METABOLISM OF CHLORPHENAMIDINE* IN LARVAE OF THE CATTLE TICK *BOOPHILUS MICROPLUS*

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[Manuscript received February 12, 1971]

Abstract

Larval ticks were immersed in aqueous preparations of chlorphenamidine labelled with ^{14}C in the 2-methyl position. The chemical penetrated rapidly and produced no mortality of larvae, but a quick knockdown from which at low concentrations they appeared to recover quickly. Metabolism of chlorphenamidine was relatively slow compared with that of organophosphorus acaricides in the same strain of larvae. Major metabolites, identified by paper chromatographic and countercurrent distribution behaviour, and by ultraviolet spectra, were *N*-formyl-4-chloro-*o*-toluidine, 4-chloro-*o*-toluidine, and a conjugated phenolic derivative. This sequence appeared to be the metabolic route. The evidence suggested that chlorphenamidine itself was the active toxicant.

I. INTRODUCTION

Roulston and Wharton (1967) showed that the organophosphorus-resistant Biarra strain of cattle tick had no cross-resistance to chlorphenamidine. As this compound represented a new class of potential acaricides whose mode of action was unknown, it was considered appropriate to investigate its metabolism in cattle tick larvae.

II. MATERIALS AND METHODS

(*a*) *Chemicals*

[^{14}C]Chlorphenamidine,‡ labelled in the 2-methyl position, and of specific activity 4·8 mCi/mmole, was dissolved in n-hexane and a polar impurity removed by four extractions with water. Chromatography in the paper-partition system used herein showed the resultant material to be radiochemically pure. Stock solutions of purified [^{14}C]chlorphenamidine and purified unlabelled chlorphenamidine in n-hexane were stored at 5°C.

N-Formyl-4-chloro-*o*-toluidine‡ and 4-chloro-*o*-toluidine‡ each gave single ultraviolet-absorbing spots after paper-chromatographic separation, and single peaks, when checked by ultraviolet spectra, after countercurrent distribution between n-hexane and 60% aqueous methanol.

Solvents were glass-distilled A.R. grade, with the exception that some operations were performed using spectroscopic grade n-hexane.

Polyethylene glycol 400, L.R. grade from British Drug Houses, was used as purchased. Permablend TMI was obtained from Packard Instrument Pty. Ltd., Melbourne.

(*b*) *Tick Larvae and Chemical Treatment*

Larvae of the Yeerongpilly strain of cattle tick, which has been maintained here as a reference susceptible strain for acaricide-resistance studies, were used 12–14 days after hatching. Treatment of larvae with [^{14}C]chlorphenamidine was done by immersion for 5 min in aqueous preparations, as in earlier investigations with coumaphos (Roulston, Schuntner, and Schnitzerling 1966).

* *N*-(2-methyl-4-chlorophenyl)-*N'*,*N'*-dimethylformamidine.

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‡ Supplied by Ciba Co. Pty. Ltd., Lane Cove, N.S.W.

Treatment of larvae with ^{14}C metabolites of chlorphenamidine was done similarly except that, after immersion, larvae were filtered on Whatman No. 41 paper, dispersed lightly with a spatula, and carefully dried between pads of Whatman No. 541 paper. This was done to minimize loss of chemicals by evaporation or entrainment during drying, which was normally effected by air draught. When high doses of [^{14}C]chlorphenamidine were used, and when ticks were treated with ^{14}C metabolites, dilutions were made with the respective unlabelled materials.

(c) *Extraction and Separation of Radioactive Compounds*

At appropriate times after treatment of larvae with radiochemicals, samples were removed from the storage incubator (30°C and 90% R.H.), washed twice with acetone to remove external radioactive deposits, and then homogenized in acetone containing 15% (v/v) methanol in the ratic of 10 ml solvent per 1 g larvae. The ticks were homogenized in an all-glass vessel cooled by ice and water in a surrounding jacket. When required for paper chromatography, external washes and extracts of 0·1-g samples of larvae were evaporated to near dryness or to an aqueous methanol stage using dry air at ambient temperature, before applying to the paper. Paper-chromatographic separations for metabolic studies were made on Whatman No. 1 paper which had been dipped in a 4% (v/v) solution of polyethylene glycol 400 and dried prior to sample application. The descending solvent was n-hexane–benzene–methanol (65:24:5 v/v). Polar metabolites were chromatographed on No. 1 paper using water-saturated n-butanol as descending solvent.

