Changes in energy metabolism due to anthelmintics in Fasciola hepatica maintained in vitro

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Abstract—Cornish R. A. and Bryant C. 1976. Changes in energy metabolism due to anthelmintics in Fasciola hepatica maintained in vitro. International Journal for Parasitology 6: 393–398. The effects of rafoxanide (RFX), nitroscanate (NSC) and mebendazole (MBZ) on oxidative pathways in whole F. hepatica maintained in a simple salt solution have been examined. The anthelmintics did not alter glucose uptake or glycogen mobilization. NSC and RFX depressed ATP and increased AMP levels. MBZ behaved similarly at first, but later depressed the total adenine nucleotides. All three drugs influenced end product formation, increasing it initially, although by different mechanisms. With NSC, early increases in lactate and acetate excretion were later abolished. With RFX, there was an initial increased production of acetate and propionate. Later, excretion of propionate was reduced and that of succinate was increased. MBZ also increased succinate excretion, but to a much greater extent. In addition, it inhibited lactate production. A number of effects of the drugs on the internal concentrations of metabolic intermediates are described. The mechanisms of action of the drugs are discussed.

INDEX KEY WORDS: Fasciola hepatica; anthelmintics; rafoxanide; nitroscanate; mebendazole; energy metabolism; in vitro maintenance.

Introduction

Fasciola hepatica is able to maintain normal energy metabolism during in vitro incubation for at least 48 h (Cornish & Bryant, 1976). It is possible to utilize the system for the analysis of anthelmintic effects on the parasite’s main energy producing pathway and perhaps, as a result, a rational approach to the discovery of new drugs can be developed. In the present study the effects of 3 anthelmintics have been examined, RFX, MBZ and NSC. RFX (3'-chloro-4'(4-chloro-phenoxy)-3,5-diido-salicylanilide) is commercially in use as a fasciolicide. Some salicylanilide derivatives have been found to be effective uncouplers in a wide range of organisms, including parasites (Corbett, 1974). MBZ (methyl 5-benzoyl-2-benzimidazolecarbamate) has been shown to have activity against Fasciola hepatica in field trials (Kelly, Chevis & Whitlock, 1975). It is reported to inhibit glucose uptake, deplete glycogen reserves and decrease the generation of energy-rich phosphate bonds in some adult nematodes (Van den Bossche & De Nollin, 1974). MBZ also causes ultrastructural changes in some nematodes (Borgers & De Nollin, 1975; De Nollin, Borgers, Vanparijs & Van den Bossche, 1974). NSC (4-isothiocyanato-4'-nitrophenylether) is effective against some nematodes and cestodes in dogs and is also a fasciolicide (Boray, personal communication). No biochemical effects of this drug have hitherto been reported.

Materials and Methods

All enzymes and cofactors except one were purchased from Boehringer, Mannheim; succinyl coA synthetase was purchased from Sigma Chemical Co., St. Louis. NSC tissue solubilizer and PCS scintillant were purchased from the Radiochemical Centre, Amersham, Bucks. MBZ was obtained from Ethnor Pty. Ltd., NSC from CIBA-Geigy Australia Ltd.; and RFX from Merck, Sharp and Dohme (Australia) Pty. Ltd. The flukes were collected and prepared for incubation as described by Cornish & Bryant (1976). Twenty milligrams of drug was used per 50 ml of media containing approximately 1 g flukes. NSC and RFX were suspended in 200 µl propylene glycol and MBZ was suspended in 200 µl Tween 80. The incubations, glycogen determinations and end-product analyses were carried out as described by Cornish & Bryant (1976) and the preparation of extracts and determination of intermediates was carried out according to Behm & Bryant (1975). 14C-3-O-methyl glucose uptake was measured on individual flukes. Each incubation contained 5 ml Hédon–Fleig plus methyl glucose. At the completion of the incubation each fluke was removed, washed gently several times, blotted, then placed in a scintillation vial. One millilitre NCS tissue solubilizer

Abbreviations used in the text. ATP, ADP, AMP adenine tri-, di- and monophosphate; F6P, fructose-6-phosphate; FDP, fructose-1, 6-diphosphate; G6P, glucose-6-phosphate; MBZ, mebendazole; NSC, nitroscanate; 2 & 3 PGA, 2- & 3-phosphoglycerate; PEP, phosphoenolpyruvate; RFX, rafoxanide; TP, triose phosphates.
was added and the tissue digest was carried out at 50°C until the samples appeared homogeneous when shaken. Thirty microlitres glacial acetic acid was added to each vial to neutralize the sample, then 10 ml PCS scintillant was added and the samples were counted in a liquid scintillation counter.

RESULTS

Glycogen levels

No significant effects on the glycogen levels in Fasciola hepatica were exerted by any drug at any time. However, to ensure that changes were detectable under experimental circumstances, flukes were incubated in Hedon-Fleig solution from which glucose was omitted. Glycogen levels dropped to 50% within 20 h from the start of the experiment and to 20% within 48 h.

