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STUDIES ON SCHISTOSOMIASIS. 4*. DIFFERENTIAL STAINING OF LIVE AND DEAD CERCARIAE AFTER IMMOBILIZATION WITH PHYSOSTIGMIN

J. A. VAN WYK, Veterinary Research Institute, Onderstepoort

ABSTRACT

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A modified eosin technique similar to that used for differential staining of live and dead spermatozoa stained dead cercariae of *Schistosoma mattheei* red while those that were alive remained colourless and transparent. Prior to exposure to the dye live cercariae were immobilized with physostigmin, which was better for that purpose than five other substances tested.

The lowest concentration of physostigmin to immobilize cercariae quickly and effectively was found to be $3{,}3 \times 10^{-6}$ M.

INTRODUCTION

Studies on the effects of ageing and physical or chemical treatments on cercariae are hampered by the fact that it is impossible to differentiate between live and dead specimens. Krakower (1940) and Ingalls (1946), amongst others, used immobility and/or reduction in refractivity and crenation as criteria of death. However, Lewert & Hopkins (1965) and Kuntz & Stirewalt (1946) found that some of these states may be transitory.

The method of differentiation described in this paper is a modification of that used for staining sheep and cattle spermatozoa (Lasley, Easley & McKenzie, 1942; Blom, 1950).

In preliminary experiments (Van Wyk, unpublished data) the smear method of differentiation after staining with eosin (Lasley *et al.*, 1942; Blom, 1950) was found unsuitable because cercariae absorbed the dye as they dried out. The method was therefore modified for examining cercariae in a wet preparation.

In such a wet preparation cercariae are, however, difficult to handle because the numbers are usually low and squash preparations under coverslips (Sadig, 1970) contain too few cercariae for accurate evaluation. Moreover, during staining live cercariae attach themselves to the sieve when the stain is removed, hence inaccurate differential counts are to be expected when using this technique.

Various methods have been tried to overcome this problem: silicone coating of glassware (Fife, Sleeman & Bruce, 1967); concentration by continuous-flow centrifuging (Olivier, 1966a) or a combination of positive phototropism, negative geotropism and temperature preference (Standen, 1950); sieving, filtering (Raybould, according to Bradley, 1967) and sedimentation at low temperatures (Pellegrino & Nunes, 1956).

As all these techniques have serious limitations it was necessary to develop one which would temporarily immobilize cercariae with a wide margin of safety.

Many workers have investigated the effects of chemical and physical agents on schistosome cercariae and have used various criteria to assess their efficacy: infectivity after treatment (Kuntz & Stirewalt, 1946); swimming or passive sinking to the bottom of the container (Krakower, 1940; Ingalls, 1946; Kuntz & Stirewalt, 1946; Lewert & Hopkins 1965); active settling-down (Bolwig, 1955); mobility, or lack thereof, once down (Krakower, 1940; Ingalls, 1946; Kuntz & Stirewalt, 1946; Bolwig, 1955; Lewert & Hopkins, 1965); reduction in refractivity (Ingalls, 1946; Kuntz & Stirewalt, 1946); crenation (Ingalls, 1946), and active shedding of cercarial tails (Bolwig, 1955).

In these investigations the above and other criteria were used to determine thoroughly the effects of six immobilizing substances on cercariae.

ORIGIN OF CERCARIAE

The strain of *Schistosoma mattheei* used was isolated from naturally infested cattle in Zululand in 1964 (McCully & Kruger, 1969). It was maintained in the laboratory in sheep and in *Bulinus* (*Physopsis*) *globosus* and *B.* (*P.*) *africanus,* the descendants of snails collected in Zululand, the Transvaal Lowveld and Pretoria District. The methods of Kruger & Heitmann (1967) and Heitmann (1969) were used for maintenance of the parasite.

COLLECTION OF CERCARIAE

The cercariae were obtained from *B.* (*P.*) *globosus* and *B.* (*P.*) *africanus* infested individually with five miracidia each. When cercariae were required, groups of 20 to 60 infested snails were placed in 500 ml glass beakers containing 250 ml filtered river water. The beakers were placed in bright, cold (fluorescent) light at 25°C for an hour, then the cercarial suspension was harvested and replaced by an equal volume of river water. Harvesting was repeated at the end of the 2nd and 3rd hour and all the cercariae were pooled in measuring cylinders. Snail faeces and other heavy impurities were removed by sedimentation. Each experiment was started within 3 hours of collection of the last cercariae

THE TESTS

I *Observations using criteria other than infectivity of cercariae*

(a) *Preliminary trial with physostigmin*

Lewert & Hopkins (1965) showed that physostigmin caused temporary immobilization of cercariae. In a preliminary trial low concentrations of this substance were tested to facilitate the handling of cercariae in subsequent tests. This was refined and will be described later [Section I (c) below].

