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STUDIES ON THE MODE OF ACTION OF METHYRIDINE

BY

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Methyridine [2-(β-methoxyethyl)pyridine] reached the blood and all regions of the alimentary canal following oral or subcutaneous administration of therapeutic doses of the drug. Concentrations in the lower gut were highest following subcutaneous administration, and there was a close association between blood and intestinal levels suggesting that the drug is excreted into the alimentary canal along its whole length. *In vitro* experiments with intact nematodes (*Ostertagia spp.* from sheep; *Nematospiroides dubius* and *Heterakis spumosa* from mice; and *Nippostrongylus muris* from rats) and with preparations of *Ascaris sp.* have demonstrated that methyridine passes through the cuticle and exerts an "irreversible" paralysing effect on the worms. This action is obtained within 1 hr at concentrations similar to peak levels shown to be present in the alimentary canal following the administration of therapeutic doses. Concentrations of the drug were found to be higher in the abomasum than in the intestine of sheep and yet the anthelmintic activity of methyridine is greatest against intestinal nematodes. No significant differences in the resistance of various nematode species to the drug were demonstrated, but the amount of methyridine entering ascarids was significantly greater at *p*H 8 than at *p*H 5 or 3. In addition, the *in vitro* anthelmintic activity of the compound was greatest in alkaline solutions, which probably accounts for the differences in anthelmintic activity noted in various parts of the alimentary canal. The paralysing effect of methyridine on nematodes is probably due to a neuromuscular block of the decamethonium type, and this can also be produced in the host by over-dosage. It is suggested that nematode tissues are more sensitive to this action than vertebrate tissues.

Experiments in laboratory animals (Broome & Greenhalgh, 1961a and b) and farm animals (Walley, 1961) have demonstrated considerable anthelmintic activity against nematode infections of the gastro-intestinal tract following treatment with the compound methyridine [2-(β-methoxyethyl)pyridine; "Promintic"]. The same reports indicate a number of features which make the drug unique among known anthelmintics. In particular, it is a liquid which is extremely water-soluble and which is most effective when administered subcutaneously. These peculiarities clearly merited this study of its mode of action. In the work described below estimations have been made of the drug concentration in various parts of the body and of the effect of the drug *in vitro* against various nematodes. The results are then correlated in an attempt to show how the *in vivo* anthelmintic activity is achieved.

METHODS

Chemical estimation of methyridine in tissues. All samples were collected and processed for estimation immediately after sacrificing the animals. They were then stored at +5° C until estimations could be made, and these were always completed within seven days of collection. Experiments showed that deterioration was not significant during this storage period.

One ml. samples (blood, gut contents, tissue homogenates, urine, etc.) were diluted with 1 ml. distilled water before precipitating the proteins with 2 ml. 0.3 N barium hydroxide and 2 ml. 5% zinc sulphate. They were then centrifuged at 3,000 rev./min for 5 min and the supernatant was removed. The precipitate was resuspended and washed with 2 ml. distilled water and a second extraction was performed. The two extracts were mixed, filtered through Whatman No. 40 filter paper, and made alkaline with 0.2 ml. 40% sodium hydroxide. Methyridine was obtained from this crude alkaline extract by evaporation *in vacuo* and collected by sublimation on a cold finger (cooled with solid carbon dioxide and acetone). I am indebted to Dr G. A. Snow for suggesting this method of extraction.

After complete evaporation the sublimate was melted from the cold finger, filtered and made acid with 0.2 ml. concentrated hydrochloric acid before measuring the optical density at 260 mμ with a Beckman spectrophotometer using 1 cm quartz cells. The methyridine concentration was then estimated from standard curves previously prepared by extracting graded amounts of the drug from the material in question.

All determinations were carried out in duplicate, and statistical analysis of the results showed the standard error between individual determinations to be ±10 to 15%. The standard error of the average of duplicate determinations is therefore ±7 to 10%. To this must be added errors due to animal variation before interpreting the results.

The analytical method estimates any pyridine compound which volatilizes under alkaline conditions and it is therefore not specific to methyridine *per se.* Studies by Duncan & Scales (1961) indicate that the only methyridine metabolite likely to be estimated by the method is α-picoline. However, extractions from samples containing added α-picoline have shown this compound to be so volatile that little is collected on the cold finger. It is therefore felt that the results provide a reliable indication of methyridine concentration.