Methyl glucose uptake

Possible effects on glucose uptake were monitored using the radioactive methyl glucose method for F. hepatica as described by Isseroff & Read (1974). Uptake was determined as counts per minute in the worm during 3 h incubation. When c.p.m./g wet weight (y axis) was plotted against time in minutes (x axis) a control curve was obtained whose equation is \( y = 21.98x^{0.58} \) with a coefficient of determination \( r^2 = 0.89 \) and 95% confidence limits 16,600 c.p.m. At 3 h, uptake was approximately 100,000 c.p.m., which was equivalent to 1.7 μmoles glucose. Curves were determined for NSC, RFX and MBZ. Of the 18 points for each curve, only 2 for MBZ and 2 for NSC fell outside the 95% confidence limits of the control. Preincubations of the flukes with drug for 16 h (NSC) produced no significant effects on methyl glucose uptake when the worms were removed to fresh medium containing the drug.

Adenine nucleotides

In this, and following tables, the effects of different drugs are quoted at different times. It is necessary because the effects of the drugs do not become apparent simultaneously. Thus, in RFX, the majority of flukes are dead within 12 h, in NSC 24 h is required and with MBZ, 60 h or more.

The effects of RFX and NSC on adenine nucleotides in F. hepatica are given in Table 1. In each case the levels of ATP fell significantly while those of AMP rose. RFX, but not NSC, caused a significant increase in ADP. Total nucleotides were unaffected and the ATP/ADP ratios dropped from about 1.4 to 0.86 (RFX) and 0.75 (NSC).

Table 1 shows the effect of MBZ on adenine nucleotides. Results for 2 incubation times are given (36 and 48 h) because 2 different effects are observed. At 36 h ATP levels had decreased significantly while AMP levels had increased. Total

| Table 1—Effect of RFX and NSC on adenine nucleotides in F. hepatica maintained in vitro (nmoles/g wet weight ± standard deviation) |
|------------------|------------------|------------------|
|                  | Control (3-9 h)  | RFX             | Control (20 h) |
|                  | n = 6            |                 | n = 6          |
| ATP*             | 1144 ± 227       | 815 ± 347       | 1229 ± 223     | 631 ± 387 |
| ADP              | 800 ± 48         | 946 ± 77        | 870 ± 156      | 846 ± 148 |
| AMP              | 303 ± 86         | 353 ± 163       | 340 ± 154      | 590 ± 163 |
| Total            | 2246 ± 136       | 2297 ± 287      | 2439 ± 425     | 2067 ± 392 |
| ATP/ADP          | 1:4:3            | 0:86            | 1:4:1          | 0:75        |

Tests for significance showed that only * the decrease in ATP with both RFX and NSC is significant, \( P < 0.01 \); † the increase in ADP with RFX is significant, \( P = 0.005 \); ‡ the increase in AMP with both RFX and NSC is significant, \( P < 0.0005 \) and < 0.05 respectively.

| Table 2—Effects of MBZ on adenine nucleotides in F. hepatica maintained in vitro for 36 and 48 h (nmoles/g wet weight ± standard deviation) |
|------------------|------------------|------------------|
|                  | Control 36 h (n = 5) | MBZ             | Control 48 h (n = 6) | MBZ             |
| ATP*             | 994 ± 212             | 668 ± 117       | 915 ± 259         | 364 ± 178       |
| ADP              | 804 ± 135             | 782 ± 81        | 778 ± 22          | 385 ± 185       |
| AMP              | 258 ± 17              | 368 ± 49        | 322 ± 49          | 347 ± 79        |
| Total§           | 2056 ± 350            | 1817 ± 186      | 2015 ± 219        | 1279 ± 383      |
| ATP/ADP          | 1:24                  | 0:85            | 1:18              | 0:62            |

Tests for significance showed that: * the decrease in ATP at both times was significant, \( P < 0.05 \) and < 0.0125 resp.; † the decrease in ADP at 48 h was significant, \( P < 0.025 \); ‡ the increase in AMP at 36 h was significant, \( P < 0.01 \); § the decrease in total nucleotides at 48 h was significant, \( P < 0.0125 \).
nucleotides were unaffected. At 48 h, however, ATP, ADP and total nucleotides were significantly depressed. ATP/ADP ratios in both cases were considerably lower than the controls.

Figure 1 shows that the changes in ATP/ADP ratios caused by the 3 drugs are progressive and continuous.

End product excretion

The effects of the anthelmintics on the excretion of end products and intermediate pool sizes of *F. hepatica* are presented in Tables 3 and 4. They are given as differences from control values because significances were established with the paired ‘t’ statistic. Paired ‘t’ works on the null hypothesis that the mean differences from the controls are zero. In Table 3, results are given from incubations at the beginning and at the end of the experiment. Intermediate times, at which consistent results were obtained, are omitted to simplify the presentation.