A concentration of $1{,}2 \times 10^{-4}$ M physostigmin was effective for quick, reversible immobilization of live cercariae, which did not attach themselves to a 37μm sieve on which they were placed while in contact with the drug. Untreated, live cercariae were able to pene-

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trate 37 μm sieve openings, but immobilized cercariae were not lost in this way.

(b) *Differential staining with eosin and physostigmin*

In a wet preparation dye added to the cercarial suspension must be removed by washing for evaluation of its staining effect on live and dead cercariae. This is most easily done by washing the cercariae on a sieve.

When a method of preventing attachment of cercariae to a sieve was found [Section I (a) above], eosin could be tested for differential staining of live and dead cercariae.

Method

After mixing as described above, 90 ml cercarial suspension was poured off into each of two 100 ml glass measuring cylinders, designated Suspensions A and B respectively.

Cercarial Suspension A was heated to 60°C for 5 minutes and then allowed to cool to room temperature. Suspension B was not heated. Subsequently 10 ml 5% eosin bluish* aqueous solution was added to each suspension, followed by 1 ml 0,012 M physostigmin (eserine)**, final concentration $1{,}2 \times 10^{-4}$ M, to immobilize cercariae which were alive at the time of staining.

After 5 minutes the suspensions were washed on a 37 μm aperture sieve to remove the eosin and physostigmin. To ensure complete collection of the cercariae, the cylinder was repeatedly washed under a strong stream of water and the washings poured out on to the sieve each time. The cercariae were collected in a glass tube (60 ml). Formol-saline was added to Suspension A to give a final concentration of 3% formalin. Suspension B was divided into 2 parts: one was formalinized as for Suspension A, while the other was left untreated after washing. Aliquots of the suspensions were examined under a stereoscopic microscope.

Results

The dead cercariae from Suspension A, when stained with eosin, were all opaque and deep red in colour (Fig. 1a & 1b).

In the formalinized portion of Suspension B 2 135 (98,8%) carcariae were transparent and colourless (Fig. 1a & 1b), while 25 (1,2%) resembled those in Suspension A. In the unformalinized portion of Suspension B the unstained cercariae soon became mobile again and were swimming within 10 minutes after removal of the chemicals.

Discussion

When using physostigmin and eosin a good colour contrast was obtained between live and dead cercariae (Fig. 1a & 1b) and the treatment did not appear to kill live cercariae.

After adding formalin it was possible to distinguish for several days between the cercariae that were alive and those that were dead when they were exposed to the stain. If formalin was not added after the suspension was washed, the live cercariae quickly recovered and rapid elution of the dye from the red (dead) cercariae took place. In a subsequent experiment it was found that cercariae killed with formalin before staining, absorbed the eosin dye poorly and hence were difficult to distinguish from cercariae which had been alive at the time of treatment (unpublished data).

During staining, many dead cercariae adhered to the container and a strong jet of water was required to remove them. This factor could affect the results obtained by this method.

This trial showed that eosin staining effectively differentiated between live and dead cercariae when combined with a certain concentration of physostigmin. Thereafter this chemical and five others were tested in detail to determine the optimal substance to use for cercarial immobilization in this technique.

(c) *Detailed trials with six chemicals*

Cercariae in suspension were tested with physostigmin (eserine), pentobarbital sodium*, chloral hydrate, ether**, halothane*** and carbon dioxide.

Method

Stock solutions of 0,012M physostigmin (brought into solution by the addition of a few crystals of citric acid), 10% chloral hydrate and saturated ether solution were prepared and diluted as required. Carbon dioxide was bubbled through the aliquots at predetermined rates of flow for 200 and 100 seconds. A constant flow of air (of which the flow rate was determined) was bubbled through the halothane and this air plus halothane mixture bubbled through the aliquots for 25, 10 and 5 seconds respectively.

The cercarial suspensions were mixed by pouring them back and forth between two containers; bulb pipettes were used to withdraw 20 ml aliquots which were placed in glass bottles (60 ml) to a depth of 2,3 cm. The experiments were initiated within 3 hours after collection of the last cercariae. The experiments described did not run concurrently, necessitating the use of different batches of cercariae. With a few exceptions, however, the different dilutions of a given substance were tested concurrently.

