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A KYMOGRAPHIC STUDY OF THE ACTION OF DRUGS ON THE LIVER FLUKE (*FASCIOLA HEPATICA*)

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The difficulty of culturing parasitic worms *in vitro* remains the major limiting factor in the development of methods for studying anthelmintic* activity directly on the parasite. This is because of the difficulty of maintaining *in vitro* a suitable environment for the different parasites. This has been achieved in a partial sense by Chu (1940) for *Chlonorchis sinensis* and *Schistosoma japonicum*, by Lamson and Brown (1936) for *Ascaris*, and by Smyth (1948) for plerocercoid larvae of *Schistocephalus solidus* and *Ligula intestinalis*, but for many other parasites methods for obtaining the same conditions do not yet exist. In their search for material which might be likely to provide alternative means, the early workers neglected the phylogenetic relationships of the different parasitic worms. Two main groups of helminth parasites exist: the nemathelminthes and the platyhelminthes.

Owing to the work of Robello and Rico (1926) and Baldwin (1943), a method exists for the *in vitro* study of anthelmintic activity on nematodes. This method, moreover, has enabled a start to be made on the mode of action of drugs on nematodes. Von Schroeder (1885) and Trendelenberg (1916) both reported that santonin, which has a high therapeutic reputation as an anthelmintic against nematodes, appears to have no effect on the activity of the whole worm, when it is tested by observing the activity of whole ascaris *in vitro*. Baldwin, however, has suggested it may bring about incoordination of movement by virtue of a simultaneous stimulation of the body musculature (intermediate preparation) and depression of central nervous control (anterior preparation). Many *a priori* reasons exist for considering the effect of substances on the movement of the parasite to be a primary mode of action. Thus we have chosen to study the effect of drugs on the liver fluke as a representative platyhelminth worm by a kymographic technique of short duration. The present paper is, therefore, concerned with a method for studying the mode of action of drugs on trematodes, and the value of the liver fluke (*Fasciola hepatica*), as a preparation for screening substances for possible anthelmintic activity against the parasitic members of both phyla, is assessed.

Collection of parasites

The flukes were obtained from the bile ducts of bovine livers and were dissected out within half an hour of the death of the host before the liver cooled to room temperature. They were washed and subsequently placed in boiling-tubes containing Ringer's solution buffered (to *p*H 8.5) at 37° C., not more than two flukes to each tube. This was found necessary in order to prevent the flukes from attacking each other, which they did when large numbers were enclosed together. Preparations were made from these flukes within six hours of collection. Satisfactory flukes always showed rippling movements of the whole body, and movement by means of the suckers, for at least 24 hours.

Methods of recording movement

Kymograph tracings were made of the movements of a fluke suspended in Ringer's solution at 37° C., the fluke being under slight tension. The attachment for the recording arm and fixed point were by means of platinum hooks passed through the body wall posterior to the ventral sucker and close to the

* The term anthelmintic is used by us to indicate that the drug has been found effective for the cure of infestations of helminth parasites either in veterinary or clinical practice.

posterior end of the body. It was found essential to keep the fluke below the surface of the Ringer's solution while these attachments were being made in the shortest possible time. The fluke was then allowed 10 minutes to recover before recording was started. after which normal movements were recorded for 15 minutes.

Method of testing drugs

Drugs soluble in water were dissolved in Ringer's solution ; those which were precipitated in this medium were separately dissolved in water to which the saline constituents were then added as this delayed precipitation for long enough to make the test (santonin, kamala, umbelliferone, and coumarine). Insoluble drugs were emulsified by the method of Baldwin. The substances were tested initially at a high concentration (1 : 1,000) and, if active, at lower concentrations suggested by the response obtained. Finally the minimal effective concentration, as judged in most instances by the disappearance of activity within 45 min. after the addition of the drug, was obtained with at least four flukes. The absence of effect at 1 : 1,000 on at least two preparations was taken to indicate that the drug was without activity.