In NSC at 3 h there was an increase in lactate and acetate excretion. However, after 20 h there was no significant difference from the controls. RFX at 3 h caused significant increases in acetate and propionate. After 9 h the effect on acetate had been abolished while succinate production was increased and propionate production decreased. With MBZ the only effect to be observed at 12 h was a very large increase in succinate. This effect was also apparent between 36 and 48 h at which time lactate production had diminished.

The total end product output of the controls is also given in Table 3. Thus, at the earlier times, the 3 drugs increased excretion by 17% (NSC), 32% (RFX) and 16% (MBZ). At the later times the effect of NSC was removed, the net effect of RFX was an 11% decrease and for MBZ an increase of 17%.

Internal pools

The effects on the internal pools in the energy producing pathway as described by Cornish & Bryant (1976) were also determined in the presence of each of the 3 drugs. They are shown in Table 4 where they are expressed as differences from the control values. In the interests of space it is necessary to mention here only that, with NSC and RFX, pool sizes in the early part of the pathway are depressed whereas those in the later part are elevated. With MBZ all differences from control values are negative.

**DISCUSSION**

There have been a number of *in vitro* studies on the biochemical effects of anthelmintics on parasites.

### Table 3—The Effects of RFX, NSC and MBZ on End Product Excretion in *Fasciola hepatica* Maintained *in vitro*

<table>
<thead>
<tr>
<th>Time</th>
<th>Lactate</th>
<th>Acetate</th>
<th>Succinate</th>
<th>Propionate</th>
<th>Total controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSC (3 h)</td>
<td>+ 10,200*</td>
<td>+ 4000*</td>
<td>ns</td>
<td>ns</td>
<td>81,500</td>
</tr>
<tr>
<td>RFX (3 h)</td>
<td>ns</td>
<td>+ 18,000*</td>
<td>ns</td>
<td>+ 6000†</td>
<td>76,000</td>
</tr>
<tr>
<td>MBZ (12 h)</td>
<td>ns</td>
<td>ns</td>
<td>+ 56,200*</td>
<td>ns</td>
<td>352,800</td>
</tr>
<tr>
<td><strong>Time 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSC (20 h)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>426,400</td>
</tr>
<tr>
<td>RFX (9 h)</td>
<td>ns</td>
<td>ns</td>
<td>+ 4400†</td>
<td>− 29,000†</td>
<td>233,400</td>
</tr>
<tr>
<td>MBZ (36–48 h)</td>
<td>− 39,200†</td>
<td>ns</td>
<td>+ 79,700*</td>
<td>ns</td>
<td>579,000</td>
</tr>
</tbody>
</table>

The individual end products are shown as the mean differences from the controls in nmoles/g wet weight. The total end products are the mean control values in nmoles/g wet weight. Number of replicates is at least 5 (lactate) and 6 or more for the other end products.

Significances obtained by paired ‘t’ test: ns, not significant; *P < 0.005, †P < 0.025

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**Fig. 1.** Changes in ATP/ADP ratio caused by the anthelmintics during incubation. The figure demonstrates that changes are continuous and progressive throughout the experiment. The points are joined to aid comparison and do not necessarily imply proper fit. n = 3 or more replicates for all points except those indicated in the figure.
They fall into two groups. In the first, an enzyme system or an organelle is isolated from the parasite and the drug is tested on it (Van den Bossche, 1972a; Prichard, 1973; Yorke & Turton, 1974). 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, 1972b). 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.

An exception is the work of Metzger & Duwel (1973) who 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 sub-cellular fractions. However, they did not measure end products, and used only a single time interval (4 h), whereas different effects may occur at different times. In the present work, detailed time courses have been carried out for as long as 48 h, although the effects of changing drug concentrations have not been studied.

Van den Bossche (1972b) has shown that, in *Ascaris* and some other nematodes, MBZ inhibits glucose uptake and depletes glycogen reserves. He suggests that this effect is ultimately responsible for lowering ATP levels within the parasites. In *F. hepatica*, MBZ, RFX and NSC do not affect glucose uptake nor the mobilization of glycogen, yet adenine nucleotide levels are depressed. An alternative explanation for the *Ascaris* results could be that MBZ brings about depletion of ATP levels which in turn diminishes the uptake of glucose. The difference between *Ascaris* and *F. hepatica* may then be explained by the difference in glucose uptake. In *Ascaris* it is an active process whereas, in *F. hepatica*, it is passive (Beames, 1971; Isseroff & Read, 1974).

