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A Method for the in vivo Radiolabelling of *Diplostomum spathaceum*, *Hypoderaeum conoideum*, Plagiorchiidae sp. and *Notocotylus attenuatus* Cercariae with Radioselenium

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Summary. Cercariae of the trematode species *Diplostomum spathaceum*, *Hypoderaeum conoideum*, Plagiorchiidae sp., and *Notocotylus attenuatus* were radiolabelled by incubation of their host snails in a medium containing radioselenium (^{75}Se-methionine). Substantial radiolabelling of all four species of cercariae was obtained from 3–18 days following the addition of the radioisotope to the medium. The levels of incorporation of radioactivity differed among the four species, but in general the levels obtained increased in proportion to the amounts of radioselenium in the medium. It was shown that the radiolabelling procedure did not interfere with the production of cercariae and the biological characteristics of the radiolabelled larvae. This makes the application of such labelled cercariae biologically acceptable.

A radioisotope system for assaying *Hypoderaeum conoideum* cercarial host-finding under various environmental conditions is described and evaluated. This system employs exposure of the second intermediate host snail, *Helisoma duryi*, to radiolabelled cercariae. A linear proportionality was found to exist between the number of penetrating cercariae and the amount of snail-bound radioactivity. Thus snail-bound radioactivity retained after exposure to radiolabelled cercariae can be used to measure cercarial host-finding capacity.

The possible application of cercarial radiolabelling in studies on the biology of larval trematodes is briefly discussed.

Introduction

Procedures designed to radiolabel *Schistosoma mansoni* cercariae were elaborated by Lewert and Para (1966), Bruce et al. (1969) using radiocarbon; by Reid et al. (1977) using ^{3}H-thymidine; by Knight et al. (1968) and Christensen (1977) using radioselenium. Recent investigations have aptly proved the feasibility of using such radiolabelled organisms in studies on *S. mansoni* cercarial host-finding (Christensen, 1978) and in immunological studies requiring differentiation of worm subpopulations (Phillips et al., 1977; Reid et al., 1977).

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Procedures for the in vivo labelling of non-schistosome cercariae appear not to have been described previously with the exception of Bibby and Rees (1971) who demonstrated the uptake of radioactive glucose in vivo and in vitro by metacercariae of *Diplostomum phoxini.*

The present study was designed to elaborate procedures for in vivo labelling of four non-schistosome species of cercariae with radioselenium. *Diplostomum spathaceum,* which penetrates fish and develops to metacercariae in the eye lens; *Hypoderaeum conoideum* which enters a suitable snail host to encyst; *Notocotylus attenuatus* which encysts on vegetation, and Plagiorchiidae sp. *N. attenuatus* was found in *Planorbarius corneus,* and the three other species were found in *Lymnaea stagnalis.*

Experiments were conducted to develop a system ensuring a useful and detectable level of incorporated radioisotope without interference with the production of cercariae and the biological characteristics of the cercariae. Furthermore, a useful and sensitive radioisotope system for assaying cercarial host-finding capacity of *H. conoideum* is worked out and evaluated.

Materials and Methods

Parasite and Snail Material

L. stagnalis and *P. corneus* were collected in the field and subsequently maintained in glass aquaria at a temperature of 26–28° C and fed blanched and dried lettuce. Two weeks after collection the patency of trematode infections was determined by screening the snails for emerging cercariae. Those snails which were found to be positive were kept individually in aquaria containing one litre of filtered pond water

Radioisotope and Radioactivity Determination

Radioselenium in the form of ^{75}Se-methionine in sterile water containing 100 µCi/ml (code S.C. IP. The Radiochemical Centre, Amersham) was used. Radioactivity determinations and statistics were performed as described by Nansen et al. (1976). A thallium-activated NaI scintillation well counter with a counting efficiency of 23% was used. All samples were counted for a period of 6 min, and correction was made for background counts (mean of background: 50 cpm).