TABLE I

STIMULANTS

Drug	Nature of preparation	Effective concentration
Carbon tetrachloride	Solution	1 : 2,000–1 : 4,000*
Tetrachlorethylene	Emulsion	1 : 2,000–1 : 5,000*
Hexachlorethane ..	,,	1 : 20,000–1 : 5,000*
β-Naphthol ..	Solution	1 : 10,000–1 : 5,000*
p-Cymene ..	Emulsion	1 : 4,000*
Strychnine HCl ..	Solution	1 : 10,000
Coumarine ..	,,	1 : 2,000
Umbelliferone ..	,,	1 : 2,000
β-Phenylethylamine HCl	,,	1 : 5,000
Ephedrine HCl ..	,,	1 : 2,000
Tyramine HCl ..	,,	1 : 1,000
Amphetamine sulphate	,,	1 : 20,000–1 : 10,000
l-Amphetamine sulphate	,,	1 : 10,000
d-Amphetamine sulphate	,,	1 : 80,000

* Indicates that the drug possesses other types of action at higher concentrations or after acting for longer periods of time.

RESULTS

Rhythmical activity was found in a high proportion of worms taken from bovine livers, but in general worms from sheep's livers could not be relied upon to give good rhythmical activity. Rhythmical activity, which was occasionally interrupted by short quiescent periods, showed a marked variation in amplitude from worm to worm, but, after the establishment of the rhythm in any one preparation, the amplitude and the tone remained approximately constant for at least 2 hours (Fig. 1).

Three types of drug activity have been found: stimulant, paralysant, and lethal.

Stimulant drugs

On addition of the substances with stimulant properties listed in Table I, some interference with the activity of the preparation occurred, and subsequently more rapid rhythmical movements were resumed with or without a change in amplitude and tone. There was also a marked difference in the duration of these effects. The two lactones, coumarine and umbelliferone, produced rhythmical movements of large amplitude and low frequency at low concentrations. These movements, however, gradually diminished within a period of half an hour, and were replaced by sustained contraction when high concentrations were tested. The stimulation produced by strychnine hydrochloride was characterized by increase in the frequency, reduction in the amplitude, and increase in the tone. The four amines all produced an increase in the amplitude and frequency after the initial contraction had subsided and produced some increase in the tone.

The chlorinated hydrocarbons with known anthelmintic potency and therapeutic value against

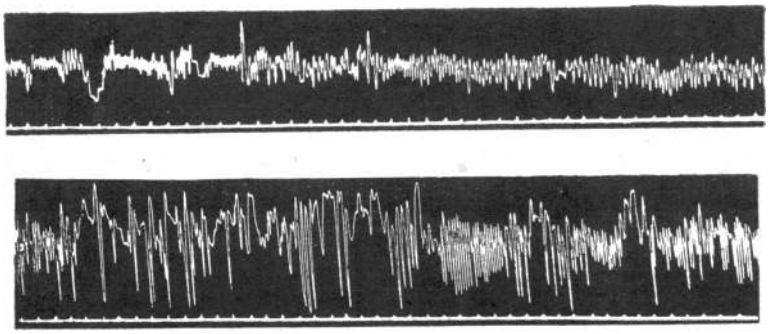

FIG. 1.—Two representative records of normal movement. (In all tracings upward stroke represents contraction. Time markings in minutes.)