Two cercarial suspensions were tested with each dilution of each substance and two served as untreated controls. One batch of each dilution, as well as its control, was left undisturbed for 90 minutes, before it was treated with eosin and physostigmin to determine the percentage of live cercariae present in the sample. The behaviour of the cercariae in the other suspension was recorded until they had all sunk to the bottom of the container; they were then observed for a further 30 seconds for spontaneous movements and their response to thigmotropic stimuli was tested. Thereafter they were washed on a 37 μm aperture sieve, re-suspended in untreated water and the rate and degree of recovery assessed. The control cercariae were not washed as they would adhere to the sieve. Twenty to 24 hours after treatment the cercariae in the treated and control suspensions were immobolized with physostigmin (final concentration 3×10^{-4} M), stained with eosin and the percentage of live cercariae determined (Section I b above).

The criteria used to assess the degrees of immobilization and the recovery rate are listed in Table 1.

Results

The criteria used for judgement of recovery and the results obtained with the different test substances are listed in Tables 1 and 2.

*Matheson, Coleman & Bell
**British Drug House

*Sagatal, Maybaker
**Anaesthetic Ether, Natal Cane Byproducts
***Fluothane, I.C.I.

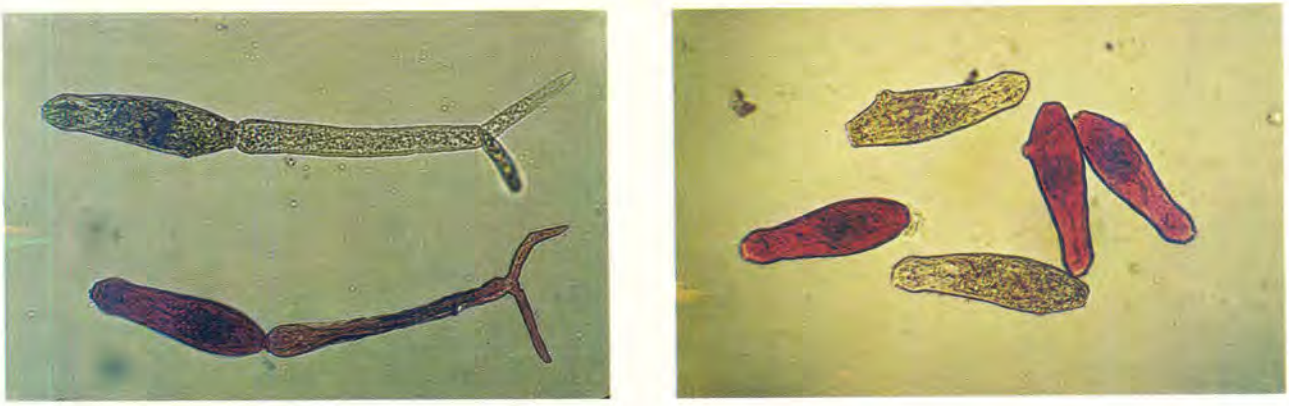

FIG. 1 a) Intact and b) tailless schistosome cercariae after exposure to eosin stain in a wet preparation. The red cercariae were dead and the light coloured cercariae alive when exposed to the stain.

Immobilization

The immobilization and subsequent recovery of cercariae usually followed a basic sequence:

(i) *Induction phase:* In most instances exposure to the drug was followed by a period of hypermotility, during which the majority of the cercariae concentrated at the surface of the water. As they became immobilized, however, they sank to the bottom. Some substances such as chloral hydrate caused intense hypermotility but others, for example certain concentrations of ether, immobilized the cercariae instantly. Intermediate reactions also occurred: alternate contraction and relaxation of the cercarial head and/or tail and cramp-like lateral contractions of the heads, giving a banana- or horseshoe-shape. Swimming either ceased abruptly [e.g. Specimen 2(a) in Table 1] or the bouts of swimming decreased in frequency until they stopped completely.

(ii) *Immobilization phase*

This phase persisted while the cercariae were in contact with the chemicals. Spontaneous recoveries occurred only at very low concentrations, when immobilization was incomplete.

The live, immobilized cercariae either had a normal refractivity (e.g. in ether), or were more opaque in appearance (i.e. translucent) than normal. The translucency was often as pronounced (e.g. with pentobarbital sodium and carbon dioxide) as in dead cercariae and was sometimes accompanied by marked crenation (e.g. in pentobarbital sodium). The cercarial heads were usually relaxed and oval in shape but in high concentrations of physostigmin more than 50% of the heads became elongated.