Incorporation of Radioselenium Into Cercariae

Experiments were conducted to determine the rate of incorporation of radioselenium into the cercariae. A separate experiment was designed for each species of cercariae.

Increasing amounts of radioselenium were added to aquaria (one litre) each containing one infected snail. Two aquaria were used for each of the radioisotope levels tested. The snails were maintained in these aquaria for two weeks and were subsequently transferred to non-radioactive aquaria. Infected non-labelled snails maintained in aquaria without isotope served as controls.

Twice weekly for a period of three weeks the following parameters were recorded: (1) snail mortality, (2) production of cercariae, and (3) cercaria-bound radioactivity.

Snails were washed three times in filtered pond water prior to photic release of cercariae. Samples of cercariae were collected and washed repeatedly and gently using low-speed centrifugation until no free radioselenium could be detected in the water. The amount of cercaria-bound radioactivity was determined using three samples of a known number of cercariae shed from each snail.

Cercariae of *N. attenuatus* were first allowed to encyst on cellophane before being removed

as metacercariae and washed to remove free radioactivity. The cercarial tails, which remained in the suspension were isolated and washed by gentle low-speed centrifugation.

Effects of Radioisotope on Biological Parameters

The effects of radiolabelling on cercarial development and their biological characteristics were evaluated by four basic criteria: (1) snail mortality, (2) production of cercariae per snail, (3) behavioural activities of cercariae, and (4) cercarial penetration and encystation capabilities.

The infectivity of radiolabelled *D. spathaceum* cercariae (9 cpm/organism) was compared with that of cercariae shed from unlabelled snails. Two groups of four *Lepistes reticulatus* (guppy fish) were exposed to 200 labelled and unlabelled cercariae per group, respectively. Twelve days later the fish were sacrificed and the number of established metacercariae in the eye lenses were recorded.

H. conoideum cercariae with mean radioactivity levels of 0, 20, 30, 85, and 115 cpm per organism were used to infect *Helisoma duryi* snails. Individual *H. duryi* (3–4 mm in shell diameter) were exposed to 20 cercariae in 10 ml water for a period of 60 min. Then snails used at each cercarial-labelling level and the number of cercariae penetrating was determined stereomicroscopically.

The encystation capacity of radiolabelled *N. attenuatus* cercariae (35 cpm/organism) was studied by comparing the number of performed encystations with the number of encystations of an unlabelled control group.

A Radioisotope System
for Assaying Cercarial Host-Finding Capacity

Radiolabelled *H. conoideum* cercariae were obtained from *L. stagnalis* according to the procedures described above.

Individual *H. duryi* (3–4 mm in shell diameter) were exposed to a suspension of radiolabelled cercariae (86 cpm/organism) in test beakers containing 100 ml filtered pond water at a temperature of 24° C and a pH of 7.8 for a period of 60 min. The number of cercariae per beaker was 10, 20, 30, and 40, respectively, and ten snails were tested at each cercarial dose level. After the exposure, the snails were individually marked, and the number of penetrating cercariae per snail was determined stereomicroscopically. The amount of snail-bound radioactivity was subsequently determined as described by Nansen et al. (1976).

Results

Incorporation of Radioselenium Into Cercariae

The rates of incorporation of radioactivity in newly emerged cercariae of the four species are shown in Figures 1 and 2.

Diplostomum spathaceum

The most heavily labelled cercariae appeared to be those which emerged between three and ten days following the addition of the isotope. The peak level was reached at day 7. The radioactivity incorporation levels obtained throughout the experimental period increased with increasing amounts of radioselenium in the culture medium. The subsequent decrease of radioactivity was rapid for high initial radioactivity levels and slower at low levels. Following the addition of the isotope in amounts of 50, 100, and 150 μCi per snail, counts for cercariae