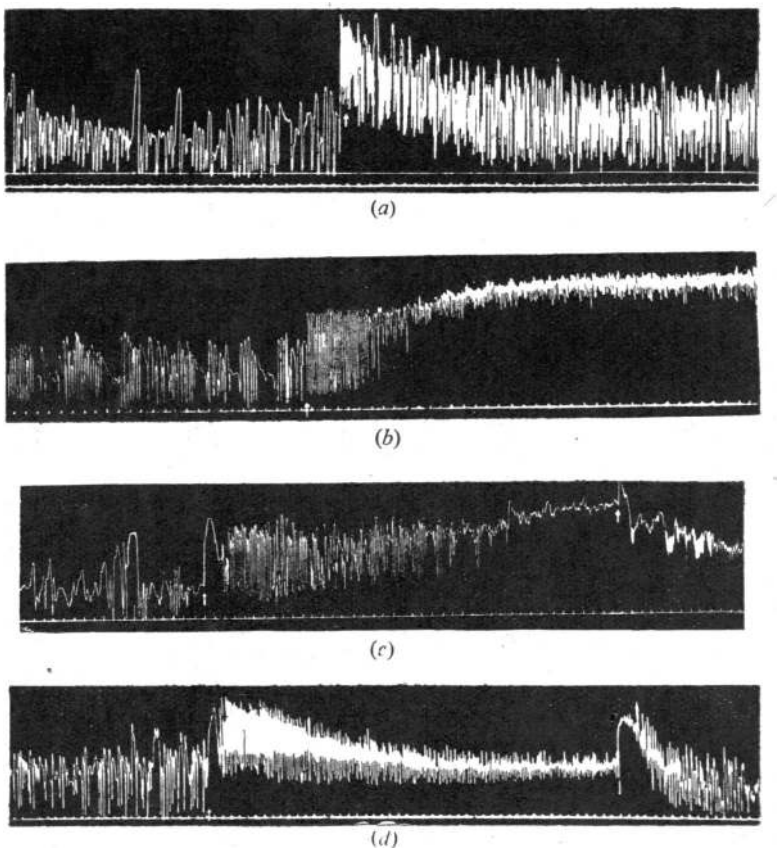

FIG. 2.—Stimulant drugs: Action on normal movement of: (*a*) amphetamine (1 : 1,000); (*b*) tetrachlorethylene (1 : 5,000); (*c*) hexachlorethane (1 : 20,000) followed by amphetamine; (*d*) coumarine (1 : 1,000) followed by amphetamine.

infestations of the liver fluke all possess stimulant properties at low concentrations. This property was exhibited to a more marked degree by hexachlorethane (Fig. 2).

From the records it will be seen that the amines have a pronounced potentiating action on the normal rhythm, increasing both the amplitude and the frequency with only slight effect on the tone of the muscle. Moreover, amphetamine was the most powerful of these potentiating amines.

Paralysant drugs

Early on in the investigation it appeared likely that amphetamine might be used to bring back

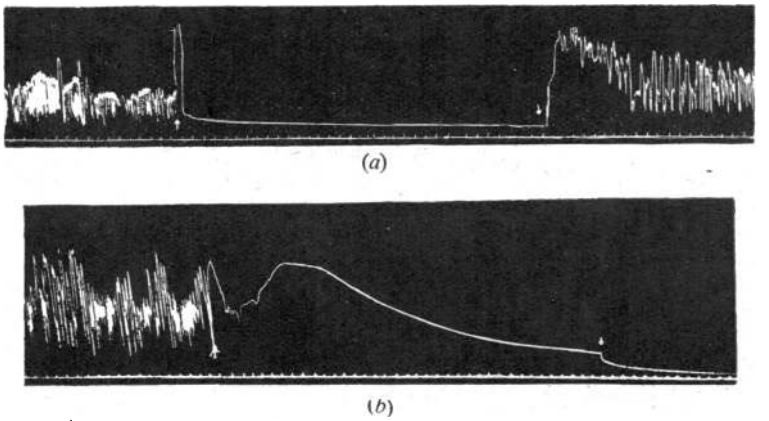

FIG. 3.—(*a*) Paralysant action of pelletierine tannate (1 : 1,000) followed by response to amphetamine. (*b*) Lethal action of hexylresorcinol (1 : 5,000) followed by absence of response to amphetamine.

TABLE II

DRUGS WHICH PARALYSE THE PREPARATION BUT RHYTHMICAL CONTRACTION IS RESTORED BY AMPHETAMINE 1 : 5,000

Drug	Nature of preparation	Effective concentration
Nicotine	Solution	1 : 200,000
Carbaminoylcholine chloride (Doryl) ..	,,	1 : 20,000
Sodium amytal ..	,,	1 : 5,000
Arecoline HBr ..	,,	1 : 10,000,000
Oil of chenopodium	Emulsion	1 : 20,000–10,000*
Pelletierine tannate	Solution	1 : 2,000
Phenylurethane ..	Emulsion	1 : 2,000*
Santonin	,,	1 : 1,000
Kamala	Filtered saturated solution	*

* Indicates that the drug possesses other types of action at higher concentrations or after acting for longer periods of time.

