to indicate anthelmintic efficacy. All three drugs cause disturbances in the concentrations of the adenine nucleotides but MBZ-Poly C and the disulphonamide treatment result in increases in the levels rather than the decreases found following RFX, NSC, MBZ and bromophenophos treatment.

The changes in adenine nucleotide levels and intermediate concentrations caused by MBZ-Poly C may reflect an early effect of the drug to increase metabolism and/or decrease ATP utilising functions in an attempt to maintain energy production. Presumably, MBZ-Poly C has the same effect as MBZ as it appears to be the 'active ingredient' of the undefined mixture for fluke (Chevis, personal communication).

Bromophenophos is thought to act as an uncoupler (van Miert & Groeneveld, 1969). The results of the in vivo study are consistent with this view and are similar to some of the effects produced by RFX, NSC and MBZ treatment, in vivo.

Trichloroethenyl-disulphonamide appears to have a different action on flukes from the other drugs studied. It causes a reduction in acetate and propionate excretion and increases in the pyruvate and succinate concentrations and succinate excretion. It is suggested that a possible effect of the drug is on CoA metabolism such that the metabolism of pyruvate to acetyl CoA and succinate to succinyl CoA is inhibited. This mode of action may explain the specificity of the
drug for *F. hepatica*. The effect of this drug on $^{14}$C-glucose metabolism by flukes incubated *in vitro*, as described earlier, should determine whether the drug causes a decrease in glucose metabolism and inhibits acetate and propionate formation from pyruvate and succinate, respectively.

The studies described in this thesis have provided information on the possible mode of action of several anthelmintics on *F. hepatica*, *in vitro* and *in vivo*. The results also suggest that adenine nucleotides may be used as indicators of anthelmintic efficacy. More work is required on different parasites and known anthelmintics to establish this hypothesis. However, the suggestion seems reasonable when it is considered that changes in adenine nucleotide synthesis as a response to adverse conditions can usually only be inferred from changes in carbon flow through the respiratory metabolic pathway. When changes in adenine nucleotide concentrations are detectable it must be assumed that the parasite can no longer adapt to the adverse conditions.
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The following references have been supplied by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, with the request that they be referred to rather than Ostlind & Mrozik, 1976.


The following references were accidently omitted from the bibliography:


APPENDIX 1

COMPOSITION OF HÉDON-FLEIG INCUBATION MEDIUM

(a) INCUBATION MEDIUM

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>7.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.3 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1 g</td>
</tr>
<tr>
<td>MgSO₄·3H₂O</td>
<td>0.3 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>0.06 units</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.10 units</td>
</tr>
</tbody>
</table>

Distilled water to give a final volume of 1 litre, pH 7.96,
(Van Noordwijk & de Wolf, 1963).

(b) HÉDON-FLEIG SALT SOLUTION

For washing the flukes, Hédon-Fleig salt solution was used. This consists of the above medium without glucose, streptomycin and benzylpenicillin.

(c) ¹⁴C-3-O METHYL GLUCOSE UPTAKE

The medium consisted of the Hédon-Fleig salt solution with streptomycin and benzylpenicillin in the concentrations given in (a) above and with 3-O-methyl glucose (2mM) and ¹⁴C-3-O-methyl glucose (0.1 μCi/ml).
(d) **IN VIVO EXPERIMENTS**

For ease of transport, stock solutions were made and diluted when required.

**Solution I:** NaCl, KCl, MgSO\(_4\), NaHCO\(_3\) and glucose at the weights shown above (a) were made up in a final volume of 100 ml distilled water.

**Solution II:** as for solution I but without glucose.

**Solution III:** CaCl\(_2\) 0.1 g in 100 ml distilled water.

For washing the flukes, i.e. Hédon-Fleig salt solution: 10 ml solution II + 10 ml solution III + 80 ml distilled water.

For incubations: 10 ml solution I + 10 ml solution III + 80 ml distilled water + streptomycin and benzylpenicillin at the same concentration as in (a) above.
SUPPLIERS OF BIOCHEMICALS

BOEHRINGER MANNHEIM, GERMANY

All enzymes except succinic thiokinase
ADP, disodium salt
ATP, trisodium salt
CoA, Grade 1
F6P, disodium salt
GTP, trisodium salt
2,3-diPGA
NAD, free acid
NADH, disodium salt
NADP, disodium salt
PEP, potassium salt
Triethanolamine-HCl buffer

SIGMA CHEMICAL CO., ST. LOUIS

AMP, sodium salt
FDP, tetradsodium salt
G6P, disodium salt
2PGA, sodium salt
3PGA, sodium salt
L-(+) lactic acid, crystalline, Grade L-1
Malic acid, crystalline
Pyruvate, sodium salt
Succinic thiokinase, lyophilised preparation
'Tween 80'

RADIOCHEMICAL CENTRE, AMERSHAM
3-O-methyl-D-(U-\(^{14}\)C) glucose
NCS, tissue solubilizer
P: S solubilizer