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Comparison of surface iodination methods by electron microscopic autoradiography applied *in vitro* to different life-stages of *Dipetalonema viteae* (Filarioidea)

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SUMMARY

Different stages of *Dipetalonema viteae* (males, females, microfilariae, and 3rd-stage larvae) have been iodinated *in vitro* under physiological conditions by chloroglycoluril, lactoperoxidase or chloramine T. The concentrations of the catalysts were correlated with the viability of the worms. Localization of the label with the different iodination methods had been visualized by electron microscopical autoradiography. Chloroglycoluril-mediated iodination is predominantly localized on the filarial cuticle. Lactoperoxidase-catalysed iodination is less specific and chloramine T catalyses iodination in a gradient decreasing from the cuticle to inner structures. It is necessary to visualize the labelling by electron microscopical autoradiography prior to biochemical and immunological experiments to avoid the extraction of structures iodinated by leakage of the catalyst into sub-cuticular regions.

INTRODUCTION

Immunological control of parasitic helminths depends mainly on the structure of the contact site with the host namely, the cuticle (Lumsden, 1975, Mackenzie, Preston & Ogilvie, 1978, Ogilvie, Philipp, Jungery, Maizels, Worms & Parkhouse, 1981). Surface antigen characterization and isolation has preferentially been carried out by using ^{125}I as a tracer. Philipp, Parkhouse & Ogilvie (1980) and Parkhouse, Philipp & Ogilivie (1981), used chloramine T and lactoperoxidase as catalysts for iodination of *Trichinella spiralis*. Chloroglycoluril was applied to *Onchocerca gibsoni* by Forsyth, Copeman, Abbot, Andress & Mitchell (1981). The only comparison of different catalysts has been reported by Hayunga, Murell, Taylor & Vannier (1979) using *Schistosoma mansoni*. Since the composition of the tegument of the helminths is largely unknown it is difficult to attribute the radioactive label to a specific surface protein. This is in contrast to the work of Markwell & Fox (1978) who used Sendai and Newcastle disease viruses to compare the specificity of iodination with different catalysts on well-characterized viral coat proteins. As a result they found that CGU-mediated iodination is slightly preferable to that by lactoperoxidase.

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The aim of our work was to evaluate with electron microscopical autoradiography the surface specificity of commonly used iodination methods with emphasis on chloroglycoluril applied to different stages of the same species.

MATERIALS AND METHODS

Host–parasite system

The filarial parasite *Dipetalonema viteae* was maintained in the jird, *Meriones unguiculatus* and the soft tick, *Ornithodorus moubata* as described by Worms, Terry & Terry (1961). Male golden hamsters (60–80 g) came from a randomly bred colony (strain LAKZ, Institut für Zuchthygiene der Universität Zürich, Switzerland).

Adult worms were harvested from hamsters according to the method described by Baschong, Tanner, Betschart, Rudin & Weiss (1982), 3rd-stage larvae (L_3) from infected ticks by the method of Gass, Tanner & Weiss (1979), and microfilariae from *in vitro*-maintained female worms after Weiss & Tanner (1979).

Iodination (Table 1)

Na ^{125}I (carrier-free, EIR Würenlingen, Switzerland) was reconstituted in 0·15 M phosphate-buffered saline (PBS), pH 7·2, to an activity of 20 mCi/ml. All iodination tests were carried out in a volume of 0·5 ml PBS at room temperature. Chloramine T and lactoperoxidase catalysed iodinations were performed in 12 ml vol. glass centrifuge tubes and chloroglycoluril-mediated iodinations in borosilicate tubes (see below).