TABLE III

DRUGS WHICH PARALYSE THE PREPARATION AND RENDER IT INSENSITIVE TO THE ACTION OF AMPHETAMINE 1 : 5,000

Drug	Nature of preparation	Effective concentration
Oil of chenopodium	Emulsion	1 : 5,000
p-Cymene ..	,,	1 : 1,000
Thymol	Solution	1 : 10,000
Hexylresorcinol ..	,,	1 : 10,000–1 : 5,000
β-Naphthol ..	,,	1 : 4,000–1 : 2,000
Carbon tetrachloride	Emulsion	1 : 1,000
Tetrachlorethylene	,,	1 : 1,000
Hexachlorethane	,,	1 : 1,000 (see text)
Ext. filix mas. ..	,,	1 : 5,000
Gentian violet ..	Solution	1 : 5,000
Phenylurethane ..	,,	1 : 1,000
Chlorbutol ..	,,	1 : 1,000
Kamala	Sat. solution	

normal rhythmical activity to preparations when the movement had been reversibly altered by a drug ; this might occur after inactivation by paralysant drugs or when the type of movement was markedly altered by other stimulant drugs. No such action would be expected after an effect which was to any extent irreversible, or after lethal drugs. It was therefore decided to add amphetamine sulphate in a concentration of 1 : 5,000 to the bath in the absence of the test substance, whenever the activity of the preparation had been obliterated or altered at the end of 45 min. This was done by replacing the solution of the test substance by Ringer's solution containing 1 : 5,000 amphetamine sulphate. By this means it was found possible to distinguish between drugs with paralysant action (listed in Table II, Fig. 3) and drugs with a lethal or probably lethal action (listed in Table III, Fig. 3). A large number of miscellaneous substances with no known anthelmintic action were tested and found to have no more than a transient action (Table IV, Fig. 4). The fluke musculature is relaxed to different degrees by parasympathomimetic drugs, except pilocarpine, and this relaxation is antagonized by amphetamine.

TABLE IV

DRUGS WHICH FAILED TO CAUSE COMPLETE PARALYSIS OF THE PREPARATION UP TO A CONCENTRATION OF 1 : 1,000 (MINIMUM 45 MIN.)

Phenothiazine	*d*-Tubocurarine chloride
Sodium tauroglycocholate	Yohimbine HCl
Ethyl alcohol (1%)	Atropine sulphate
Sulphanilamide	Hyoscine HBr
Sulphathiazole	Histamine acid phosphate
Penicillin (1,340 i.u./c.c.)	Cocaine HCl
Neosalvarsan	Quinine sulphate
Emetine HCl	Morphine HCl
Acetylcholine chloride	Caffeine
Acetyl-β-methylcholine chloride*	Guanidine HCl
Pilocarpine nitrate	Sodium bromide
Eserine sulphate*	Phenyl urea

* Transient depression

Lethal drugs

The chlorinated hydrocarbons which in low concentrations stimulate the fluke are lethal at concentrations ranging between 1 : 5,000 and 1 : 1,000. Hexachlorethane has exceptional properties which make it difficult to obtain known concentrations of it above 1 : 5,000 ; it is insoluble in water and sublimes below the boiling point of water. Thus, although sufficient substance was added to make an emulsion at 1 : 1,000 with alcohol some of the substance was precipitated. The emulsion made in this way, however, obliterated all movement in 90 min., after which amphetamine was without effect. The 1 : 5,000 solution

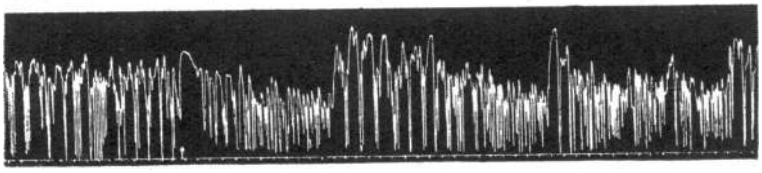

FIG. 4.—Absence of effect of morphine sulphate (1 : 1,000).

almost completely eliminated the activity, and correspondingly its action was only slightly affected by amphetamine. It would therefore appear to be a lethal drug in low concentrations, possibly with greater effect than the other chlorinated hydrocarbons. Ext. filix mas. is lethal at the same concentration in a shorter time. This is also true of gentian violet.

Ineffective substances

Phenothiazine was the only inactive anthelmintic with the exception of drugs active on blood flukes.

