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Characterization of Chemical Stimuli for the Penetration of *Schistosoma mansoni* Cercariae

II. Conditions and Mode of Action

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Abstract. The mode of action of chemical substances which trigger the penetration of *S. mansoni* cercariae into agar substrata is studied. The effectiveness of these substances is largely independent of their polarity and water solubility. Thus, they do not seem to act by a passive membrane permeation process, but they may interact with specific receptor sites, which are characterized. The receptor sites seem to respond to the following chemical characteristics of the stimulating aliphatic hydrocarbon chain: Carboxylic end group, lipophilic end group, chain length, *cis*- double bond. The penetration stimulating substances cause, even in cercariae in free water, a transformation of the tegument, manifested as a reduction of the Cercarienhüllen-Reaktion and a loss of osmotic protection.

Introduction

In previous papers (Haas and Schmitt 1978, 1981) it was shown, that the penetration of *Schistosoma mansoni* cercariae into agar substrata is triggered by molecules with specific characteristics. Thus, only aliphatic hydrocarbon chains with a hydrophilic as well as a lipophilic end group stimulate penetrations. The effectiveness of saturated substances is, at neutral pH, limited to chain lengths between 10 and 15 carbon atoms. Unsaturated substances work at longer chain lengths, and their effectiveness increases with an increase in the number of double bonds in the *cis* position. All penetration stimulating substances killed cercariae in free water and the question arises as to whether such substances may be used for a specific control of cercariae.

In this study the action mechanisms of the stimulating molecules on the penetration behaviour and the lethality of the cercariae will be discussed. Do the penetration stimulating substances have a more general effect on the cercarial tegument and thus damage the cercariae directly, or do they stimulate physiological processes via specific receptors, that cause the lethality? Information on such receptor sites might help to find maximal stimulating molecules.

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Material and Methods

A *Schistosoma mansoni* laboratory strain from Belo Horizonte, kept in *Biomphalaria glabrata*, and in some experiments cercariae from natural infections of *B. pfeifferi* from Cairo, Egypt (indicated in the tables) were used as in our previous paper (Haas and Schmitt 1981). The methods for preparing the agar substrata, incubation with the cercariae, and evaluation of the results were also described in our previous paper (Haas and Schmitt 1981).

Results

Effect of the pH on Penetration and Lethality

The influence of changes in the polarity of stimulating molecules was tested in substances offered to the cercariae at different pH conditions. The experiments of Table 1 show that in acid pH conditions the penetration stimulating effectiveness is increased. This is true for all substances that are effective at neutral pH. However, fatty acids are exceptionally effective at low pH. Short chain carboxylic acids, which have no effect at neutral pH, stimulate penetrations at pH 5.5, whereas the short chain aliphatic alcohols, nitrile, thiol and amino acids are ineffective.

Is the pH of carboxylic acids important because of ionization? In the acid pH range the amount of unionized substance is increased. Thus the relationship of $RCOO^-$ to RCOOH shifts in butanoic acid (ionization constant $1.54 \cdot 10^{-5}$; Kortüm et al. 1961) from 4,870 at pH 8.5, to 154 at pH 7.0, and to 4.9 at pH 5.5. At pH 5.5, the amount of R–COOH is 30-fold higher than at pH 7.0.

Table 1. Influence of pH on the penetration stimulatory effect of some substances. pH adjusted using 40 mM phosphate buffer + NaOH or HCl. Pen. = penetration rate, Leth. = lethality of the cercariae that had not penetrated. For each substance, separate cercarial populations have been used

	Conc. in agar (mM)	pH 5.5		pH 7.0		pH 8.5		No. replicates[b]
		Pen. %	Leth. %	Pen. %	Leth. %	Pen. %	Leth. %	
Control (phosphate buffer)	40	0	3.6	0	3.2	0	2.9	50
1-Dodecanol	0.03	19.8	36.4	8.9	20.4	0	10.3	10
1-Hexanol	10	0	0.9	0	1.8	0	1.4	10
Hexanenitrile[a]	5	0	1.3	0	1.5	0	1.0	10
1-Hexanethiol[a]	5	0	0.6	0	0.3	0	0.6	5
Norleucine[a]	10	0	4.4	0	2.1	0	3.6	5
Butanoic acid	10	22.0	100.0	0.3	4.3	0	2.4	10
Hexanoic acid[a]	5	36.7	97.2	0.1	2.0	0	1.3	10
Decanoic acid	0.3	7.3	95.0	7.1	17.4	0.2	5.7	10
Tetradecanoic acid	0.3	11.3	84.5	4.4	20.5	0.3	13.0	10
Hexadecanoic acid	5	3.4	66.7	0.1	4.6	0.4	5.3	10
9, 12, 15-Octadecatrienoic acid	0.0075	33.1	98.8	5.0	70.0	1.3	34.7	15