Table 1. *Experimental conditions for the* in vitro *labelling of different* Dipetalonema viteae *life-stages in* 0·5 *ml PBS, pH* 7·2, *at* 20 °C

Number and stage of parasites	Chloroglycoluril*		Chloramine T†		Lactoperoxidase‡		Control*
	μg	μCi Na ^{125}I	ng	μCi Na ^{125}I	pmole	μCi Na ^{125}I	(μCi Na ^{125}
2 Females	10 100	200 200	125	200	50	200	200
4–5 Males	10 100	100 100	125	100	50	100	200
50000 Microfilariae	100§	200	125 1250	200 500	not done		500
1000 Third-stage larvae	5 100§	500 200	125	200	50	200	500

* Incubation time 10 min.
† Incubation time 3 min.
‡ Incubation time 4 min, with addition of 25 μl of H_2O_2 (0·03 %) after 1, 2 and 3 min.
§ Organisms which did not survive iodination procedure.

Chloramine T

Na ^{125}I and chloramine T were reacted with the parasites for 3 min (McConahey & Dixon, 1980). L-tyrosine (Merck, Darmstadt, BRD) at a concen-

tration of 15 mM in PBS/mCi of Na ^{125}I was added in a final volume of 5 ml. After 1 min, adult *D. viteae* were transferred in a new tube and washed twice with 5 ml of 5 mM Na I in PBS (pre-warmed to 37 °C). Microfilariae and L_3 larvae were centrifuged for 10 min at 300 *g* after addition of L-tyrosine in PBS, the supernatant fluids were discarded and the pellets washed twice with 5 ml of PBS/Na I solution. Until EM-fixation the worms were kept in RPMI at 37°C.

Lactoperoxidase

Lactoperoxidase-mediated iodination was initiated by adding 2 μl of H_2O_2 (33 %) freshly diluted 1 : 1000 after 1, 2 and 3 min to the reaction mixture (Morrison, 1980) After 4 min the reaction was stopped with tyrosine.

Chloroglycoluril

Chloroglycoluril-mediated iodinations (Fraker & Speck, 1978; Markwell & Fox, 1978) were carried out in round-bottomed borosilicate tubes (13 × 100 mm, Corning, New York, USA) coated with 1,3,4,6-tetrachloro,3α,6α-diphenyl-glycoluril (trade name IODO–GEN™, Pierce Chemicals Co., Rockford, Illinois USA) dissolved in CH_2Cl_2, by drying the solution under a stream of nitrogen. Immediately before use the coated tubes were rinsed with iodination buffer to remove any flakes of the catalyst.

The biological material was placed in 500 μl of PBS in coated glass tubes and, after addition of the iodine, were incubated for 10 min with occasional stirring. L-tyrosine at a concentration of 15 mM in PBS/mCi was added to a total volume of 5 ml and after 1 min adult worms were transferred to 5 ml of pre-warmed 5 mM NaI in PBS and washed as above. Iodination of microfilariae and 3rd-stage larvae was stopped with 15 mM L-tyrosine/mCi suspended in 2 ml of PBS by incubation for 1 min. The contents of the reaction vial were transferred to a 12 ml vol. glass centrifuge tube. To this tube 2·5 ml of PBS was added. After centrifugation the supernatant fluid was discarded and the remainder washed twice as above.

Electronmicroscopy and autoradiography

Two hours after labelling, the worms were fixed with 4 % (v/v) glutaraldehyde in 0·1 M Na-cacodylate buffer (pH 7·4) for 90 min at room temperature. After fixation for 30 min, adult worms were cut into pieces (1–3 mm length) and further processed for EM in specimen tubes. Third-stage larvae were processed either as clumps formed by living larvae or by centrifugation in 10 % BSA followed by cross-linking with glutaraldehyde or as free-floating worms in cavity blocks. Preparation of microfilariae and processing of all specimens after fixation was done as described by Rudin, Tanner, Bauer & Weiss (1980).

Ultrathin sections (50–80 nm) were cut on a LKB Ultratome III and transferred to parlodion-coated grids. For electron meiroscopical autoradiography the loop method of Bienz (1977) was used. The Ilford L4 coated grids were exposed at 4 °C in a dry atmosphere for 14–133 days. For the first chloroglycoluril series the exposure time was estimated approximately by light microscopical autoradiography

on semi-thin sections. For electron microscopy the physical development and post-staining of the grids was done according to Bienz (1977).