Blood and gut concentrations of methyridine in mice. Groups of 20 mice were dosed with the optimum therapeutic dose of methyridine 400 mg/kg orally and subcutaneously (Broome & Greenhalgh, 1961b). After the appropriate time interval all animals were sacrificed and their blood was collected together with material from various regions of the alimentary canal. The samples were pooled to provide sufficient material for the chemical estimations of drug concentration.

Blood and gut concentrations of methyridine in sheep. Blood levels of the drug were followed in groups of two sheep, bled from the jugular vein at hourly intervals after dosing with either 150 or 200 mg/kg given subcutaneously or orally. Blood and gut levels were also determined in groups of two sheep, given the optimum therapeutic dose of 200 mg/kg subcutaneously (Walley, 1961) and sacrificed after the appropriate time interval. Finally, a comparison between blood and gut drug concentrations following oral and subcutaneous administration of 150 mg/kg was made in two sheep.

In vivo *anthelmintic activity.* This was estimated in mice by the method previously described (Broome & Greenhalgh, 1961b).

In vitro *anthelmintic activity.* Serial dilutions of methyridine and some of its metabolites were prepared in simple Ringer solution or in filtered gut contents from untreated sheep. They were placed in 10 cm petri dishes and warmed to 37° C before adding the nematodes *Heterakis spumosa* and *Nematospiroides dubius* (from mice), *Nippostrongylus muris* (from rats) and where possible *Ostertagia spp.* (from sheep). After incubating for the required time interval all worms were examined for motility. In the experiments to determine the speed at which the drug causes "irreversible paralysis" to *H. spumosa* the worms were allowed a 30 min recovery period in fresh Ringer solution before estimating anthelmintic activity by

the percentage immobile worms. Finally, the survival of worms in filtered gut contents taken from sheep 2 hr after dosing with 200 mg/kg of methyridine subcutaneously was examined.

Effect of pH on in vitro *anthelmintic activity. In vitro* survival studies were carried out as previously described but with the serial dilutions of the drug prepared in Ringer solution buffered with citrate-phosphate buffer. At the termination of each experiment the *p*H of all test solutions was checked by means of a glass electrode.

The effect of pH on the uptake of methyridine from solutions by Ascaris sp. *from pigs.* A 2×3 factorial experiment was used to study the uptake of methyridine by *Ascaris sp.* over periods of 3 and 6 hr at *p*H's of 3, 5 and 8 respectively. About 75 g ascarids were mopped with filter-paper to remove excess water and they were then divided into six approximately equal groups on the basis of individual worm size. Each group was randomly assigned to one of the treatments and allowed to equilibrate for 15 min in Ringer solution buffered at the appropriate *p*H. Solutions containing 100 μg/ml. of drug were prepared in Ringer solution buffered at the required *p*H by means of a citrate-phosphate buffer. After equilibration the worms were transferred to their respective methyridine solutions allowing 1 g/4 ml. solution. Duplicate 1 ml. samples were immediately taken from all solutions containing ascarids and also from identical control solutions with no ascarids. After incubation for 3 hr at 37° C the appropriate tubes and their respective controls were sampled in duplicate. In addition each worm was weighed and homogenized individually. Similar samples were collected from the remaining tubes and their controls after 6 hr incubation at 37° C. Chemical estimations on the ascarid homogenates were carried out by the method previously outlined. The *p*H of all solutions was checked at the end of the experiment by means of a glass electrode. The concentration of drug in the samples from all solutions was estimated from optical density measurements made after diluting with 4 ml. N/100 hydrochloric acid to remove any effect of *p*H.

Oxygen consumption of ascarids. The effect of methyridine on the oxygen consumption of pig ascarids was examined with the Warburg manometric technique outlined by Lasser (1944). These experiments were kindly performed by Dr J. F. Ryley. Comparisons were made between results obtained with whole worms and worms divided into 0.5 in. segments. Any effect of delayed drug absorption by the worms was thereby examined.

Neuromuscular blocking activity. These studies were also carried out with pig ascarids using isolated neuromuscular preparations as described by Baldwin (1943). In comparative experiments with other compounds known to affect cholinergic nerve endings or acetylcholine, a modification of the exposed neuromuscular preparation (Baldwin & Moyle, 1947) was used. This involved making a single slit along the lateral line of each preparation to allow the entry of compounds otherwise unable to penetrate the cuticle.

