Table IV. Aphicidal effect of Hoe 25682 by topical application to adult apterous virgines

Aphid strain	Amount of Hoe 25682 per aphid ($\mu g \cdot 10^{-3}$)	No. of aphids	Kill corrected after Abbott (%)	Weighted average of % kill
Aphis craccivora	0.01	97	24.74	
	0.015	124	57.25	
	0.02	103	48.22	
	0.025	84	78	
	0.04	71	92.95	
Aulacorthum circumflexum	0.025	84	43.9	42.26
	0.025	88	40.7	
	0.033	40	57.76	
	0.04	74	33.89	60.2
	0.04	110	77.99	
	0.05	84	79.35	
Myzus persicae (susceptible)	0.02	81	8.64	
	0.05	87	45.08	34.52
	0.05	99	22.87	
	0.05	47	39.53	
	0.1	105	62.67	68.75
	0.1	63	78.89	
Myzus persicae (OP-resistant)	0.05	86	31.89	
	0.07	86	56.1	
	0.1	102	73.6	

0.2 μl of butanone solution applied with a microsyringe. Mortality was checked after 20 h.
Mortality in the control never exceeded 14%, otherwise experiments were discarded.

Table V. The toxicity of Hoe 25682 to adult apterous aphids

Species	LD_{50} (10^{-3} μg/mg weight)	Slope of the regression line
A. craccivora	0.02	2.05
A. circumflexum	0.04	1.86
M. persicae (susceptible)	0.20	1.76
M. persicae (OP-resistant)	0.21	1.98

insecticide appeared on the leaf surface (Table III). Most of the aphids were killed or were affected and dropped on the ground. Results of the topical application experiment are shown in Tables IV and V in terms of LD_{50}.

The symptoms of intoxication by Hoe 25 682 in aphids were multifold: 1. knockdown was followed by excitation and motor hyperactivity. 2. The aphids showed stumbling movements. 3. Aphids responded only feebly to stimuli (adynamic phase[12]). 4. They culminated in death.

These results were similar to those observed by HOLTGRÄWE[13] for the organophosphorus compound triazophos, 1-phenyl-1.2.4-triazolyl-3-(0.0 diethylthionophosphate). The only exception was the observation of repeated short-term recovery in aphids, especially in *A. craccivora*, which were exposed to Hoe 25 682. This can be explained by the rapid metabolic destruction of carbamates[14]; however, recurring penetration of toxicant will finally kill the insects.

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Metabolically Regulated Cyclical Contractures in Microinjected *Spirostomum:* a Pharmacological Study

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Summary. Spirostomum was treated extracellularly and intracellularly with a range of metabolites to investigate the intracellular regulation of cyclic calcium movements. The results indicate close links between calcium movements and mitochondrial metabolism.

Externally applied pharmacodynamic stimuli may trigger cyclic myonemal contractures in the heterotrich ciliate *Spirostomum*[2]. Studies with detergent-extracted cell models[3], intracellular aequorin luminescence[4] and CaEGTA/EGTA buffer microinjection[5] concur that stimulus-contracture coupling is through an increase in the cytoplasmic free Ca^{2+} concentration. Although contraction and re-extension of extracted cells does not require exogenous metabolites[3], and so resembles other myonemal contractile mechanisms[6], the role of nucleotide phosphate in vivo remains unclear[7]. Because Ca^{2+} to trigger contracture is released from intracellular stores, not derived extracellularly[7], and because the release is not mediated by cell surface membrane depolarization[5,8],

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Spirostomum is well suited to the study of nonelectrogenic pharmacodynamic coupling through intracellular Ca^{2+} release. In this study we have microinjected normal cell metabolites intracellularly into *Spirostomum* to investigate interactions between metabolism and intracellular Ca^{2+} movements.

Materials and methods. *Spirostomum ambiguum* were cultured at 20 °C in 2 m*M* KCl, 0.5 m*M* NaCl and 0.5 m*M* $CaCl_2$ in distilled water. Wheat grains were added to support the bacterial flora on which *Spirostomum* feeds. The response of *Spirostomum* to extracellularly administered compounds is very variable, even within cells of the same clone. We obtain repeatable results when cells are subcultured every 2 days and then left overnight in fresh medium without food organisms. It is not known whether injected cells are similarly variable: all cells used in the present study were cultured as above.

The apparatus for microinjection has been described before[9]. Glass micropipettes with tip diameters 3–6 μm were used throughout. Cells were immobilized for microinjection with 4% gelatin in culture medium, which restricted locomotion while permitting contractures to be observed. All compounds were dissolved in culture medium and neutralized. All solutions were membrane filtered before use. The volume microinjected could not be controlled precisely but was in the range 5–10% of cell volume: the active intracellular concentration may therefore have been 10–20 times less than the concentration injected. Artifactual contractures associated either with cell damage or mechanical stimulation during manipulation were readily distinguished and discounted. Provided that dessication of the preparation was avoided, successfully injected cells still showed ciliary activity and myonemal contractures in reponse to mechanical stimulation at least 30 min post-injection.