RESULTS

Motility of untreated control worms was taken as a parameter to assess the viability of the worms. Reagents were applied at concentrations at which no change in motility could be detected by eye (for males and females) or microscopically (for 3rd-stage larvae and microfilariae) until fixation for electron microscopy. Na I and tyrosine had no effect on the motility of any stage, whereas chloroglycoluril and chloramine T at high concentrations were toxic for the worms. Lactoperoxidase at the concentrations used did not seem to affect the viability of the organisms (Table 1).

Chloroglycoluril iodination

Chloroglycoluril-mediated iodination of adult females and males showed cuticular specificity at both concentrations used (Pl. 1A, B). The catalyst concentration (10 or 100 μg/tube) did not significantly influence the specificity and intensity of the labelling. At a concentration of 100 μg, the incubation of male worms was reduced to 5 min. Longer exposure to the catalyst immobilized the worms. The shorter reaction time resulted in a lower intensity of labelling.

Iodination of *D. viteae* 3rd-stage larvae and microfilariae was performed at the lethal concentration of 100 μg of chloroglycoluril/tube. In both larval stages this lethal concentration resulted in a loss of specificity (Pl. 1C, D) or cuticular labelling. Third-stage larvae iodinated with 10 μg of catalyst/tube retained their motility and showed efficient surface labelling of the cuticle (Pl. 1D).

As a control, the 4 stages were incubated for 10 min with the appropriate amount of Na ^{125}I without any catalyst. No detectable iodination occured in any of the 4 stages (Pl. 1F). Addition of tyrosine to the organisms after iodination did not result in any difference in labelling by autoradiography compared to those without tyrosine. Thus, a significant incorporation of radioactive tyrosine can be excluded.

Lactoperoxidase iodination

Lactoperoxidase-mediated iodination (50 pmole lactoperoxidase) labelled less intensively than chloroglycoluril iodination. This was expressed as a longer exposure time for the autoradiographs to reach a sufficient grain density. Adult *D. viteae* showed rather specific cuticular labelling (Pl. 2A) with occasional scattering into inner structures.

As in the adult worms the intensity of lactoperoxidase-catalysed iodination of 3rd-stage larvae (Pl. 2B) was at a low level compared to the chloroglycoluril reaction. In contrast to the adult worms the label was not concentrated on the cuticle.

Chloramine T iodination

At chloramine T concentrations which did not influence the motility of the worms (125 ng for males, females and 3rd-stage larvae, 1250 ng for microfilariae)

the least specific and weakest iodination occurred in our experiments. Adult male and female *D. viteae* showed an even distribution of the marker over the whole filarial profile (Pl. 2C(i) or a density gradient from the surface to inner structures of the worm (Pl. 2C(ii)). In no experiment with microfilariae and 3rd-stage larvae could a preference for the cuticle be assigned (Pl. 2D).

DISCUSSION

The specificity of labelling of helminths as a tool to trace surface material of the parasites depends on the nature of the surface covering the species under investigation and on the method and conditions of iodination. The *D. viteae* system allowed investigation of iodination of cuticles of different thickness and antigenically different structures (Weiss & Tanner, 1981).

We limited our studies to the iodination of different life-stages of *D. viteae* by chloramine T, lactoperoxidase and chloroglycoluril using methods previously applied to labelling of antigenic surface material as recently published (Forsyth *et al.* 1981; Parkhouse *et al.* 1981; Philipp *et al.* 1980).

Our aim was to label living organisms and localize surface staining by electron microscopy. Worms which died during iodination using chloroglycoluril as catalyst showed a less specific surface localization of the label than living ones. This could be explained by assuming that the relatively low solubility of chloroglycoluril (8×10^{-6} M, Fraker & Speck (1978)) results in its accumulation in the hydrophobic layer of the cuticle. To avoid possible toxic effects of $Na_2S_2O_5$ (for chloramine T) or enzyme inhibitors (for lactoperoxidase), L-tyrosine was added in excess to terminate the reaction by binding any free activated iodine. In the case of chloroglycoluril, L-tyrosine was added to produce similar conditions in all three labelling procedures. Chloramine T and chloroglycoluril at high concentrations seem to interfere with the motility of almost all stages of *D. viteae*. Microfilariae did not show signs of lower motility at a concentration of 1250 ng in 0·5 ml PBS, a fact which might be due to a rather different structure of the cuticle. In the case of lactoperoxidase, only the concentration of H_2O_2 seemed to interfere with the viability of microfilariae and 3rd-stage larvae.