In the case of chloral hydrate, the posterior suckers of the cercariae became relaxed and protruded when the cercariae were immobilized.

Recovery

The degree of reversibility of immobilization depended on the chemical used, its concentration and the time of exposure.

Recovery usually followed the same pattern:

(i) *Movements in stationary cercariae:* When cercariae began to recover, they contracted and extended their heads and/or tails, curled and uncurled their furcae and bent their heads and tails laterally either slowly and deliberately (e.g. in physostigmin) or with infrequent flicks alternated with periods of quiescence (e.g. in chloral hydrate). As recovery progressed, these movements increased in tempo and intensity. The whiplike oscillation seen in normal cercariae was not, however, noted during the initial stages of recovery.

(ii) *Swimming:* The period of slow oscillation was followed by attempts at swimming off the bottom of the container. In some instances (e.g. in chloral hydrate) recovering cercariae swam from a relaxed position without passing through a period of oscillation. The first swimming motions were very sluggish and the intervals between swimming bouts longer than usual and cercariae would swim off the bottom and then settle again. The swimming movements became stronger and were jerky initially but later became smoother until they almost resembled the usual movements of normal cercariae.

(iii) *Crawling:* Cercariae could swim before they were able to crawl or creep along the bottom of the container.

This crawling was preceded by extension and relaxation of the cercarial head and by protrusion and retraction of the posterior sucker. Crawling increased in intensity until cercariae were able to adhere actively to the container when the water was swirled. Tailless cercariae usually started crawling before intact ones.

When ether, halothane and carbon dioxide were used there was an apparent adherence during the period of immobilization. This appeared to be passive, however, as the tips of the heads of the cercariae and not the suckers were in contact with the container and the cercariae swayed to and fro with the current.

Loss of cercarial tails

Many cercariae lost their tails in the higher concentrations of chloral hydrate, ether, pentobarbitalsodium and halothane. With ether some of the tails appeared to be shed actively. Cercariae which had lost their tails died before the intact ones.

Ageing or normal (control) cercariae

As cercariae aged their behaviour changed as follows:

(a) *Swimmers:* Swimming actions became jerky instead of smooth, as in newly emerged cercariae, and later became sluggish.

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TABLE 1 Criteria used to assess the effect of immobilizing chemicals on cercariae