Results and discussion. The aim of this study was to identify compounds active in eliciting cyclic myonemal contractures from *Spirostomum*. After SLEIGH[2], we have adopted contracture frequency as a measure of efficacy, expressing the results as the number of contractures during the first minute post-injection. This was usually also the total number of contractures. To define an active compound, the frequencies obtained experimentally, many of which are listed in the Table, were pooled. The frequency distribution obtained was clearly bimodal with modes at 0.4 and 4.1 contractures min^{-1}. We have therefore operationally defined active compounds as those triggering more than 3.0 contractures min^{-1}, inactive compounds as those triggering less than 1.5 contractures min^{-1}. Compounds active extracellularly were defined as those eliciting more than 20 contractures in the first 10 min application, inactive compounds elicit less than 5 contractures during the same interval.

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The results of microinjection into *Spirostomum*

Active	Inactive
Oxidized gluthathione 4.3 (26)	Cysteine-glutathione 0.61 (20)
$+10^{-2}$ *M* amytal 4.1 (18)	Reduced glutathione 0.38 (20)[d]
$+10^{-5}$ *M* atractylate 4.6 (16)	Pyruvate 0.28 (25)
α Ketoglutarate 3.9 (20)	Acetoacetate 0.45 (16)
$+10^{-2}$ *M* amytal 4.4 (20)	Oxalacetate 0.29 (17)
L-glutamate 4.1 (17)	α-Glycerophosphate 0.39 (24)
$+10^{-5}$ *M* avenceolide 4.0 (20)	Isocitrate 0.60 (20)
$+10^{-2}$ *M* *methyl* glutamate 4.4 (20)	Malate 0.76 (20)
$+10^{-2}$ *M* amytal 3.7 (20)	Malonate 0.31 (18)
Succinate 4.0 (18)	L-glutamine 0.22 (20)
$+10^{-5}$ *M* oligomycin 4.5 (15)	Dihydroxyacetonephosphate 0.80 (20)
$+10^{-5}$ *M* atractylate 4.1 (25)	Coenzyme A 0.46 (21)
$+10^{-2}$ *M* amytal 0.3 (20)	Palmitoyl CoA 0.50 (20)
$+10^{-3}$ *M* KCN 4.4 (30)[c]	10^{-2} *M* Methyl glutamate 0.44 (20)
β-Hydroxybutyrate 3.9 (25)	10^{-2} *M* Amytal 0.16 (20)
$+10^{-2}$ *M* amytal 4.1 (30)	10^{-5} *M* Bongkrekic acid 0.4 (15)
Phosphoenolpyruvate 4.7 (20)	10^{-5} *M* Avenceolide 0.6 (20)
$+10^{-5}$ *M* atractyloside 0.66 (20)	10^{-5} *M* Oligomycin 0.15 (18)
Adenosine 5′-diphosphate 5.5 (30)	10^{-5} *M* Atractyloside 0.24 (16)
$+10^{-5}$ *M* oligomycin 4.5 (20)	3×10^{-4} *M* ruthenium red 0.47 (24)
$+10^{-2}$ *M* amytal 5.5 (21)	KCN 0.46 (20)[c]
$+10^{-5}$ *M* bongkrekic acid 0.61 (18)	10^{-2} *M* NAD 0.18 (20)
$+10^{-5}$ *M* atractyloside 0.71 (26)	10^{-2} *M* NADH 0.31 (20)
Guanosine 5′-diphosphate 3.2 (20)	10^{-2} *M* NADP 0.36 (20)
10^{-4} *M* Phenazine methosulphate 4.2 (20) [a,b]	10^{-2} *M* NADPH 0.22 (20)
$+10^{-2}$ *M* amytal 0.31 (20)	Adenosine 5′-triphosphate 0.88 (16)
10^{-6} *M* Menadione 4.7 (20)	Guanosine 5′-triphosphate 0.72 (20)
$+10^{-2}$ *M* amytal 0.80 (20)	Inosine 5′-diphosphate 0.55 (15)
	Adenosine monophosphate 0.90 (15)
	Adenosine-3′, 5′-cyclic monophosphate 1.05 (20)
	culture medium 0.13 (23)

Compounds are classified as either active or inactive as described in the text. Activities are expressed as the mean number of contractures elicited in the first minute post-injection. The sample size in each case is given in parenthesis. Unless stated, the concentration microinjected was 10^{-3} *M*.

[a] Active extracellularly: all others were active only when microinjected.

[b] Gave a single irreversible contracture at 10^{-3} *M*.

[c] Microinjected after 5–10 min in gelatin plus 10^{-3} *M* KCN.

[d] Microinjected together with 10 m*M* sodium borhydride.