Chloramine T, the small water-soluble catalyst might be adsorbed to the cuticle or even incorporated through the cuticle into inner structures; a fact which could explain the low specificity of this catalyst. Lactoperoxidase is a large molecule which will not penetrate the membrane as easily (Philips & Morrison, 1971) and thus catalyses surface iodination more specifically. Chloroglycoluril creates active iodine at the surface of the solid catalyst and/or by the few molecules soluble in the reaction solution. The possibility that active iodine is incorporated is therefore low.

Recently, Schlager (1979) reported that lactoperoxidase does not only mediate classical iodination of tyrosine and histidine in proteins but also certain lipids. The mechanism of the reaction remains unclear. The extraction of the PBS and PBS-NaOH treated sediments of *D. viteae* homogenates (Baschong *et al.* 1982) by *n*-butanol resulted in a concentration of approximately 10% of total radioactivity in the butanol layer (unpublished results), a fact which leads to the assumption that chloroglycoluril-mediated iodination is capable of labelling structures with lipophilic character.

It seems that the thick cuticle of females is the best barrier for the marker and the catalyst during the iodination procedure. This results in a more or less surface-specific, lactoperoxidase-mediated iodination compared to other stages and the highest resistance against toxic effects of the catalysts. In the larval stages, the cuticle is approximately 10 times thinner and there is no surface specificity after lactoperoxidase-mediated labelling. The loss of specificity with higher chloroglycoluril concentrations might indicate a higher sensitivity to the toxic effect of the catalyst as compared to adult worms.

The physical development of the autoradiographs with Agfa Gevaert Developer (Kopriwa, 1975) produces compact, rounded silver deposits whose size can be influenced by the developing time, resulting in relatively good resolution. Nevertheless, a certain dimension of the structures is required to attribute the label to specific parts of the cuticle. The successful labelling of female and male worms allows us to associate the marker on the autoradiographs with parts of the cortical layer of the nematode cuticle (Bird, 1980). The thin cuticles of microfilariae and 3rd-stage larvae, however, exclude localization of the marker within the cuticle.

In conclusion, it can be said that for the *D. viteae* system and probably other filarial systems, chloroglycoluril at low concentrations seems to be the most useful of the catalysts tested for cuticular iodination.