RESULTS

The distribution of methyridine in the blood and alimentary tract of mice and sheep

Blood and gut methyridine concentrations in mice. Analytical results of blood and gut contents taken from mice, given a single therapeutic dose of methyridine 400 mg/kg subcutaneously or orally, are summarized in Fig. 1. This shows that the drug is absorbed and metabolized rapidly with maximum blood concentrations 1 hr after dosing and that little remains 5 hr later. (Excretion in mice amounts to only 1 to 2% of the dose during the 48 hr following administration.) There appears to be a close similarity between drug concentrations in the blood, small intestine, caecum and large intestine. The stomach, however, has much higher and more persistent levels than the other regions of the body.

Drug concentrations in the blood, small intestine, caecum and large intestine are higher following subcutaneous administration than following oral administration,

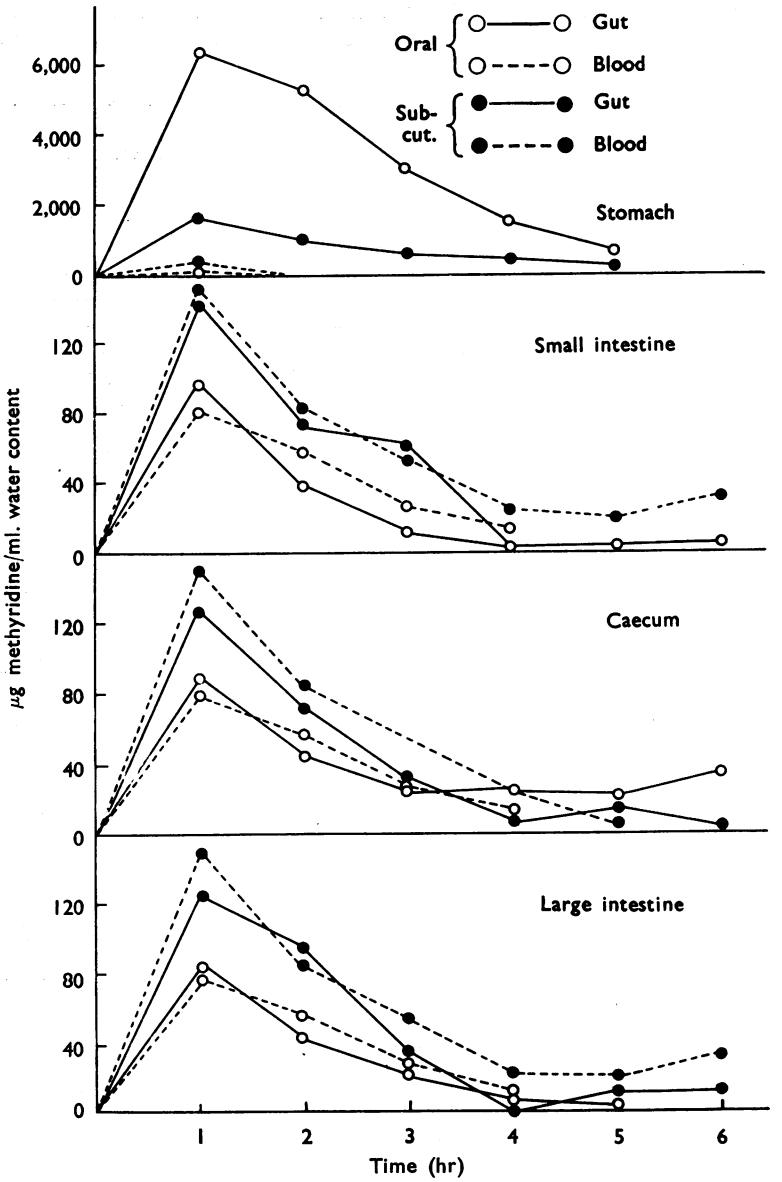

Fig. 1. Blood and gut concentrations of methyridine in mice following subcutaneous and oral dosing with 400 mg drug/kg.

but stomach levels are greater when the drug is given orally. Examination of the relationship between maximum blood levels following oral, and subcutaneous, administrations indicates a ratio of 1/1.67 respectively.