Chemical and Concentration	Effect of chemical: Induction phase: Hyperactivity	Induction phase: Sucker movement	Induction phase: Head telescoping & Furcae movement	Immobilization phase: Down by (time)	Immobilization phase: Mobile	Immobilization phase: Translucency	Immobilization phase: Thigmotropism reaction	Immobilization phase: Adhere to container	Washed at (time) (min)	Recovery of cercariae after removal of chemical: First observation after washing (time of return of criteria: min): Furcae movement	Head movement	Slow flexing	Stationary oscillation	First swimmers hovering	First swimmers well afloat	Crawling	Adhere to container	Second observation: Time after washing (min)	Second observation: Swimmers: % (Estimate)	Second observation: Swimmers: Sluggish	Second observation: Swimmers: Jerky	Second observation: Swimmers: Smoother	Second observation: Non-Swimmers: Crawlers	Second observation: Non-Swimmers: Adhere to container	Third observation: Time after washing (h)	Third observation: Swimmers: % (Estimate)	Third observation: Swimmers: Sluggish	Third observation: Swimmers: Jerky	Third observation: Swimmers: Smoother	Third observation: Non-Swimmers: Crawlers	Third observation: Non-Swimmers: Adhere to container	% alive at 20–24 hours
1. PHYSOSTIGMIN (a) $1{,}2 \times 10^{-3}$ M	+	A	•	9	O	++	P	O	14	6	16	6	10	14	19	No	No	74	0	O	O	O	O	O	22	10	M	F	O	O	O	94,2
(b) 3×10^{-4} M	+	+	++	6	O	++	P	O	8	•	33	14	19	19	20	No	No	116	70	F	M	O	F	F	22	90	F	N	N	O	O	94,1
(c) $1{,}5 \times 10^{-4}$ M	++	+	+	5	F	++	P	O	17	2	•	2	7	9	13–15	23	No	68	•	•	•	•	F	F	22	90	F	N	N	O	O	93,3
(d) 6×10^{-5} M	+++	A	+	5	N	++	P	O	17	•	24	4	10	12	14–18	23	No	71	50	F	N	N	F	F	22	90	F	N	N	F	O	91,6
(e) 2×10^{-5} M	+++	A	++	10	N	++	P	O	16	•	•	2	7	11	17	24	No	76	90	F	N	N	F	F	22	90	F	N	N	F	O	92,1
(f) 5×10^{-6} M	+++	A	A	13	N	+	P	O	18	•	16	1	2	3	10–12	•	No	72	80	N	N	N	F	O	22	80	F	N	N	O	F	92,7
(g) $3{,}3 \times 10^{-6}$ M	+++	A	++	16	N	+	P	O	21	•	•	0–1	1	3	4–9	•	No	70	90	F	N	N	•	O	22	80	F	N	N	O	F	95,4
(h) $1{,}65 \times 10^{-6}$ M	+++	A	A	>19	M	+	P	O	24	•	•	0–1	1	1	6	•	No	64	90	F	N	N	O	F	22	80	F	N	M	•	F	91,8
(i) Control	A	A	A	—	M	A	•	N	—	—	—	—	—	—	—	—	—	—	95	O	N	N	N	F	—	90	F	N	M	F	F	94,8
2. ETHER (a) 50% saturated solution	A	A	A	3	O	A	•	N	3	5	1	2	•	No	No	•	5	5	0	O	O	O	O	N	22	0	O	N	O	O	O	0,0
(b) 33% saturated solution	A	A	A	6	F	A	P	N	15	•	—	<1	1	2	6	50	No	100	90	N	M	F	N	O	24	40	F	N	M	O	O	34,0
(c) 20% saturated solution	+	A	++	•	N	A	P	N	12	•	—	•	•	<2	2	52	<52	78	60	F	M	N	O	O	22	40	F	M	M	O	O	46,4
(d) Control	A	A	A	—	M	A	•	N	—	—	—	—	—	—	—	—	—	—	95	O	N	M	F	O	—	95	N	M	F	O	O	73,0
3. PENTOBARBITAL (a) $1{,}2 \times 10^{-1}$ M	A	A	A	•	O	+++	A	O	8	3	2	3–8	8	17	No	No	19	55	<1	M	O	O	O	N	21	0	O	F	N	O	F	0,5
(b) $4{,}8 \times 10^{-2}$ M	++	A	++	8	F	+++	P	O	8	3	1	1–3	3	1	3	4	7	110	90	N	N	M	N	F	21	80	F	N	N	F	F	89,3
(c) $2{,}4 \times 10^{-2}$ M	++	A	++	24	M	+++	A	O	25	4	4	<3	3	6	•	5	7	62	80	N	N	F	•	•	21	40	F	N	N	F	F	62,5
(d) $1{,}2 \times 10^{-2}$ M	++	A	++	>85	M	++	P	•	85	•	•	<1	<1	<1	<1	•	•	20	90	N	N	F	•	•	21	40	N	N	N	F	O	79,7
(e) $3{,}6 \times 10^{-3}$ M	++	•	•	>80	M	+	•	•	80	•	•	<1	<1	<1	<1	<1	•	10	95	F	N	M	•	•	21	95	F	N	N	O	F	91,3
(f) Control	A	A	A	—	M	A	•	N	—	—	—	—	—	—	—	—	—	—	90	O	N	M	N	N	—	80	F	O	N	O	F	95,0
4. HALOTHANE (a) Bubble 420 ml gas	A	A	A	6	F	++	P	F	11	No	No	No	No	No	No	No	No	2	0	O	O	O	O	O	20	0	O	O	F	O	O	0,0
(b) Bubble 170 ml gas	A	+	++	5	N	++	P	N	12	•	1	<1	<1	<1	1	1	1–9	98	20	F	M	F	N	N	20	20	M	F	O	O	O	28,1
(c) Bubble 85 ml gas	A	+	++	4	N	+	P	N	15	•	1	<1	<1	<1	1	2	<82	82	40	F	M	O	N	F	20	40	N	N	O	O	O	49,6
(d) Control	A	A	A	—	M	A	•	N	—	—	—	—	—	—	—	—	—	—	70	O	N	M	N	N	—	70	N	N	F	O	O	78,9
5. CO_2 (a) Bubble 3600 ml gas	A	A	A	8	M	+++	•	N	12	•	•	<1	<1	<1	<1	5	<1	91	70	O	N	N	N	N	22	40	N	N	M	F	F	49,3
(b) Bubble 1800 ml gas	A	A	A	10	M	+++	•	N	13	•	•	<2	<2	<2	<2	4	3	92	80	O	N	N	N	N	22	40	N	N	M	F	F	51,5
(c) Control	A	A	A	—	M	A	•	N	—	—	—	—	—	—	—	—	—	—	70	•	•	•	N	N	—	80	O	N	N	O	F	94,3
6. CHLORAL (a) $9{,}1 \times 10^{-2}$ M	+++	++	++	6	O	++	A	O	9	6	9	7	•	21	26	24	No	86	60	N	M	F	O	O	21	30	M	N	N	O	O	59,6
HYDRATE (b) $5{,}5 \times 10^{-2}$ M	+++	++	•	±10	N	++	P	O	11	•	3	3	•	6	7	15	No	80	60	O	M	F	O	O	21	80	N	M	N	O	O	80,4
(c) $4{,}2 \times 10^{-2}$ M	+++	++	++	>23	M	++	P	O	27	4	3–12	•	•	6	7	No	No	60	20	F	O	M	F	O	21	60	M	N	N	O	F	35,4
(d) Control	A	A	A	—	M	A	•	N	—	—	—	—	—	—	—	—	—	—	80	O	N	M	N	N	—	90	F	N	N	O	O	76,1