Larger samples of chlorphenamidine-treated ticks were homogenized at 5°C in an all-steel ball mill using 70% (v/v) aqueous methanol in the proportion of 5 ml solvent per 1 g larvae. The clear supernatant obtained by centrifuging a homogenate was extracted successively with four equal volumes of n-hexane and four equal volumes of benzene. The methanol was evaporated from the remaining extract at 40°C in a dry nitrogen stream. The combined hexane extracts were extracted with a small volume of 0·1N HCl, which quantitatively removed radioactive materials. The acid extract was then neutralized in the presence of an equal volume of spectroscopic grade n-hexane and the resulting two phases used as a sample for countercurrent separation in system A (as described below). The benzene extracts were evaporated separately to small volumes, using dry air at ambient temperature, combined, and made to a volume suitable for countercurrent separation in system B. The aqueous residue, after methanol evaporation, was used directly as a sample for system C.

In countercurrent distribution system A spectroscopic grade n-hexane was used as upper phase and 60% (v/v) aqueous methanol as lower phase. Benzene extracts were separated using benzene as upper phase and 60% aqueous methanol as lower phase (system B). In system C n-butanol was used as upper phase and 0·01M sodium phosphate buffer (pH 7·0) as lower phase. The apparatus used was an automatic Gallenkamp–Towers instrument with upper and lower phase volumes of 3 ml.

Measurement of radioactivity on chromatograms, after covering them with 25-gauge Melinex to avoid contamination of the counter window by relatively volatile radioactive materials, was made with a B.T.L. multi-Geiger chromatogram counter. Liquid samples were prepared in a toluene solution containing 0·55% Permablend TMI, with addition of ethanol where necessary for miscibility, and counted in a Packard scintillation spectrometer.

Ultraviolet spectra were obtained using a Perkin Elmer model 350 spectrophotometer.

III. Results

(a) *Deposition of Chlorphenamidine and Toxic Effects*

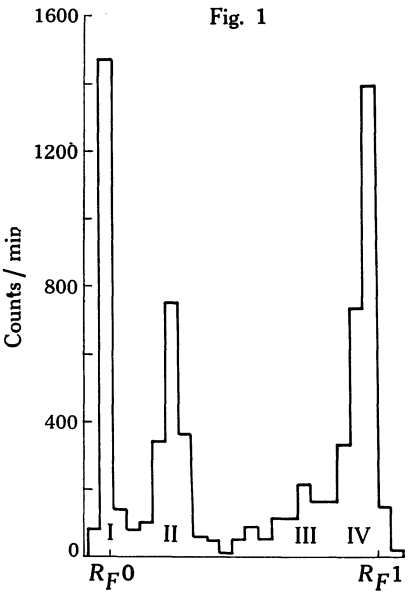
Only 10% of the [^{14}C]chlorphenamidine in 0·05 and 0·2% aqueous preparations of the acaricide used to treat larvae was deposited on the larval cuticle. Chlorphenamidine-treated larvae were "knocked down" during treatment and showed no movement when dried after immersion. At 0·05% recovery was apparent about 1 hr after treatment, but at 0·2% none of the larvae fully recovered.