Blood and gut methyridine concentrations in sheep. Fig. 2 shows the results of analysis of blood and gut material collected from a total of six sheep killed after receiving a single dose of 200 mg/kg subcutaneously. As in mice, it is

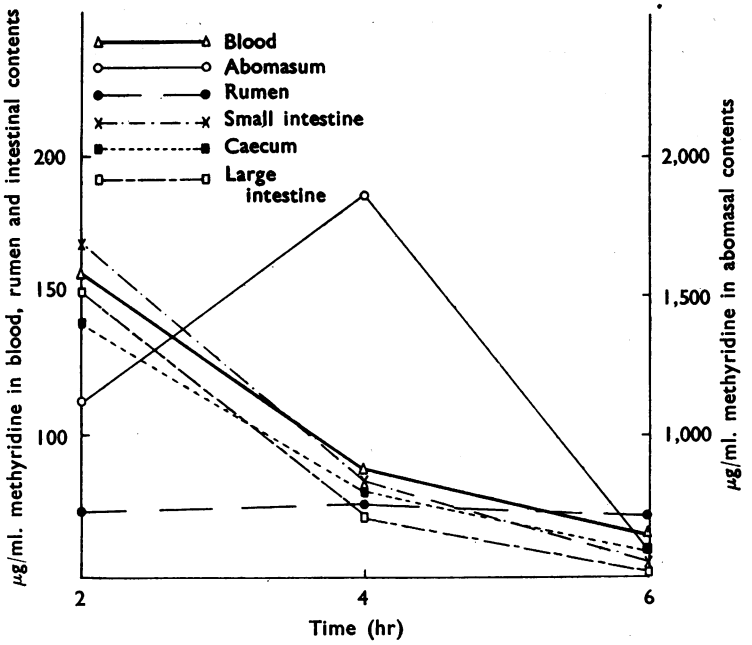

Fig. 2. Analysis of blood and gut contents from sheep given 200 mg methyridine/kg subcutaneously.

apparent that methyridine is absorbed and metabolized rapidly and maximum blood levels are attained approximately 2 hr after dosing (Fig. 3). The drug concentration in all regions of the alimentary canal except the abomasum is closely related to the corresponding blood level. Levels in the abomasum are much higher and more persistent than elsewhere. Thus, the distribution of methyridine along the alimentary tract is similar in both mice and sheep. The effect of oral and subcutaneous dosing on blood concentrations is shown in Fig. 3. The ratio between

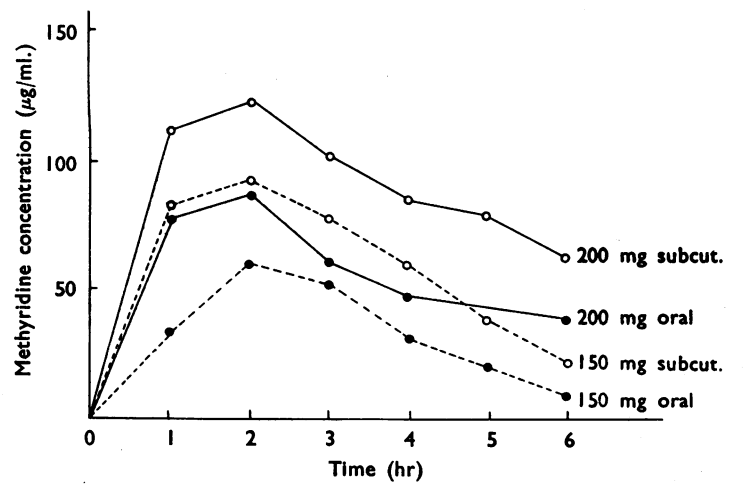

Fig. 3. Concentration of methyridine in sheep blood following oral and subcutaneous dosing.

maximum blood levels is 1/1.47 and that for the areas under the curves is 1/1.42, for oral and subcutaneous dosing respectively. Analysis of blood and gut contents from the sheep, treated with 150 mg/kg orally or subcutaneously, indicated that these differences in blood level are probably reflected in all regions of the alimentary canal except the rumen and abomasum.

Having shown that methyridine reaches the gut in most regions, it became important to know whether physiological concentrations of the drug had any anthelmintic effect on nematodes *in vitro*. In addition the anthelmintic activity of its most likely metabolites was examined both *in vivo* and *in vitro*.