O = nil, F = few, N = numerous (<50%), M = majority (>50%), • = no observation, — = not applicable, No = no recovery to this extent, P = present, A = absent, + = slight, ++ = medium intensity, +++ = prominent

TABLE 2 Differential staining of live and dead cercariae

Chemical and concentration	Unwashed (1,5 hours)					Washed (20-24 hours)	
	Stained				Unstained	Stained	Unstained
	% White & Red or LT Pink	% Red & Pink or Dark Pink	% Red	Total % Stained (Dead)	% White (Live)	(Dead) %	(Live) %
1. PHYSOSTIGMIN (a) $1,2 \times 10^{-3}$M	*•	•	62,4	62,4	37,6	5,8	94,2
(b) 3×10^{-4}M	•	•	19,2	19,2	80,8	5,9	94,1
(c) $1,5 \times 10^{-4}$M	•	•	7,6	7,6	92,4	6,7	93,3
(d) 6×10^{-5}M	•	•	1,0	1,0	99,0	8,4	91,6
(e) 2×10^{-5}M	•	•	2,0	2,0	98,0	7,9	92,1
(f) 5×10^{-6}M	•	•	2,7	2,7	97,3	7,3	92,7
(g) $3,3 \times 10^{-6}$M	•	•	2,7	2,7	97,3	4,6	95,4
(h) $1,65 \times 10^{-6}$M	•	•	2,0	2,0	98,0	8,2	91,8
(i) Control	0,0	0,0	1,7	1,7	98,3	5,2	94,8
2. ETHER (a) 50% saturated solution	0,0	0,0	100,0	100,0	0,0	100,0	0,0
(b) 33% „ „	1,0	41,6	51,3	93,9	6,1	66,0	34,0
(c) 20% „ „	0,0	1,4	3,3	4,7	95,3	53,6	46,4
(d) Control	0,0	0,0	3,8	3,8	96,2	27,0	73,0
3. PENTOBARBITAL SODIUM (a) $1,2 \times 10^{-1}$M	•	•	92,0	92,0	8,0	99,5	0,5
(b) $4,8 \times 10^{-2}$M	•	•	2,7	2,7	97,3	10,7	89,3
(c) $2,4 \times 10^{-2}$M	•	•	4,3	4,3	95,7	37,5	62,5
(d) $1,2 \times 10^{-2}$M	•	•	3,8	3,8	96,2	20,3	79,7
(e) $3,6 \times 10^{-3}$M	•	•	2,0	2,0	98,0	8,7	91,3
(f) Control	0,0	0,0	1,7	1,7	98,3	5,3	95,0
4. HALOTHANE (a) Bubble 420 ml gas	0,0	0,0	100,0	100,0	0,0	100,0	0,0
(b) „ 170 ml gas	17,3	0,0	70,0	87,3	12,7	71,9	28,1
(c) „ 85 ml gas	0,0	0,0	97,0	97,0	3,0	50,4	49,6
(d) Control	0,0	0,0	1,5	1,5	98,5	21,1	78,9
5. CO_2 (a) Bubble 3600 ml gas	15,0	6,7	0,4	22,1	77,9	50,7	49,3
(b) Bubble 1800 ml gas	2,7	0,0	0,5	2,7	96,8	48,5	51,5
(c) Control	0,0	0,0	0,7	0,7	99,3	5,7	94,3
6. CHLORAL (a) $9,1 \times 10^{-2}$M	1,0	97,8	1,0	99,8	0,2	40,4	59,6
(b) $5,5 \times 10^{-2}$M	43,1	38,8	5,4	87,3	12,7	19,6	80,4
(c) $4,2 \times 10^{-2}$M	45,8	7,7	4,1	57,6	42,4	64,6	35,4
(d) Control	0,0	0,0	2,4	2,4	97,6	23,9	76,1