Table 1 suggests that both NSC and RFX affect ATP synthesis similarly. The total concentrations of nucleotides are not altered but, in each case, ATP is diminished and AMP is increased. It has been reported that RFX uncouples oxidative phosphorylation (Corbett, 1974) and inhibits $^{32}$Pi incorporation in *Ascaris* mitochondria (Van den Bossche, 1972a). These studies on sub-cellular preparations are consistent with the evidence reported here. It therefore seems that RFX is capable of traversing the body or intestinal walls of the fluke and impairing the efficiency of the ATP synthesizing system in the whole animal as well as in isolated mitochondria. Metzger & Duwel (1973) have reported a similar phenomenon with another salicylanilide.

The effect of MBZ on adenine nucleotides is more complex (Table 2). At 36 h it apparently uncouples, and by 48 h, the total adenine nucleotides are substantially decreased. Van den Bossche (1972a) has shown that $^{32}$Pi incorporation by *Ascaris* mitochondria is inhibited by MBZ, which agrees with this observation. The depressed total of adenine nucleo-
tides indicates an effect on adenine nucleotide turnover, which may be due to diminished synthesis of precursors because of insufficient ATP. (If this is so, then lower concentrations of RFX and NSC may exert the same effect, over longer time periods.)

The effects on adenine nucleotides must have profound metabolic consequences for the intact animal. Changes in ATP/ADP ratios will have secondary effects which must not be confused with those that occur as a direct result of the drug. Thus, a drug induced change in the internal concentration of a metabolite may arise by direct inhibition of an enzyme or, it may follow from the alteration of the concentration of an allosteric regulator such as ATP.

The 3 drugs significantly decrease ATP/ADP ratios within the worm (Fig. 1). Decreased ATP/ADP ratios lead to increased carbon flow through the pathways of energy metabolism (Newsholme & Start, 1973). If this were the only effect of the drugs, then carbon flow, as indicated by end-product formation, should increase in each case, although the magnitude of the change might vary.

Table 3 shows that the changes caused by the drugs depend on the time of measurement and on the drugs used. It is therefore likely that the anthelmintics exert effects in addition to those on the ATP synthesizing machinery. A substantially increased flow of carbon through the pathways occurs at time 1 in each case, which conforms with prediction. With NSC at the earlier time, total end product formation is not significantly different from the control. However, internal pool sizes (Table 4) show that there is still substantial internal accumulation of lactate and pyruvate. NSC is the only drug which also caused a significant increase in FDP. It is therefore suggested that the diminished ATP/ADP ratio causes increased activity of phosphofructokinase; the resultant increase in FDP concentration increases the activity of pyruvate kinase (Prichard, 1976) leading to the production of more pyruvate and lactate. If the internal pyruvate pool increases it is not surprising that excretion of acetate also increases at some stage. All effects of NSC can therefore be explained in terms of an inhibition of ATP synthesis.

With RFX, at the early stage, the flow through the acetate and propionate producing branches is increased. At time 2, acetate excretion is not significantly different from the control, propionate production is markedly diminished and succinate production is increased. The internal acetate pool remains high and there is a significant increase in the internal succinate pool (Table 4). There is thus a block between succinate and propionate. The apparently tight stoichiometry between acetate and propionate production observed earlier (Cornish & Bryant, 1976) is disrupted, perhaps as a consequence of the reputed uncoupling effect of the drug. RFX, therefore, appears to diminish ATP synthesis, causing increased carbon flow. There follows a depletion of some of the internal pools in the pathway to the level of FDP. Later, as an independent effect the further metabolism of succinate is inhibited. The latter is important because it normally yields ATP.

The effect of MBZ on end production formation (Table 3) is to increase the excretion of succinate substantially at both times. At the second time, also, the excretion of lactate is markedly inhibited suggesting that the carbon flow is diverted to the acetate and propionate producing branches of the pathway. The increased succinate production suggests that the conversion of succinate to propionate is inhibited, especially as the internal pool of propionate is decreased whereas that of succinate is not changed. All significant changes in internal pool sizes are negative, which is difficult to interpret. It seems, however, that MBZ exerts effects which override those which may be induced by changes in the ATP/ADP ratio. These effects would seem to be at some point between PEP and lactate and between succinate and propionate.

The energy producing pathway is essential for the maintenance of the integrity of the parasite and has considerable capacity to adjust (Cornish & Bryant, 1976). If the system described here, which is easy to manipulate, to sample, and to assay (many can be performed sequentially in the same assay system), is to be used in the evaluation of drug action, this caveat must be clearly recognized.

This study, and that of Metzger & Duwel (1973), shows that effects of anthelmintics on energy metabolism can be detected in a simple in vitro system. The most important indicators appear to be the concentrations of adenine nucleotides and end products. Changes may occur in the concentrations of internal intermediates, but it is difficult to determine whether or not they are 'primary' or 'secondary'. Experiments are now in progress to confirm these findings on parasites obtained from treated hosts and to test the predictions in enzyme systems isolated from the parasite.

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