[a] Egyptian strain of *S. mansoni*
[b] about 100 cercariae per agar plate

Table 2. Influence of pH and concentration of unionized butanoic acid (R – COOH) on penetration rate (Pen.) and lethality rate (Leth.). 20 mM phosphate buffer + NaOH. All experiments done simultaneously with the same cercarial populations. Means of 15 replicates with about 100 cercariae each. The penetration rates in the two pH ranges differ significantly from each other ($p < 0.001$, χ^2-Test)

Conc R – COOH mM	pH 5.5			pH 7.0		
	Total conc. butanoic acid, mM	Pen. %	Leth. %	Total conc. butanoic acid, mM	Pen. %	Leth. %
0.06	0.3	3.7	4.0	10.0	0.4	6.1
0.20	1.0	4.0	12.7	30.0	1.4	13.7
0.60	3.0	10.8	64.1	100.0	1.9	29.0

However this increased amount of nonionized acid proved to be not the stimulating factor. When at both pH ranges the same concentration of RCOOH was chosen the cercariae responded more intensely at pH 5.5 (Table 2). Even when in the experiments of Table 2 at pH 7.0 the R – COOH-concentration was 10 times higher than at pH 5.5 (this implies a 300 times higher total concentration of butanoic acid), the cercariae showed significant higher penetration rates in the acid pH conditions. Thus the acidic pH influences the response to short chained carboxylic acids independent of the concentration of undissociated acid.

Is the carboxylic end group the unique requirement for the stimulating activity of molecules in acidic pH conditions? This was examined by testing different short chain substances with carboxylic groups (5 mM at pH 5.5). Our experiments showed that the carboxylic groups in amino acids were not effective: Glycine, asparagine, aspartic acid, and even norleucine with its saturated aliphatic C4-hydrocarbon chain were ineffective at pH 5.5. Only substances with CH, CH_2, or CH_3 beside the carboxylic group and with a lipophilic end group were effective (substance concentration in agar 5 mM, pH 5.5, 10 replicates. PR = penetration rate, LR = lethality rate): methanoic acid PR 3.0%, LR 45.5%, 2-propenoic acid PR 2.5%, LR 54.7%, 2-butenoic acid PR 1.2%, LR 26.1%, benzoic acid PR 3.3%, LR 59.1%. When the short chain substances lacked a lipophilic end group, they were ineffective at pH 5.5: Thus the dicarboxylic acids ethanedioic, butanedioic, and hexanedioic acid and the amino acids with a terminal carboxylic group aspartic and glutamic acid did not stimulate penetration at a concentration of 5 mM.

Influence of Osmotic Conditions on Lethality

All penetration stimulating substances, including human skin surface lipids, kill cercariae rapidly. Is this lethality of the cercariae caused by an osmotic sensibility, which appears during the transformation cercaria – schistosomulum? Our experiments of Table 3 show that this lethal effect of the penetration stimulating substances is at least reduced in isotonic solutions of electrolytes or sugars. Yet, under isotonic conditions cercariae have reduced activity and the penetration rates are diminished.

Table 3. Effect of osmotic conditions on penetration and lethality of the cercariae that had not penetrated. Penetration stimuli in the agar plates are: 0.0075 mM linolenic acid (*cis*-9,12,15-octadecatrienoic acid), 5 mM hexanoic acid at low pH, ether washings of human skin surface (1 mg/ml). Electrolytes and sugars are included in the agar plates and the cercarial suspensions. Experiments using different cercarial populations are separated