*• = Not determined separately (included with % red count)

(b) *Non-swimmers:* In freshly collected specimens the cercariae on the bottom of the container crawled around or vigorously whipped their tails from side to side. Older cercariae became less active, moved slowly and their side-to-side movements were less freqeunt. Towards the end of the observation period many cercariae rested on the bottom of the container and swam to the surface only sporadically.

When the water was swirled most of the older cercariae could not remain attached to the container.

Discussion

Differential staining of live and dead cercariae

From the data presented in Table 2 it is apparent that in those cercariae which absorbed the eosin stain, the colouring was not always uniform. Following chloral hydrate or ether treatment a few cercariae stained either red or pink posteriorly at the point of attachment of the tail but did not stain anteriorly; some became dark pink or red posteriorly and light pink anteriorly; some were uniformly red and some remained completely unstained. When pentobarbital sodium and carbon dioxide were used, some cercariae became uniformly pink (included with the red count in pentobarbital sodium), some were red and some were colourless or translucent.

The cercariae which partially absorb eosin should be classified as dead. Those with small red areas posteriorly and colourless anterior ends were probably dying (e.g. No. 6 in Table 2, where the percentage of red-and-pink cercariae decreased while the white-and-red and unstained cercariae increased with decreasing concentrations of chloral hydrate) and it is unlikely that they would be infective.

Death of cercariae

The criteria used by previous workers (Krakower, 1940; Ingalls, 1946; Kuntz & Stirewalt, 1946; Muftic, 1970) for defining the death of cercariae did not take into account the transitory nature of some of the reactions. In this investigation some cercariae which could have been considered dead according to their definitions, for example immobility (Krakower, 1940; Ingalls, 1946; Muftic, 1970), crenation (Ingalls, 1946) or reduction in refractivity (Ingalls, 1946; Kuntz & Stirewalt, 1946), recovered after removal of the drugs by washing.

The criteria used in this investigation aimed at depicting the process of immobilization of cercariae and determining the degree and rate of recovery after removal of the drugs tested. The most useful criterion was found to be eosin-staining, while the presence or absence and type of swimming movements and the ability of the cercariae to adhere to the container might also be useful.

Physostigmin

It was found that physostigmin not only gave more consistent results than the other substances used to

immobilize cercariae but also had a wider safety margin. It was active in very low concentrations: 1 ml 0,012 M physostigmin will immobilize all the cercariae in 4 to 6 l water. At low concentrations its effect was reversible if it was removed completely. Lewert & Hopkins (1965) found that cercariae of *Schistosoma mansoni* recovered spontaneously from the paralysing effect of 0,0012 M ($1,2 \times 10^{-3}$ M) physostigmin. In this investigation, however, (Table 1, specimen 1 a) the effect of the drug at this concentration was irreversible when the *S. mattheei* cercariae remained in contact with it, and recovery was incomplete if they were placed in clean water after 14 minutes of exposure. However, the 62,4% dead cercariae found after 90 minutes' contact with $1,2 \times 10^{-3}$ M physostigmin (Table 2) probably do not give a true indication of the effect of this concentration; in later tests 10,4% and 11,0% dead cercariae respectively were found after similar treatments. Since pure physostigmin is only slightly soluble in water, the actual concentration used by Lewert & Hopkins (1965) may have been lower than 0,0012 M.

Ether

According to Khayyal (1965), general anaesthesia with ether vapour and other anaesthetics relaxes the schistosomes in the mesenterial blood vessels and causes a shift to the liver. In this laboratory it was found that adult parasites anaesthetised with ether immediately they were collected and then killed by heating to 60°C are relaxed and suitable for morphological studies.

This study shows that ether does immobilize the cercariae but it is unpleasant to handle, its safety margin is relatively small and high concentrations of the chemical are necessary to get the required effect.