The anthelmintic activity of methyridine and some of its metabolites

The *in vitro* survival studies were disappointing in that motility—the end point for estimating anthelmintic activity—could not be assessed with sufficient precision to allow an accurate quantitative evaluation of the results. Figures quoted in the tables should only be regarded as a qualitative guide to the relative effectiveness of compounds tested.

In vivo *and* in vitro *anthelmintic activity of methyridine and some of its metabolites*. The results from these studies are presented in Table 1, from which

TABLE 1

THE ANTHELMINTIC ACTIVITY OF METHYRIDINE AND SOME OF ITS POSSIBLE METABOLITES

Anthelmintic test:	Minimum drug concentrations "killing" worms at 2 hr *in vitro* (mg/ml.)				*In vivo* mouse test (using maximum tolerated dose)		
Nematode species:	*H. spumosa*	*N. muris*	*N. dubius*	*Ostertagia spp.*	*H. spumosa*	*N. muris*	*N. dubius*
Compound							
Methyridine	0·06	0·06	0·06	0·06	Active	Active	Active
2-Pyridyl acetic acid hydrochloride	1	10	10	10	No action	No action	No action
Pyridyl ethanol	10	10	10	10	No action	No action	No action
α-Picolinic acid	0·5	40	40	40	No action	No action	No action
α-Picoline	10	10	10	10	No action	No action	No action
Formaldehyde	1	10	10	No test	No action	No action	No action
Formic acid	1	10	10	No test	No action	No action	No action

it is clear that methyridine possesses more anthelmintic activity than its metabolites (identified by Duncan & Scales, 1961) both *in vivo* and *in vitro*. Furthermore, the concentrations of methyridine required for *in vitro* activity are in the same range as those previously shown to be present in the gut contents of animals treated with the drug. The similarity in the results with all the nematode species studied suggests very little species variation in susceptibility to the drug. Here it may be noted that the apparent susceptibility of *H. spumosa* to α-picolinic acid and 2-pyridyl-acetic acid hydrochloride probably arises indirectly as a result of the low *p*H of these solutions.

Experiments in which filtered gut contents from untreated sheep served as the diluent indicated that they did not diminish the *in vitro* activity of methyridine. Tests with material obtained 2 hr after dosing sheep with 200 mg/kg subcutaneously indicated activity similar to that anticipated from analytical results.

Having shown that the peak drug concentrations in the gut contents of treated animals are sufficient to affect nematodes *in vitro*, it was necessary to find out if they are also sufficiently prolonged to remove the worms. Studies were therefore undertaken to find the time required by solutions of various concentrations to produce " irreversible paralysis " in *H. spumosa.*

The onset of methyridine-induced paralysis in the nematode H. spumosa. " Irreversible paralysis " is defined here to mean paralysis which could not be reversed by a 30-min recovery period in fresh Ringer solution. The outstanding feature of these results was the rapidity with which methyridine affected the test nematodes. Thus all worms in contact with solutions of peak physiological concentrations were irreversibly paralysed after approximately 1 hr. In more dilute solutions those worms, which were going to succumb, did so within 2 hr ; after which there was no further effect over a 5-hr test period.

These results indicate that the concentrations of drug in the alimentary canal of mice and sheep following therapeutic doses are sufficiently prolonged to account for its *in vivo* anthelmintic activity. The results obtained by Walley (1961) indicate that it is less efficient in the abomasum than in the small intestine of sheep despite the fact that concentrations were higher in the abomasum. The effect of a number of factors which might modify its activity in the abomasum was therefore examined in an attempt to provide an explanation for these apparently anomalous results.

The effect of pH *on the* in vitro *anthelmintic activity of methyridine.* Preliminary studies with Ringer solution of varying *p*H showed the nematode *N. muris* to be the most suitable for these studies, as it survived the widest range of *p*H. Table 2

TABLE 2

THE EFFECT OF *p*H ON THE *IN VITRO* ANTHELMINTIC ACTIVITY OF METHYRIDINE AGAINST *N. MURIS*

N.A.=No anthelmintic activity, Sl.A.=slight anthelmintic activity, A=anthelmintic activity, estimated at 2 hr

Drug concentration	*p*H				
	3	4	5	7	8
1,000 μg/ml.	N.A.	Sl.A.	A	A	A
200 μg/ml.	N.A.	Sl.A.	A	A	A
100 μg/ml.	N.A.	N.A.	A	A	A
50 μg/ml.	N.A.	N.A.	Sl.A.	A	A
25 μg/ml.	N.A.	N.A.	N.A.	N.A.	N.A.