	Penetrations		Lethality		No. replicates[c]
	Mean %	χ^2-Test p	Mean %	χ^2-Test p	
Linolenic acid	19.7		96.0		5
		<0.001		<0.001	
Linolenic acid + 0.9% NaCl	3.0		10.7		5
Linolenic acid	11.7		96.9		15
		0.4		<0.001	
Linolenic acid + 50% Ringer, $^1/_2$ isotonic	12.8		35.5		15
		0.02		<0.001	
Linolenic acid + Ringer, isotonic	10.0		6.3		15
		<0.001		0.2	
Control (H_2O)	0.1		5.1		15
		0.99		0.01	
50% Ringer, $^1/_2$ isotonic	0.1		3.1		15
		0.08		0.6	
Ringer, isotonic	0.4		3.5		15
Linolenic acid	18.9		98.9		10
		<0.001		<0.001	
Linolenic acid + mannitol, isotonic	6.9		3.1		10
		<0.001		<0.001	
Mannitol, isotonic	0.4		0.7		10
		0.05		<0.001	
Control (H_2O)	0		3.0		10
Linolenic acid[a]	36.2		100.0		10
		<0.001		<0.001	
Linolenic acid + glucose, isotonic[a]	5.6		4.5		10
				0.1	
Glucose, isotonic[a]	0		2.8		10
				0.1	
Control (H_2O)[a]	0		1.5		10
Hexanoic acid, pH 5.5[b]	22.3		93.3		5
		<0.001		<0.001	
Hexanoic acid, pH 5.5[b] + 50% Ringer, $^1/_2$ isotonic	35.5		27.3		5
		<0.001		<0.001	
Control (H_2O, pH 5.5)[b]	1.6		2.4		5
Human skin surface lipids	36.6		100.0		5
		<0.001		<0.001	
Human skin surface lipids + Ringer, isotonic	5.3		19.3		5
		<0.001		<0.001	
Ringer, isotonic	0		1.4		5

Ringer, contents (mval/l): Na^+ 147.1, K^+ 4.0, Ca^{2+} 4.5, Cl^- 155.6
Isotonic means isotonic to mammalian blood, 276 mosm/l

[a] Egyptian strain of *S. mansoni*
[b] Experiments with hexanoic acid with 40 mM phosphate buffer, all other experiments at pH 7.0 with 5 mM phosphate buffer
[c] about 100 cercariae per agar plate

Table 4. Cercarienhüllen-Reaktion (CHR) in serum of schistosomiasis-patients with cercariae after incubation with a penetration stimulating substance (*cis*-9,12,15-octadecatrienoic acid, 0.01 mM/l). Incubation period in serum: 2h at room temperature

Incubation 2 h (30° C) in	No. Cercariae	% Cercariae with CHR	χ^2-Test p
Ringer, isotonic +octadecatrienoic acid	765	29.8	<0.001
Ringer, isotonic	684	73.7	<0.001
Water	710	92.4	

Influence of Penetration Stimuli on CHR

Cercariae show in the serum of schistosomiasis-patients a Cercarienhüllen-Reaktion, CHR (Vogel and Minning 1949a, b), whereas transformed schistosomules do not (Stirewalt 1963). Our experiments with cercariae that had been stimulated with octadecatrienoic acid show that the CHR in these parasites is much reduced (Table 4). The so-treated cercariae very often have a CHR around the tail, whereas the body itself does not show the reaction. In the experiments of Table 4 only CHR of the body was recorded. The penetration stimulus seems to influence only the tegumental transformation of the body.

Discussion

Mode of Action of Stimulating Substances

Do the penetration stimulating substances have a more general effect on the cercarial tegument (by a passive permeation) or do they interact with specific receptor sites which can be characterized? As only relatively nonpolar substances are effective, a passive uptake of these substances by the cercarial body wall could be supposed to act as a triggering factor. Then the partition of the stimulating substances between the aquatic medium and the membrane (the glycocalyx) is determinant. This depends, among other things, on the relative polarity of the substances and the membrane, and the solubility of the substances in the water phase and the membrane (Dietschy 1978).

Indications that the polarity of the stimulating molecules might have an influence are shown by the carboxylic acids. The saturated carboxylic acids have an effect on the penetration behaviour at neutral pH only at chain lengths between 10 and 15 carbon atoms, thus at a relatively limited polarity. Short chain carboxylic acids are ineffective, as long as they are present in a neutral or alkaline pH range. However, they have a strong penetration stimulating effect, when offered in a more nonpolar form at a lower pH. Although these short chain substances are, even in unionized condition, far more polar than the ionized long chain carboxylic acids (C10–C14), their similar effectiveness as penetration stimulus via a passive permeation of the membrane might be

explained as follows: These short chain substances are more water soluble and show (compared with the longer chained fatty acids) "anomalously" high rates of monomolecular diffusion into biological membranes (Dietschy 1978; Sallee 1979).