Discussion

The value of any particular approach to the study of anthelmintics can best be understood when it is realized that the host-parasite relationship is a special instance of the organism-environment relationship which forms the background to all biological studies. Veterinary practice possesses a variety of methods for combating parasitic infestations which act in one of four ways on the biological system comprising the host-parasite relationship. The first is by alterations in the relationship of the host to its environment; the second is by effecting changes in the internal environment of the host; the third is by affecting the relationship of the parasite to the internal environment of the host; and the fourth is by altering the internal environment of the parasite itself. The pharmacology of anthelmintics should be concerned with all four divisions, but in practice the first division is usually considered a separate field of study in the domain of ecology. Within the three remaining fields which are concerned with the administration of substances to infested animals or man the study of anthelmintics has been approached either as a chemotherapeutic problem or as if it were a pharmacological study on isolated organ systems of vertebrates. In fact both aspects must be integrated to provide a satisfactory approach to the problem. Chemotherapeutic tests are primarily effective for the detection of substances of therapeutic value, because micro-organisms are primarily dependent on a continuous process of reproduction for their distribution and for the location of the reproductive phase of their life cycle. Substances, therefore, which interfere with metabolic processes by whatever means are effective in reducing the distribution and influencing the location of micro-organisms. In parasitic worms, however, the distribution and the location of the adult reproductive worm are differentiated functions and depend on the separate physiological processes of reproduction and movement. Distribution is largely dependent on reproduction though movement plays some part in it, but the location of the adult is almost wholly dependent on movement which appears to be influenced by environmental factors. Elimination of the parasite, therefore, is dependent upon the drug affecting either the mechanism by which location is achieved and maintained or by suppressing reproduction. It is at present impossible to study the effect of drugs on reproduction *in vitro* because of difficulties of culturing them, but certain pharmacological techniques have been applied to the study of movement in parasitic worms, as in the present paper.

We require to know the effect of any drug on the components of the fluke's movement and on the co-ordination of these in its behaviour. Provided that the same effects are produced *in vivo* as *in vitro* we can then hope to get some idea of the possible modes of action of anthelmintics which affect movement. At present, we have only taken a step in this direction. The kymographic technique, which we have used, has enabled us to distinguish three different types of action affecting movement from which a classification of drugs can be made.

Some drugs are stimulants as defined earlier; these are the amines, the lactones, and strychnine. Other drugs are stimulants at low and lethal at higher concentrations—namely, the halogenated hydrocarbons, *p*-cymene and β-naphthol. This combined action may well be the reason why the halogenated hydrocarbons are effective on the liver fluke itself *in vivo*, whereas gentian violet, which *in vitro* is more lethal but has no stimulant action, is ineffective.*

Tetrachlorethylene has been shown by Rogers (1944) to have a stimulant effect on *Nippostrongylus muris*; this appears to be an exaggeration of normal movement since it caused the parasite to leave the mucous lining of the intestine. Halogenated hydrocarbons, therefore, as a group are likely to be stimulant to both phyla. Baldwin's (1943) technique using segments of *Ascaris* would appear to be inadequate, therefore, to reveal all types of stimulant action probably because his preparations are only part of the whole animal.

Finally there are drugs with paralysant action at low concentrations which become lethal as the concentration is raised—e.g., phenylurethane and oil of chenopodium.

Because major systematic differences such as those between phyla are most likely to be associated

*Private information from Dr. E. L. Taylor.

with major biochemical differences it is to be expected that comparison between members of the two differential phyla will reveal the presence or absence of selective* action within the helminths when tested *in vitro*. It is therefore worth while comparing our results with those of Baldwin.

As might be expected, the liver fluke is killed by all the protoplasmic poisons which affect *Ascaris*. Moreover, such comparison demonstrates that the liver fluke is affected by lower concentrations of the drugs which are lethal to both preparations. The liver fluke is also affected by some drugs which are not effective on *Ascaris*—i.e., the fluke preparation is sensitive to a wider range of known anthelmintic drugs than the *Ascaris* preparation. It should here be noted that Baldwin's preparation is insensitive to the action of gentian violet, which is active on threadworms *in vivo*. This may be due to an *in vitro* selective action of gentian violet on threadworms, but it might equally well be due to the fact that Baldwin's preparation does not allow direct access of the substance to the gut of the *Ascaris* preparation. The evidence available suggests that the cuticle is a more effective, and probably also a more selective, barrier to the penetration of drugs in *Ascaris* than in the liver fluke. Comparative tests on flukes with intact cuticles, and cuticles pierced at the point of attachment, reveal that the intact fluke is only slightly more resistant to all the drugs which we have tested. On the other hand some drugs are without effect on *Ascaris* because of fundamental differences in the neuromuscular mechanism. An explanation of the different responses to pelletierine and arecoline should be sought in one or both of these mechanisms. By contrast, those drugs which are effective for the elimination of cestodes from the intestine *in vivo* are also active on the liver fluke *in vitro*; a result which is consistent with the systematic relationships of the two parasites. The paralysant action of arecoline, which relaxes the muscle, parallels the results obtained by Betham (1946) on segments of *Taenia*.