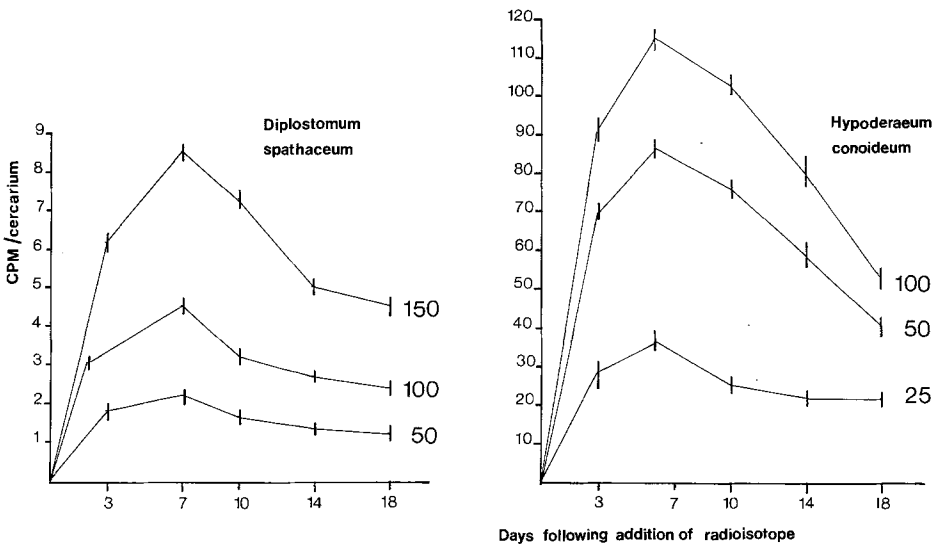

Fig. 1. Time course study of radioisotope incorporation. Radioactivity confined to *Diplostomum spathaceum* and *Hypoderaeum conoideum* cercariae derived from *Lymnaea stagnalis* exposed to different levels of radioselenium. Average radioactivity (net counts ± SD) cpm confined to cercariae from two snails at each dose level

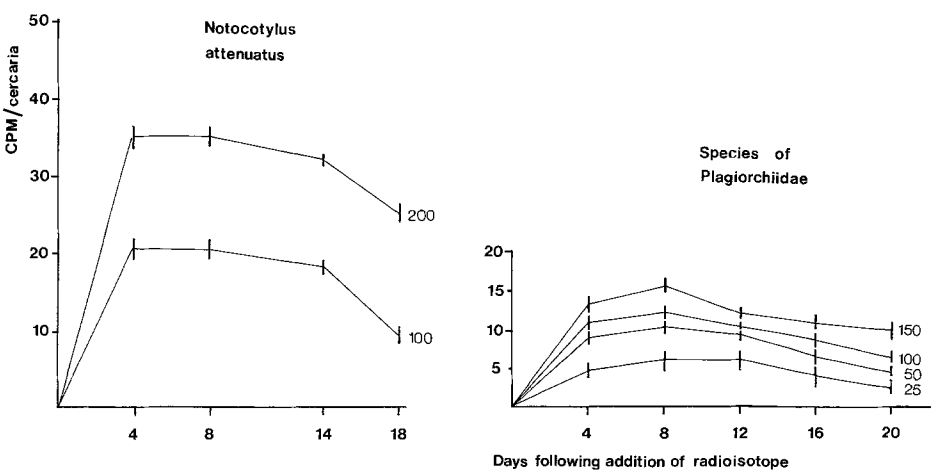

Fig. 2. Time course study of radioisotope incorporation. Radioactivity confined to Plagiorchiidae sp. and *Notocotylus attenuatus* cercariae shed from *Lymnaea stagnalis* and *Planorbarius corneus*, respectively. Average radioactivity (net counts ± SD) cpm confined to cercariae from two snails at each dose level

obtained at day 18 represented 86%, 55%, and 53%, respectively, of the peak levels obtained at day 7.