TABLE 1

PENETRATION OF [^{14}C]CHLORPHENAMIDINE INTO LARVAE AFTER IMMERSING THEM IN 0·05 AND 0·2% AQUEOUS PREPARATIONS FOR 5 MIN

Concentrations outside and inside larvae given as μg/g larvae

Time after Immersion (hr)	0·05% Preparation		0·2% Preparation	
	Concn. Outside	Concn. Inside	Concn. Outside	Concn. Inside
0·25	225	—	990	—
2	22	185	185	800
6	11	170	65	885
12	10	195	36	1130
24	9	70	85	330
48	9	55	70	235



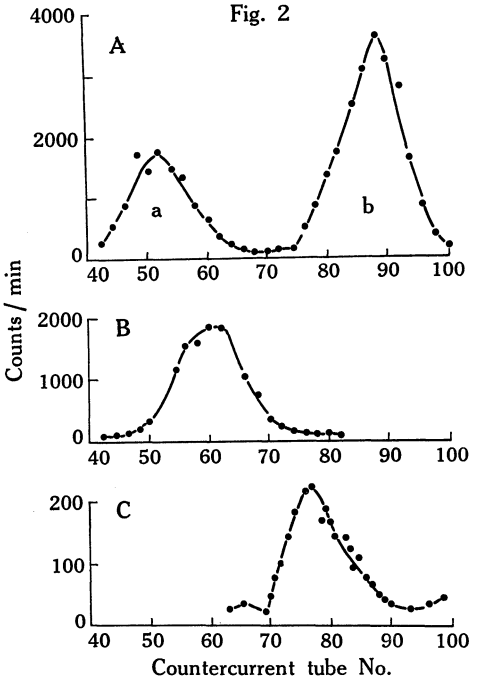


Fig. 1.—Radiochromatogram of [^{14}C]chlorphenamidine (IV) and its metabolites (I, II, and III) produced in cattle tick larvae 48 hr after immersion in 0·05% aqueous colloid of the acaricide, as separated by paper partition chromatography.

Fig. 2.—Peaks in radioactivity in countercurrent distribution separations of liquid–liquid extracts from larval cattle ticks 72 hr after treatment with 0·05% [^{14}C]chlorphenamidine. The number of transfers were: *A*, 111; *B*, 88; *C*, 100. Aliquots (1%) of upper and lower phases were counted together in the liquid scintillation system.

(*b*) *Penetration and Metabolism of Chlorphenamidine*

Table 1 shows that the penetration of chlorphenamidine into larvae was quite rapid, and comparable to that of organophosphorus acaricides (Schuntner, Roulston, and Schnitzerling 1968). In paper-chromatographic separations of extracts of larvae treated with [^{14}C]chlorphenamidine, radioactivity was distributed as in Figure 1. The fractions were subjected to further clean-up procedures. Identifiable ultraviolet spectra were obtained for metabolites II, III, and the parent compound IV, but final yields were less than 5% of the paper-chromatogram fractions.

Countercurrent distribution of hexane and benzene extracts and of the aqueous residue of an 8-g sample of [^{14}C]chlorphenamidine-treated larvae gave the results shown in Figure 2.

Two compounds were present in the hexane extract (Fig. 2*A*). Fraction *b* was identified by ultraviolet spectrum as chlorphenamidine (Fig. 3, IV) while fraction *a*,

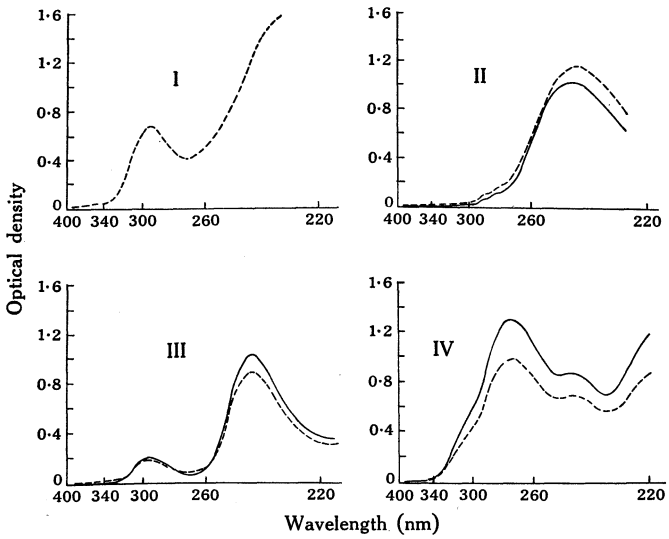


Fig. 3.—Ultraviolet spectra of reference compounds, and of metabolites separated by counter-current distribution. I, probable phenolic conjugate. II, *N*-formyl-4-chloro-*o*-toluidine. III, 4-chloro-*o*-toluidine. IV, chlorphenamidine. ——— Reference compound. - - - - Metabolite.

following acidic extraction and basic back-extraction into spectrographic grade n-hexane with overall volume reduction, was shown to have the spectrum of 4-chloro-*o*-toluidine (Fig. 3, III).