shows that the strongest solution of drug (1,000 μg/ml.) had no effect on *N. muris* at *p*H 3, but as the solutions became more alkaline activity increased so that 50 μg/ml. paralysed worms at *p*H 7 and 8. A similar effect was seen with *Ascaris sp.* from pigs and to a more limited extent with *Ostertagia spp.* (which do not tolerate the more alkaline conditions). No effect of *p*H was seen with *H. spumosa* because the worms died in solutions of low *p*H as a result of acidity *per se.*

The uptake by pig ascarids of methyridine from solutions of varying pH. Before analysing the uptake of drug by *Ascaris sp.* from the various test solutions, adjust-

ments were made for any change in drug concentration which occurred in corresponding controls. In addition, the relationship between individual worm size and the methyridine concentration of appropriate homogenates was examined. No statistically significant effect of worm size was evident, and it was therefore unnecessary to make adjustments for any variation in individual worm weights between treatments.

Since ascarids do not appear to metabolize methyridine (Duncan, unpublished data), it is considered that Fig. 4 shows the effect of *p*H on the ability of the drug

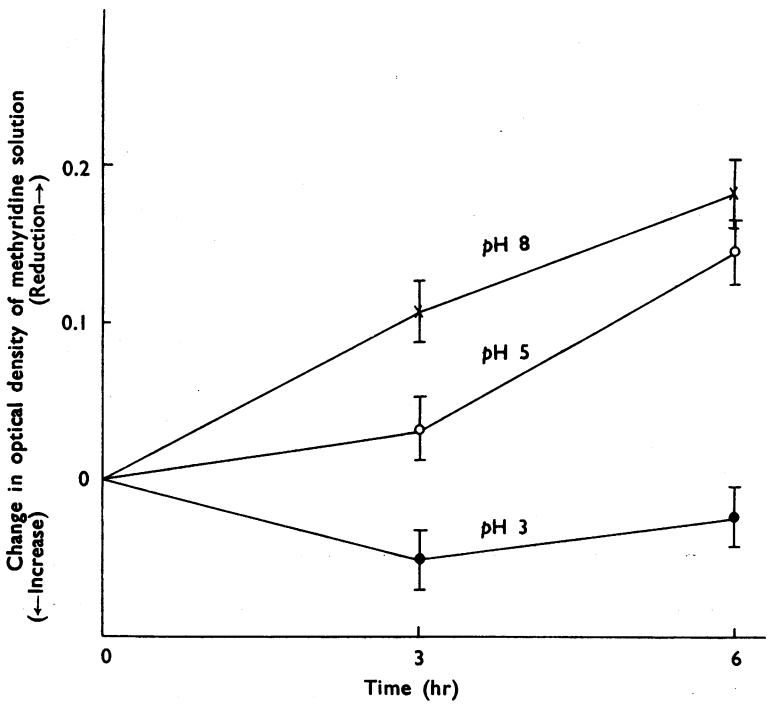

Fig. 4. Uptake of methyridine from solutions of differing *p*H by *Ascaris sp.* from pigs.

to enter the nematodes. A definite effect of *p*H is shown by more drug entering the worms maintained under alkaline conditions. The apparent increase in the methyridine content of solutions at *p*H 3 is not statistically significant. There appears to be a constant relationship between drug uptake and time.

Chemical analysis of worm homogenates showed that the concentration of methyridine within the worms after 6 hr at *p*H 8 was approaching that in the outside solution. Thus it appears that the drug passes into nematodes according to the concentration gradient and that preferential absorption does not occur.

The effect of methyridine on parasitic nematodes

Another important aspect of this study on the mode of action of methyridine is a consideration of the way in which the drug actually affects the parasitic

nematodes. The investigations reported below concern its action on aerobic metabolism and neuromuscular activity in *Ascaris sp.* from pigs.

Studies on the aerobic metabolism of Ascaris sp. The results of this experiment are presented in Table 3, which shows that methyridine does not significantly inhibit the aerobic metabolism of this nematode.