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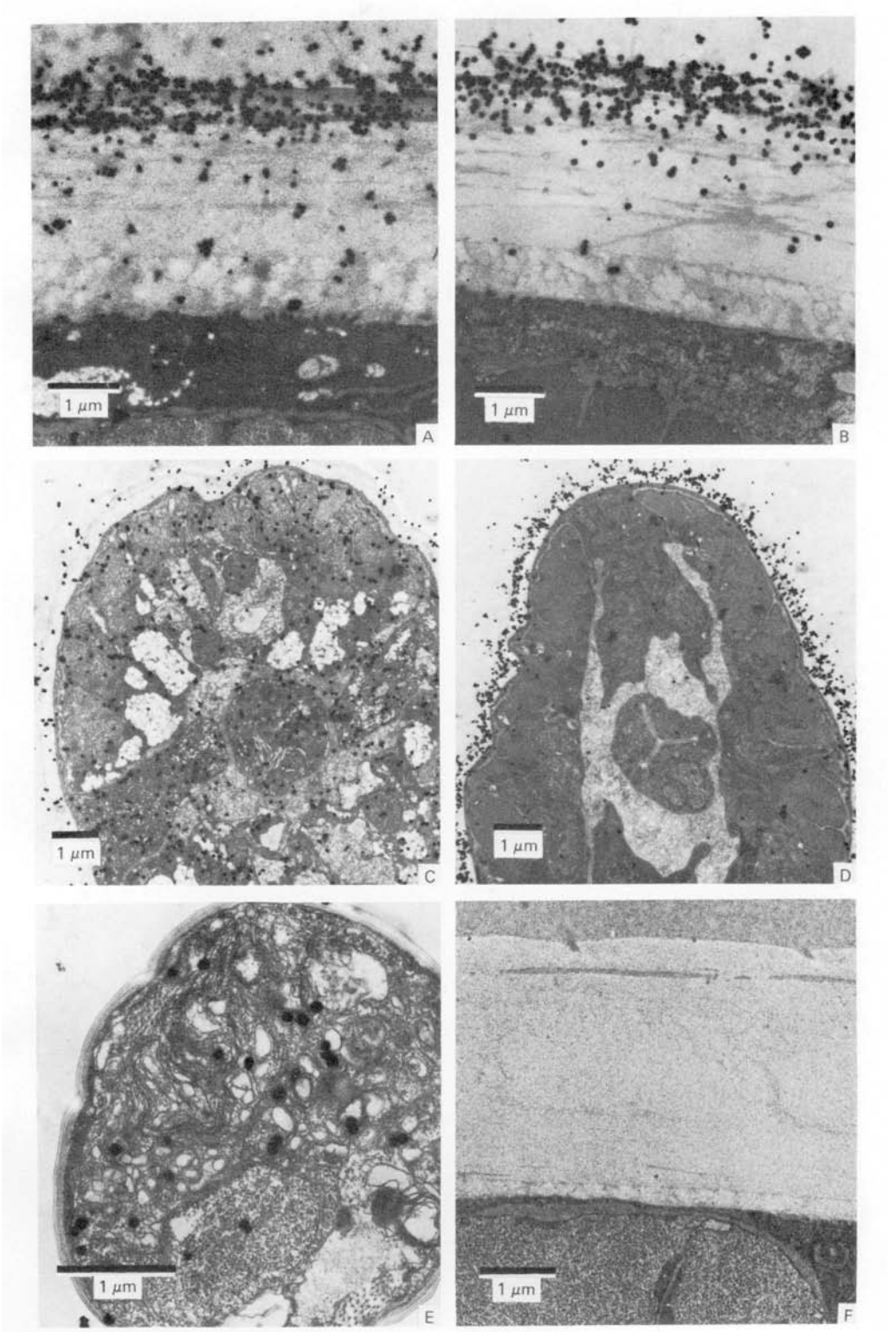