The countercurrent separation of the benzene extract (Fig. 2*B*) produced one main fraction. Evaporation of the separated upper phase left a small residue which when dissolved in methanol had a spectrum resembling *N*-formyl-4-chloro-*o*-toluidine. Upon making the methanol solution slightly aqueous and extracting with spectrographic n-hexane, some clean-up of the methanol solution was obtained to give the spectrum shown in Figure 3, II.

The aqueous residue also contained only one compound following counter-current separation (Fig. 2*C*), and ultraviolet examination of the butanol phase

showed a uniform spectrum throughout the radioactivity peak with close correspondence of absorbance and radioactivity (Fig. 3, I). It was initially thought that the ultraviolet spectrum was that of the metabolite but absorbance was far too strong for the amount of radioactive material present. Further attempts at clean-up using Sephadex LH20 columns with methanol and with water as solvents failed to change the ultraviolet spectrum of the radioactivity peak material. Infrared examination using the KBr pellet technique did not yield a meaningful spectrum. Hydrolysis of metabolite I with 0·5N HCl at 100°C for 1·5 hr was followed by n-butanol extraction of radioactive material. The latter was chromatographed in the water-saturated n-butanol system and the radioactive spot, which had a much higher mobility than the original metabolite, was eluted with methanol and examined for ultraviolet spectral characteristics. Making the solution alkaline resulted in a marked shift of the peak to a longer wavelength (from 290 to 345 nm), and acidification resulted in reversion to the original spectrum. This phenomenon is consistent with a phenolic structure. However, a number of reference compounds examined failed to provide a matching spectrum. Recoveries of metabolites were as shown in Table 2.

TABLE 2

RECOVERY OF METABOLITES OF [^{14}C]CHLORPHENAMIDINE PRODUCED IN LARVAE, FOLLOWING LIQUID–LIQUID AND COUNTERCURRENT DISTRIBUTION SEPARATIONS

^{14}C Metabolite	Phase	Recovery from Homogenate (%)
4-chloro-*o*-toluidine (III)	Hexane extract and countercurrent A fraction *a*	98
	Ultraviolet spectroscopy of cleaned-up fraction	90
N-formyl-4-chloro *o*-toluidine (II)	Benzene extract	99
	Countercurrent B fraction	61
	Ultraviolet spectroscopy of cleaned-up fraction	49
Probable phenolic conjugate (I)	Aqueous residue after evaporation of methanol	91
	Ultraviolet spectroscopy on countercurrent fraction (upper phase)	91

The metabolism of penetrated chlorphenamidine was comparatively slow and was still proceeding 24 hr and more after treatment of larvae (Fig. 4), whereas organophosphorus acaricides were largely metabolized in about 6 hr (Schuntner, Roulston, and Schnitzerling 1968; Roulston, Schnitzerling, and Schuntner 1968; Roulston, Schuntner, and Schnitzerling 1969). Larvae treated at the two dose levels gave qualitatively similar metabolic results up to about 24 hr, but thereafter larvae treated at the higher dose showed a marked buildup of metabolite II and some decrease in the other two metabolites.