TABLE 3

THE EFFECT OF METHYRIDINE ON THE OXYGEN CONSUMPTION OF *ASCARIS SP.*

Treatment (methyridine/ml.)	μl. oxygen utilized by 1.1 g worm tissue/hr	
	Whole worms	Cut worms
Control	126	111
10 mg	100	103
3·3 mg	121	99
1.1 mg	135	92

Studies on neuromuscular inhibition. The effect of the drug on segments of ascarid worms with the cuticle intact is illustrated in Fig. 5. This shows that extremely rapid paralysis with contraction follows the addition of small amounts of drug to the tissue bath. A comparison of the methyridine-effect with that of

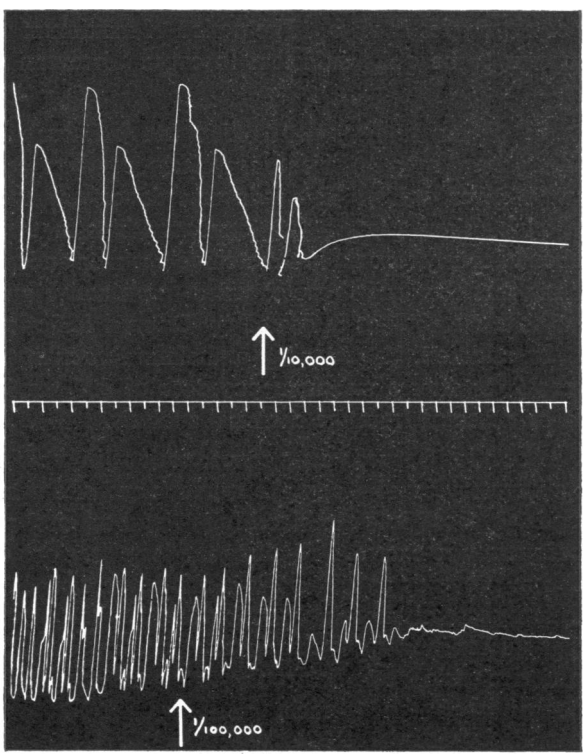

Fig. 5. Effect of methyridine on intact segments of *Ascaris sp.* Time marker 30 sec.

D-tubocurarine using slit preparations is presented in Table 4. For completeness the results of Norton & De Beer (1957) with piperazine and decamethonium are also included in the table.

TABLE 4

COMPARISON OF NEUROMUSCULAR BLOCKING AGENTS IN SPLIT *ASCARIS* PREPARATIONS

The results for piperazine and decamethonium were taken from Norton & De Beer (1957)

	Effective concentration	Effect on *Ascaris* preparations	Effect of acetylcholine chloride (10 μg/ml.)
Tubocurarine chloride	5 μg/ml.	Flaccid paralysis	Antagonism
Methyridine	10 μg/ml.	Partially contracted paralysis	No effect
Piperazine citrate	250 μg/ml.	Flaccid paralysis	Antagonism
Decamethonium bromide	40 μg/ml.	Contracted paralysis	No effect

DISCUSSION

The chemical analyses of blood and gut contents from mice and sheep show that methyridine behaves very similarly in both species. These will therefore be considered together whenever possible. It is clear from Figs. 1 and 2 and Table 1 that when the drug is given in therapeutic doses administered either orally or subcutaneously it reaches all regions of the alimentary canal in concentrations sufficient to affect a variety of nematodes *in vitro*. The period of maximum gut concentration is short, but it appears to be quite sufficient to cause "irreversible paralysis" to *H. spumosa*.

Anthelmintic studies in laboratory animals (Broome & Greenhalgh, 1961b) have shown that the drug is a more efficient anthelmintic when administered subcutaneously than when given orally. This is in accordance with the results of these chemical analyses, which show greater drug concentrations in the gut contents of animals treated subcutaneously (except in the stomach, rumen and abomasum). The alternative hypothesis that the drug is metabolized to an active anthelmintic seems unlikely, because all the metabolites indicated by Duncan & Scales (1961) are less active (Table 1). In addition, the *in vitro* anthelmintic activity of gut contents collected from sheep 2 hr after a subcutaneous dose of 200 mg/kg of drug is no greater than would be expected from the chemical analysis.

There is a close association between drug concentrations in the blood and contents of all parts of the alimentary canal except the stomach and abomasum. This seems to indicate the drug is absorbed into the blood following both oral and subcutaneous dosing, and is subsequently re-excreted all along the lower regions of the alimentary canal. As metabolism proceeds and blood levels fall, the drug is re-absorbed and gut levels fall. This hypothesis is supported by the results (Raventós & Broome, unpublished data) of studies in rats showing that methyridine readily passes from the blood to the gut, and vice versa, according to the concentration gradient, in all regions of the alimentary canal except the stomach.