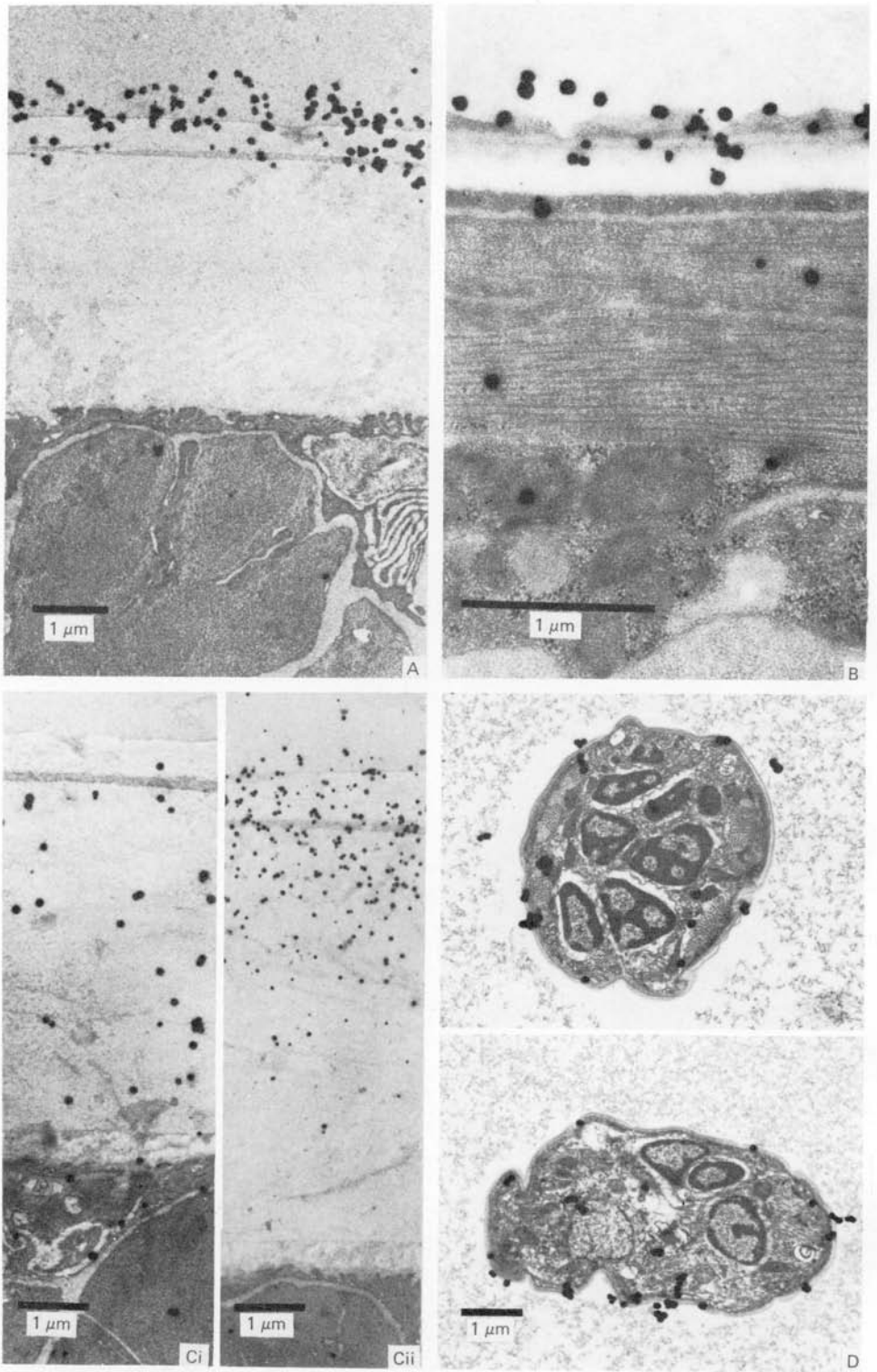

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EXPLANATION OF PLATES

Plate 1

A–E. Chloroglycoluril (CGU)-mediated iodination of *Dipetalonema viteae*.

A. Female, 100 μg CGU/200 μCi Na ^{125}I, exposure time 14 days.

B. Female, 10 μg CGU/200 μCi Na ^{125}I, exposure time 21 days.

C. Third-stage larva, 100 μg CGU/200 μCi Na ^{125}I, exposure time 16 days.

D. Third-stage larva, 5 μg CGU/500 μCi Na ^{125}I, exposure time 28 days.

E. Microfilaria, 100 μg CGU/200 μCi Na ^{125}I, exposure time 16 days.

F. Male *D. viteae* incubated with 200 μCi Na ^{125}I without catalyst, exposure time 21 days.

Plate 2

A and B. Lactoperoxidase (LPO)-mediated iodination of *Dipetalonema viteae*.

A. Male, 50 pmole LPO/100 μCi Na ^{125}I, exposure time 51 days.

B. Third-stage larva, 50 pmole LPO/200 μCi Na ^{125}I, exposure time 27 days.

C and D. Chloramine T(Chl T)-mediated iodination of *D. viteae*.

C. (i) Male, 125 ng Chl T/100 μCi Na ^{125}I, exposure time 21 days.

C. (ii) Male, 125 ng Chl T/100 μCi Na ^{125}I, exposure time 133 days.

D. Microfilariae, 125 ng Chl T/100 μCi Na ^{125}I, exposure time 27 days.

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