(c) *Metabolism of Intermediate Metabolites*

The solutions of metabolites obtained from countercurrents A(*a*) and B were each reinforced with 2·5 mg of the respective reference compound before evaporating

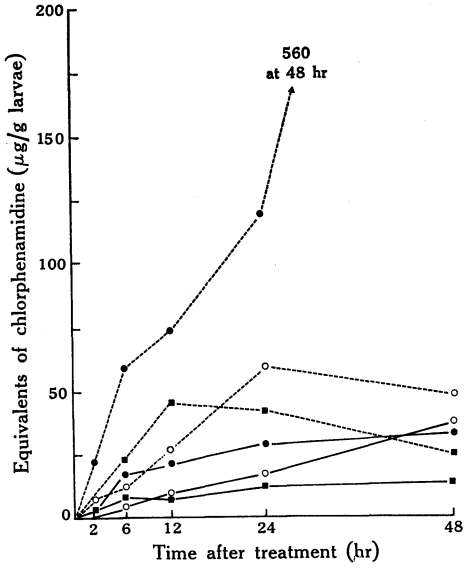


Fig. 4.—Metabolism of [^{14}C]chlorphenamidine in larval cattle ticks following their treatment with 0·05% and 0·2% aqueous preparations of the chemical, as indicated by paper chromatography. ○ Metabolite I. ● Metabolite II. ■ Metabolite III. ——— 0·05% [^{14}C]chlorphenamidine. - - - - 0·2% [^{14}C]chlorphenamidine.

the solvents. The residues were used in aqueous preparations to treat larvae for the metabolic studies, the results of which are shown in Figure 5. Both compounds were

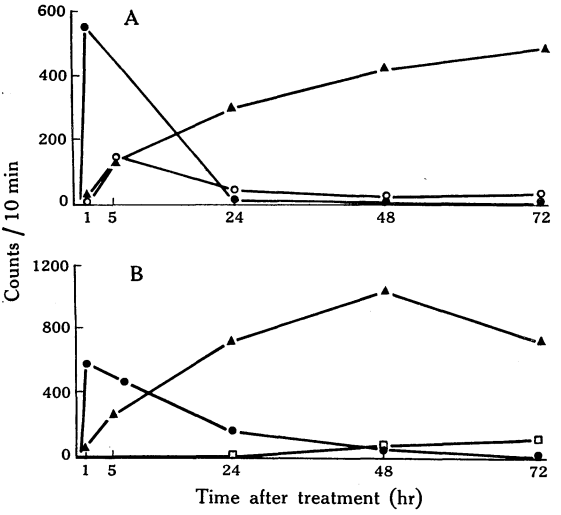


Fig. 5.—Metabolism of [^{14}C]*N*-formyl-4-chloro-*o*-toluidine (*A*) and [^{14}C]-4-chloro-*o*-toluidine (*B*) in larval cattle ticks treated with 0·05% aqueous preparations of the radiochemicals, as indicated by paper chromatography.
● Parent radiochemical inside larvae.
▲ Polar metabolite inside larvae.
○ Non-polar metabolite inside larvae.
□ Polar metabolite outside larvae.

metabolized at similar rates to polar material which had no mobility in the polyethylene–glycol chromatographic system. Chromatography of these materials in the water-saturated butanol system showed that both polar metabolites had the R_F value of metabolite I produced from chlorphenamidine. This identity was confirmed by ultraviolet spectral scans of methanol elutes of the radioactive spots.

A relatively non-polar intermediate metabolite produced in larvae treated with metabolite II chromatographed like 4-chloro-*o*-toluidine in the polyethylene–glycol system. Washes of the treated larvae at the various sampling occasions showed that both compounds (II and III) penetrated readily but there was an increase in external radioactive material at 48 and 72 hr on larvae treated with [^{14}C]4-chloro-*o*-toluidine. This material did not move on chromatography in the partition system and was not further investigated. Washes of larvae treated with [^{14}C]*N*-formyl-4-chloro-*o*-toluidine contained only the parent compound.

IV. Discussion

The identification of metabolites II and III following paper-chromatographic separation and liquid–liquid clean up, although supported by evidence of co-chromatography with reference compounds, was based on recoveries below 5%. Hence the possibility of their being minor metabolites in initially mixed fractions could not be ruled out. Reliable identification of the metabolites was obtained following countercurrent distribution separations and liquid–liquid clean-up with minimal evaporative steps. Here recoveries ranged from 49 to 92% of initially extracted radioactive materials, sufficient to classify the metabolites as major ones. There was rapid knockdown of larvae following immersion in aqueous chlorphenamidine preparations, which indicated that the parent compound was the toxicant.