This mechanism, by which the compound reaches the intestinal regions of the digestive system, appears unique among known anthelmintics, and it helps to explain

certain uncommon features associated with the drug's activity. Firstly, its concentration and its persistence in all regions of the lower gut appear to depend largely on the level in the blood rather than on the rate at which food passes through the alimentary canal. Furthermore, it seems to pass from the blood to the gut along the whole length of the alimentary canal. This allows the drug intimate contact with all worm species, even if they are embedded in the intestinal mucosa or lying beneath the mucous lining of the digestive tract. The worms therefore receive no protection from their varying habitats, a factor which undoubtedly contributes to the broad spectrum of anthelmintic activity following treatment with methyridine.

The analytical results of material from the stomach (mice) and abomasum (sheep) show that their drug concentrations do not follow this " generalized intestinal plan." Thus it can be seen from Figs. 1 and 2 that the concentrations in these regions are extremely high and bear little or no relationship to the corresponding blood levels. Using rats as experimental animals Raventós & Broome (unpublished data) have shown that methyridine passes rapidly into the stomach against the concentration gradient and that conversely absorption from the stomach is extremely slow. This effect appears to be related to the acidic secretions of these organs which presumably cause the ionization of the basic methyridine molecule. The latter is thereby effectively removed from any equilibrium controlling the passage of drug into the gut.

Although there is a high concentration of drug in the abomasum, Walley (1961) has shown it to be less effective against abomasal than intestinal parasites. The data of Walley also indicate that if abomasal nematodes inhabit the small intestine they are just as susceptible to treatment as are normal intestinal species. This suggests that the relatively poor anthelmintic effect reported by Walley against abomasal parasites is not the result of a differential species susceptibility to the drug. The effect of *p*H on the *in vitro* anthelmintic activity (Table 2) appears to offer a pharmacological explanation of Walley's results. As *p*H is lowered the activity decreases until at a *p*H of 3 the drug is ineffective at physiological concentrations. Abomasal *p*H lies between 3.5 and 4.5 according to Masson & Phillipson (1952). The most likely reason for this *p*H effect is that the unionized molecule is the active component since it alone can penetrate the lipid barrier of the nematode cuticle. Acidity favours ionization of the molecule and thus decreases its lipid solubility which slows down its entry into the worms. When the *p*H falls below approximately *p*H 3 the molecule is completely ionized and does not enter the worm to produce its toxic effect. Thorp (unpublished data) estimates the pK_a of methyridine as approximately 5.5.

The experimental results illustrated in Fig. 4 show that acidity does, in fact, significantly reduce the uptake of methyridine by nematodes of the genus *Ascaris*. Whether this is the only reason for the relative inefficiency of the drug in the abomasum can only be conclusively proved by *in vivo* experiments involving modifications of abomasal *p*H.

The final aspect of this work concerns the manner in which the drug produces its toxic effect in parasitic nematodes. The chemical similarity between methyridine and nicotinic acid or nicotinamide suggested the drug might affect aerobic metabolism. However, Warburg studies (Table 3) revealed no evidence to support this hypothesis.

Nevertheless the results were interesting because they showed the nematodes were still " alive " even though completely paralysed.

Investigations into the nature of methyridine-induced paralysis indicated a type of neuromuscular inhibition which cannot be antagonized by acetylcholine. This suggests a block of the decamethonium type which had previously been demonstrated by my colleague, Dr. J. Raventós, to be caused by the drug in vertebrates. Despite the similar pharmacological activity of methyridine in the host and its nematode parasites, the drug can be used as a safe and effective therapeutic agent (Broome & Greenhalgh, 1961 ; Walley, 1961). Since similar amounts of methyridine appear to reach the tissues of both host and parasite, it seems likely that the usefulness of the drug must depend on the greater sensitivity of nematode than vertebrate tissue to its action. Indirect evidence in support of this hypothesis is available because concentrations of 10 μg/ml. will completely paralyse intact ascarid segments (Fig. 5) whilst blood levels of over 100 μg/ml. have not caused fatalities in mice, rats and sheep. The therapeutic usefulness of the drug therefore appears to depend on quantitative differences between nematode and vertebrate neuromuscular transmission.

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