Hypoderaeum conoideum

Markedly higher radioactivity incorporation levels were obtained with these cercariae than with those of *D. spathaceum*. The maximum cercaria-bound ra-

dioactivity was obtained 7 days following the exposure of snails to radioselenium and the radioactivity levels obtained throughout the experimental period increased with increasing amounts of radioselenium in the water. The peak at day 7 was followed by a rapid and almost linear decrease of cercarial labelling. At the lowest incubation level the decline following the peak level proceeded at a somewhat slower rate than at high levels. However, for all incubation levels detectable amounts of cercaria-bound radioactivity could be demonstrated for up to 18 days.

Plagiorchiidae sp.

Cercariae of this type exhibited relatively higher radioactivity levels than *D. spathaceum*, but considerably lower levels than *H. conoideum*. The maximum labelling was obtained between four and 12 days following administration of the isotope, and the peak level obtained during this period increased with increasing amounts of radioselenium in the water. Following the peak level the radioactivity per organism declined slowly during the remainder of the experimental period.

Notocotylus attenuatus

The maximum cercarial labelling was obtained by day 4 after the administration of radioselenium and this initial high level persisted until day 14, followed by a slow decline. The amount of cercarial tail-bound radioactivity constituted 6–9% of the total cercaria-bound radioactivity.

Effect of Radioisotope on Biological Parameters

No difference could be detected between mortality rates of labelled and unlabelled snail groups, respectively, and no interference with the production of any of the four species of cercariae could be demonstrated. At any applied dose level of radioselenium the production of cercariae from labelled snails was roughly comparable with that of unlabelled snails, and a continuous production of labelled cercariae was observed for a period of at least 18 days. The daily production of cercariae per snail was as follows: *D. spathaceum*, 400–800; *H. conoideum*, 50–100; Plagiorchiidae sp., 100–150; *N. attenuatus*, 150–200.

With regard to behavioural activity and longevity no difference could be observed between labelled and unlabelled cercariae of any of the four species.

No difference existed in the infectivity of labelled and unlabelled *D. spathaceum* cercariae. The number of metacercariae recovered at autopsy from the two groups of *L. reticulatus* was 40 and 42%, respectively. There also appeared to be no difference in the penetration capacity of labelled and unlabelled *H. conoideum* cercariae. The number of cercariae which failed to penetrate was less than 5% for all groups. Similarly, no difference could be detected in the encystation capacity of labelled and unlabelled *N. attenuatus* cercariae. In both groups, only 10% of the cercariae failed to encyst.

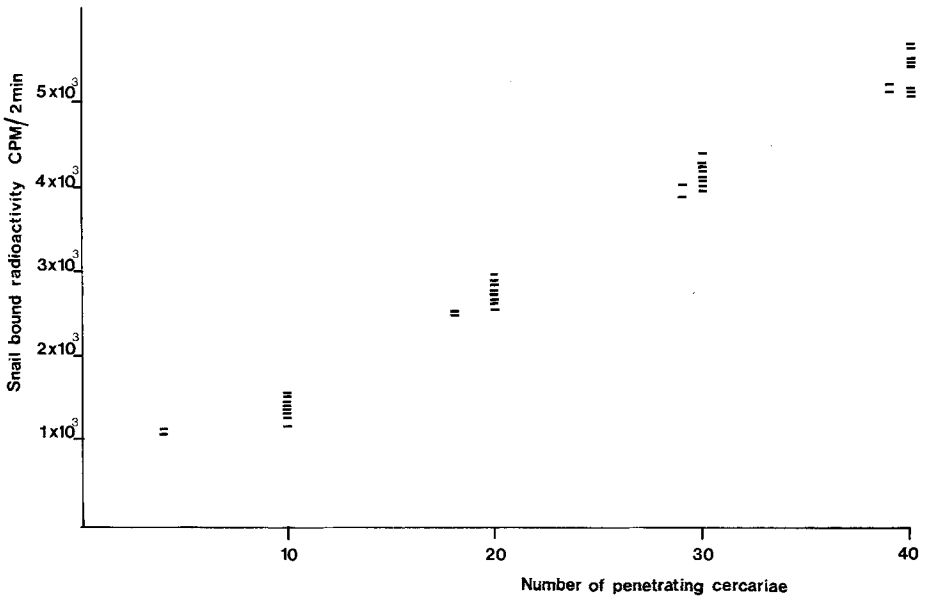

Fig. 3. Radioactivity (net counts/2 min) confined to *Helisoma duryi* in relation to the number of penetrating *Hypoderaeum conoideum* cercariae. Ten snails at each cercarial dose level