Pentobarbital sodium

Ritchie, Garson & Knight (1963) added pentobarbital sodium to the perfusion fluid used to collect adult *S. mansoni*. In studies on *S. mattheei* the author obtained consistently good results when this chemical was added to the perfusion fluid (Earle's saline) as well as to the parasites after collection.

However, pentobarbital sodium only immobilizes cercariae at high concentrations and the reversibility of its effects is poor.

Other chemicals

The other three substances tested, i.e. CO_2 (routinely used for immobilizing insects) and halothane and chloral hydrate (used as general anaesthetics in vertebrates), were found to be unsuitable because their safety margins are relatively low and the gases are difficult to apply quantitatively.

II. *Infectivity of Cercariae Revived after Immobilization*

Cercariae which are motile are not necessarily infective. Therefore, as a final test of recovery from immobilization the infectivity of two lots of treated cercariae was compared with that of untreated (control) cercariae of the same age.

Method

The cercariae in two samples were immobilized with 3×10^{-5} M physostigmin and $2,4 \times 10^{-2}$ M pentobarbital sodium respectively and the chemicals removed by washing (see above) 13 and 25 minutes respectively after addition to the cercarial suspensions. A third sample of cercariae served as an untreated control. With the aid of a stereoscopic microscope six lots of 100 cercariae each were removed from each of the three main samples of cercarial suspension by suction via a rubber tube on a glass pipette and transferred to 18 different test tubes. The cercariae were counted as they were drawn into the pipette.

After removal of the immobilizing chemicals, the treated cercariae were allowed 2 hours in which to recover. Thereafter 18 *Praomys* (*Mastomys*) *natalensis* were anaesthetized with pentobarbital sodium and exposed percutaneously (skin of tail) for half an hour to the different groups of cercariae (Purnell, 1965).

After exposure of the *P.* (*M.*) *natalensis* the numbers of cercariae remaining in the test tubes were counted and the three original cercarial suspensions (two treated and one control) examined to determine the percentage of live cercariae (Section 1 b above).

Fifty-eight days after infestation the surviving *P.* (*M.*) *natalensis* were killed by intra peritoneal injection of pentobarbital sodium and the worms recovered by perfusion as described by Duvall & De Witt (1967).

Results

The results are summarized in Table 3.

At the time of infestation the percentages of live cercariae remaining in the three samples from which cercariae for infestation were taken were 75,0% in the control (untreated) sample and 98,6% in each of the treated samples.

The worm recovery data are given in Table 3.

Because of the large variations in individual worm burdens and the small number of experimental animals used, statistical analysis of the results are unsatisfactory. Nevertheless, it can be seen that many treated cercariae in both test groups were able to penetrate the definitive host and develop to adult worms.

Discussion

Recovery after immobilization with physostigmin and pentobarbital sodium was very good as some

TABLE 3 *S. mattheei* worms recovered from *P.* (*M*) *natalensis* exposed to untreated or to treated-and-revived cercariae

Cercariae	No. of animals	No. of cercariae per animal	Mean % cercariae which penetrated	Worm recoveries (%)		
				Of total number of cercariae		Of number of cercariae which penetrated
				Mean %	Range	
Untreated (controls)	6	100	70,5	27,2	16–37	38,3
Treated with Physostigmin (3×10^{-5}M)	6	100	73,9	17,0	7–26	22,9
Pentobarbital Na ($2,4 \times 10^{-2}$M) . .	*4	100	55,75	22,0	20–25	39,5

*Two *P.* (*M.*) *natalensis* died from anaesthesia during exposure to cercariae

cercariae were infective thereafter. This was surprising, however, because sucker function, seen in their ability to crawl and adhere to the container (Table 1) was regained slowly in comparison with the other functions and it was considered unlikely that cercariae with relatively inactive suckers could remain in contact with the host long enough to penetrate the skin. No explanation can be offered for the fact that a lower percentage of control than of treated cercariae was alive at the time of infestation.

Conclusions

A problem which has been encountered by various workers (Krakower, 1940 and Kuntz & Stirewalt, 1946) is that different batches of cercariae harvested in the same way may vary markedly in viability. These findings are confirmed in the present investigations.

The eosin method of selective staining of dead cercariae, when combined with physostigmin immobilization, was shown to be a useful method for determining the viability of batches of cercariae before they are used in experiments. Because live cercariae are not necessarily infective, however, (Olivier, 1966b) selective staining of cercariae cannot replace animal viability controls, but merely complements them.

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