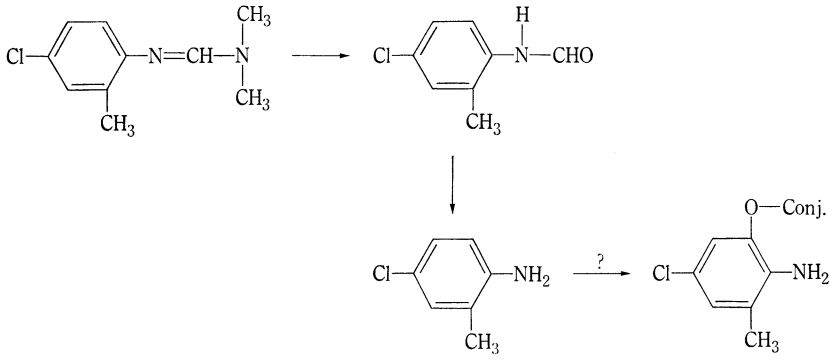


Fig. 6.—Probable metabolic route for degradation of chlorphenamidine in larval cattle ticks.

This contention is supported by the relatively slow metabolism of chlorphenamidine in larval ticks. Larvae treated at 0·05% chlorphenamidine produced metabolites II and III before the polar metabolite I, and the steady increase in the latter, combined with the flattening of the curves representing II and III, indicated that the latter two compounds were intermediates and metabolite I was the end-product (Fig. 2). Similar metabolism was observed in larvae treated with 0·2% chlorphenamidine, for up to 24 hr after treatment, after which time there was a dramatic increase in the level of metabolite II and a slight decrease in the polar metabolite level. This, coupled with the downward trend in the metabolite III curve, suggested that compound II was the precursor of I and III and that a biochemical block had occurred at this dosage. It is concluded that the sequence of chlorphenamidine degradation in larvae is as shown in Figure 6.

No investigation was made into the further metabolism of the polar metabolite I in larval ticks, because the linear build-up shown in larvae dosed at 0·05% chlorphenamidine and also in larvae dosed at 0·01% (unpublished data) indicated that this compound would degrade slowly in ticks. It seemed also that such a polar compound would penetrate the lipoid barrier in larval cuticle at a very slow rate after dosage by immersion. Following treatment of larvae with [^{14}C]4-chloro-*o*-toluidine it was shown that the main degradation was to metabolite I, while some polar material appeared in external rinses at 48 and 72 hr after treatment. It seems likely that the latter was produced from some of the 4-chloro-*o*-toluidine during its passage through the cuticle, and remained in this site accessible to the acetone used for washing larvae. The metabolism study using [^{14}C]*N*-formyl-4-chloro-*o*-toluidine indicated that larvae also degraded this compound to metabolite I through 4-chloro-*o*-toluidine as intermediate, the results thus agreeing with conclusions drawn from chlorphenamidine metabolism. The two identified metabolites of [^{14}C]chlorphenamidine produced in larvae were also found upon photodecomposition of chlorphenamidine in solvents and in thin-layer chromatography plates (Knowles and Sen Gupta 1969) and as major metabolites in the white rat (Knowles and Sen Gupta 1970), dog, and goat (Sen Gupta and Knowles 1970). These authors separated several unidentified degradation products, one of which was postulated to be 2-methyl-4-chloro-6-hydroxyaniline. It is possible that metabolite I, produced by chlorphenamidine metabolism in ticks, could be a conjugate of this or a related phenol. This was the only evidence of conjugation in larval ticks treated with chlorphenamidine, whereas the above authors found that most of their radioactive chlorphenamidine was rapidly eliminated in the urine of the three mammals, largely as conjugated metabolites.

V. Acknowledgment

Financial support for this study from the Australian Meat Research Committee is gratefully acknowledged.

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