A Radioisotope System for Assaying Cercarial Host-Finding Capacity

The radioactivity confined to *Helisoma duryi* exposed to radiolabelled *H. conoideum* cercariae is shown in Figure 3. Only minor individual variations could be demonstrated in the amount of snail-bound radioactivity after exposure to identical numbers of radiolabelled cercariae (maximum SD $\pm$ 9%).

A linear relationship was found between the calculated number of penetrating cercariae and the amount of snail-bound radioactivity. The amount of radioactivity confined to the snails corresponded to approximately 80% of the total cercaria-bound radioactivity in a given test beaker. Within the limits of cercarial dose levels used in the present experiment a direct proportionality existed between the number of cercariae available and the amount of radioactivity taken up by exposed snails.

Discussion

It has become increasingly evident that radioisotope tracer techniques are essential for throwing light on several important aspects of parasitic infections and parasite ecology. A number of recent studies show that the application of radiolabelled *Schistosoma mansoni* cercariae may provide valuable quantitative and qualitative information on the host-parasite relationship which is difficult to obtain by more traditional techniques (Phillips et al., 1977; Christensen, 1978).

The present experiments were performed to develop procedures for radiolabelling non-schistosome cercariae without interfering with the biological characteristics of the host-parasite relationship. Furthermore, the practical application of such radiolabelled organisms in studies on cercarial ecology was exemplified.

Although the radioactivity levels obtained in different species of cercariae varied considerably, the pattern of radioactivity incorporation was roughly comparable for *D. spathaceum, H. conoideum,* and Plagiorchiidae sp. cercariae. The maximum amount of cercaria-bound radioactivity was achieved 7–8 days after exposure of infected snails to the radioisotope, and this initial high level increased in proportion to the amount of radioselenium in the exposure medium. Detectable levels of cercaria-bound radioactivity were achieved for a period of at least 18 days following the addition of the radioisotope.

With the exposure levels used no interference could be demonstrated with snail survival or with the production of cercariae of any of the four species and apparently the radiolabelling did not influence the biological characteristics of the labelled larvae. Thus, cercarial swimming behaviour and longevity, penetration and encystation capabilities, and cercarial infectivity were within the limits defined with unlabelled control larvae.

The apparent lack of effect of radiolabelling upon cercarial development and cercarial behaviour makes the application of such labelled cercariae biologically acceptable. Cercariae obtained during repeated sheddings show normal biological activity while continuing to demonstrate relatively high radioactivity levels. This indicates that it is possible to make multiple use of a single isotope administration for different experiments.

The radioisotopical system for assaying the host-finding capacity of *H. conoideum* cercariae described in this paper seems to overcome some of the problems encountered in more commonly used methods. Similar systems for assaying *Fasciola hepatica* miracidial and *S. mansoni* cercarial host-finding have been described earlier (Nansen et al., 1976; Christensen et al., 1977a). Applying these assay systems, a considerable amount of information has been obtained concerning miracidial and cercarial host-finding in relation to various biological and environmental characteristics (Christensen et al., 1976a, b, 1977b; Christensen, 1978), and we suggest that the assay system described here will be able to provide further valuable information on larval ecology of other trematode species.

Studies should also be conducted to evaluate the possible application of this radiolabelling methodology in studies on immunological, pathophysiological, and migrational aspects of these trematode infections in intermediate and final hosts.

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