When the results of extracellular and intracellular application are compared, each compound can be placed into one of three general classes, active both extracellularly, and when microinjected, active only when microinjected and always inactive. No compound active extracellularly was inactive when microinjected. We therefore find no evidence of a cell surface mediated coupling mechanism, and in 8 of 10 cases action must be intracellular. In those cases where activity was both extracellular and intracellular, drug action at the cell surface cannot be excluded because the injected compound may only act after diffusing out from the cell.

To identify more precisely the intracellular sites of drug action, metabolic inhibitors were injected along with active materials. A cautionary note should be sounded about the interpretation of such results. For some inhibitors, we have demonstrated both positive and negative controls, for example amytal blocks succinate but not glutamate activity. Although kinetic differences could underly such distinctions, for example glutamate may act faster than succinate and so avoid inhibition, it is more plausible that such results reflect underlying differences in sites of action between active compounds, and we have interpreted them accordingly. Other inhibitors, for instance avenceolide and KCN, gave only negative results. Even though such results may be significant, numerous explanations are always possible and great caution must be exercised in their interpretation.

A wide range of cell metabolites were microinjected but only eight triggered cyclicity: adenosine 5′-diphosphate (ADP), guanosine 5′-diphosphate (GDP), phosphoenolpyruvate (PEP), succinate, L-glutamate, β-hydroxybutyrate, α-ketoglutarate and oxidized glutathione.

Of the nucleotide phosphates tested, only ADP and GDP proved active. When ADP was studied in more detail, it was found that activity was abolished by the inclusion of either 10^{-5} *M* atractyloside or 10^{-5} *M* bongkrekic acid. Neither inhibitor blocked oxidized glutathione or succinate activity. Both atractylate and bongkrekate are very specific inhibitors of mitochondrial adenine nucleotide translocase[10], so it seems that ADP acts intramitochondrially. It is interesting that PEP activity is likewise sensitive to atractylate and presumably therefore also acts intramitochondrially because in vitro PEP can discharge stored Ca^{2+} from mitochondria[11]. In vitro it appears that it is the PEP-adenine nucleotide exchange (possibly PEP-adenosine 5′-triphosphate) which releases Ca^{2+} rather than PEP uptake per se[12]. This being the case, it is plausible that a sudden rise in cytosolic ADP or GDP might have a similar transient effect. It must be recalled, however, that ADP application does not release Ca^{2+} from mitochondria in vitro.

Succinate is also apparently active intramitochondrially because its action is amytal sensitive, implying the involvement of reverse electron flow through mitochondrial complex 1[13]. Succinate activity was not abolished by KCN, but neither has cyanide been shown to act positively in this system. However, because the extracellular application of 10^{-4} *M* KCN results in loss of ciliary beating and slow reversion to the contracted morphology[14], the continued succinate activity in the presence of both extracellular and microinjected KCN may be significant. Again it should be recalled that steady state succinate has not been shown to potentiate mitochondrial Ca^{2+} release in vitro.

These results suggest that cyclic myonemal contractures and thus cyclic Ca^{2+} movements may be closely linked to mitochondrial metabolism. This is fully supported by the activities of α-ketoglutarate and β-hydroxybutyrate, especially as the only known metabolism of β-hydroxybutyrate is intramitochondrial. To assign sites of action to the other two metabolites, L-glutamate and oxidized glutathione, is more difficult.

To test the possibility that glutamate acts intramitochondrially, 2 inhibitors of mitochondrial glutamate transport, γ-methyl glutamate[15] and avenceolide[16], were injected. Neither blocked glutamate cyclicity which implies, taken at face value, that glutamate acts cytosolically. However, as these inhibitors have no positive controls, it may simply be that both are inactive or inefficient here. Cytosolic glutamate action need not preclude mitochondrial involvement; action could be through α-ketoglutarate synthesis.

Oxidized glutathione is likewise equivocal. Enzymic action is suggested because the slowly metabolized analogue cysteine-glutathione is inactive. Oxidized glutathione may be reduced both cytosolically and intramitochondrially. When microinjected, neither NADP nor reduced glutathione is active. Therefore we suggest either that oxidized glutathione acts to deplete cytosolic NADPH or that it acts intramitochondrially. In the latter case, the glutathione dehydrogenase reaction products would be ineffective when microinjected because mitochondria are permeable to neither.

In addition to normal metabolites that are active, 2 active non-metabolites are reported here, phenazine methosulphate and menadione, both well-known as electron acceptors from complex 1. Both actions are amytal sensitive, again pointing to mitochondrial involvement.

The links described between mitochondrial metabolism and cyclic movements of intracellular Ca^{2+} in *Spirostomum* make it plausible that mitochondrial Ca^{2+} storage is actively involved; but no link has been explicitly demonstrated. Ruthenium red, commonly used to block mitochondrial Ca^{2+} movements, does not induce cyclicity but at 10^{-3} *M* it produces irreversible cell contracture. The results are all compatible with drug actions via complex 1, used in the widest sense to include mitochondrial phosphate potential, NADPH oxidation and transhydrogenase activity[13]. The ways in which complex 1 might modulate mitochondrial Ca^{2+} handling are presently under investigation.

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