The kymographic test of Betham does not allow any strict comparison to be made between the action of drugs on trematodes and cestodes, owing to the restricted use to which it was put. The only drug used as an anthelmintic with selective action directly on nematodes is santonin. Pelletierine and ext. filix mas., because they are active only on the fluke *in vitro* but not on *Ascaris* and because they are used exclusively against cestodes in the intestine, are likely to prove selective for platyhelminthes. Gentian violet, on the other hand, although it does not affect Baldwin's preparation, does eliminate nematodes (threadworms) from the intestine and is therefore almost certainly active on both phyla. In this connexion it should be noted that, of the two lactones which are stimulant to the fluke, coumarine paralyses *Ascaris* (posterior segment) and umbelliferone is without effect. This is also true of strychnine. The mode of action of phenothiazine and drugs active on blood flukes is still obscure.

Stephenson (1947) measured the effect of carbon tetrachloride, tetrachlorethylene, and gentian violet on the survival time of the liver fluke *in vitro* and was unable to detect any action of the chlorinated hydrocarbons, but obtained some effect with gentian violet, thus demonstrating that his method was less sensitive than the one under discussion. We attribute this to the difference in viability of the preparation in the two types of test. In an acute test, using as a measure of activity the effect on movement, the viability of the preparation is assured. This is not so in a test based on survival time, as the viability of the preparation falls off rapidly towards the end of the experimental period when the preparation is required to be most sensitive to differences in activity of the drugs under test. Moreover, the method led to paradoxical results in the hands of Chu (1940), who showed that *Chlonorchis sinensis* survived longer in higher concentrations of certain drugs (e.g., methylene green and trypan blue) than at lower concentrations; that in effect there was a reverse relationship between dose and effect which was attributed to a reduction in oxidation rate preventing the parasite *in vitro* from poisoning itself with its own excreta.

The comparisons we have made suggest that the majority of drugs used as anthelmintics are protoplasmic poisons acting on both phyla and also on the host. The apparent selectivity in the anthelmintic activity of the protoplasmic poisons *in vivo* does not therefore reflect a selective action on different parasites but rather the importance of different biochemical and physiological conditions in the immediate neighbourhood of the parasite affecting the action of these poisons. This emphasizes the need for tests capable of distinguishing the selective action of drugs *in vitro*, if safer anthelmintics are to be found. This does not mean that we require to test drugs on isolated members of each species but that we require to assess the significance of systematic differences revealed by tests on representative members of each phyla.

* The word selective is used by us to indicate that drugs act exclusively on a few parasites. It is qualified by the adverbs *in vitro* and *in vivo*. (Specificity should be reserved for testing the action of drugs on different parts of an organism. In this way its use in this field is consistent with the rest of pharmacology.)

SUMMARY

1. It is possible to obtain rhythmical kymographic records from fresh bovine flukes suspended in Ringer's solution at a *p*H range from 6.5–8.5. These movements are maintained for a period of at least 2 hours and frequently as long as 6 hours.

2. The effect of known anthelmintics and of a number of other drugs, particularly those affecting the neuromuscular mechanism of vertebrates, have been tested on this preparation by allowing them to act for a maximum period of 45 min.

3. At the end of this period the addition of amphetamine restores rhythmical activity provided the drug has had only a paralysant action on the fluke. In this way it has been possible to distinguish between stimulant, paralysant, and lethal drugs.

4. It is suggested that the possible reason why the chlorinated hydrocarbons are so effective as anthelmintics against *Fasciola hepatica* is that they combine the stimulant action at low with the lethal at higher concentrations. A number of other anthelmintics have been shown to have other types of combined action.

5. Comparison with the similar test on *Ascaris* segments (Baldwin, 1943) reveals that the liver fluke as a representative of the platyhelminthes is sensitive to all the drugs which affect *Ascaris* and in addition to umbelliferone, pelletierine, extract filix mas., and gentian violet. The significance of this comparison is discussed.

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