Further arguments seem to support the view that the stimulating molecules exert a more general effect on the body surface of the cercariae: some of the cercarial behaviour influencing properties of the stimulating molecules also have a bactericidic or fungicidic effect (review of the literature: Novak et al. 1961). Thus the maximum activity of saturated monocarboxylic acids against diverse microorganisms occurs at a chain length of around 12 carbon atoms (Tetsumoto 1933; Hoffman et al. 1939; Kodicek 1948). This effect increases with the increase in the number of double bonds (Hoffman et al. 1939; Wyss et al. 1945; Kodicek 1948, 1956), and *cis* isomers are effective, but *trans* are not (Kodicek 1948, 1956). Moreover, short chain carboxylic acids show this activity mainly under acid conditions, and long chain acids also under alkaline pH conditions (Hoffman et al. 1939; Wyss et al. 1945).

However, a passive uptake of the stimulating molecules by the surface of the cercarial body or the membrane of a receptor may be considered at best as a factor of secondary importance; an action via specific receptor sites is more probable. This is supported by the following arguments:

1. The influence of the pH on the effectiveness of the substances cannot be explained by a change of polarity and solubility of the stimulating molecules. Carboxylic acids, which are too nonpolar due to their too long hydrocarbon chains and therefore cannot permeate a membrane, may be made more polar by buffering them at an alkaline pH. When only their polarity is determinant, then they could become effective. However, this does not hold in our experiments. Thus the relative too nonpolar, because it has too long a chain, tetradecanoic acid has in our experiments at pH 8.5 no effect on the cercariae, but an intensive effect at pH 5.5, although the substance is far more polar at pH 8.5 than at pH 5.5. The partition ratio between heptane and buffer at pH 8.5 is nearly 1,000 times lower than at pH 5.5 (Simpson et al. 1974).

A more intense reaction would also be expected in alkaline conditions for many other reasons: (a) The water solubility of the stimulating carboxylic acids increases in the alkaline range, e.g. in tetradecanoic acid from pH 5.5 to pH 8.5 by a factor of about 100 (Nyren and Back 1958). (b) The enzymes of the penetration glands of the cercariae should be more effective under alkaline conditions: Their maxima of activity lie between pH 8.5–8.8 (Gazzinelli et al. 1966; Dresden and Asch 1972; Campbell et al. 1976). (c) The pH-optimum for in vitro transformations from cercaria to schistosomule in the tissue culture medium TC-199 is at pH values above 8.0 (Ramalho-Pinto et al. 1974, 1975). (d) The longevity of cercariae is increased in alkaline conditions (Asch 1975).

Nevertheless, under alkaline conditions fewer cercariae penetrate both in vivo (Asch 1975) and in our in vitro experiments using different chemical penetration stimuli. Yet in acidic pH conditions the penetration stimulating effectiveness of all those substances increases, that are also effective at neutral pH. This also holds for the relatively nonpolar and poor water soluble long chain fatty acids, which become still more nonpolar and more water-insoluble in acidic

conditions. Thus the pH influences penetration rate and lethality rate independent of the polarity of the stimulating substances.

Acidic pH conditions have a special influence on the effectiveness of substances with terminal carboxylic groups: They also help to make short chain fatty acids effective, obviously independent of the amount of unionized substance. The finding that short chain substances with other hydrophilic end groups remain uneffective supports the idea that there exists a specific receptor site for the carboxylic group. Whether the pH alters receptor sites in such a way that the stimulating molecules release a different response or if it works via other inputs, cannot be decided yet.

2. Double bonds in the alkyl chain have a far greater effect on the cercarial penetration behavior, as one would expect, when only considering their increasing influence on the polarity (of long chain fatty acids and alcohols). Thus for example a double bond in a fatty acid decreases the partition coefficient between polyethylene (as the organic phase) and buffer, and between biologic membranes and buffer, as much as by removal of 1–2 CH_2-groups of the alkyl chain. However, the effect of the double bond on the cercarial penetration rate is far greater. Octadecatrienoic acid which has a great influence on cercarial behaviour has an even higher partition rate between n-heptane and buffer (Sallee 1978a, b; Simpson et al. 1974) than the so far less effective tetradecanoic acid. Hence, double bonds do not seem to work via a passive membrane permeation process, but they might interact with a specific receptor site.

3. Substances other than fatty acids have an effect on the cercarial behaviour at very different relative polarities. Thus, saturated alcohols work at pH 7.0 in the same chain length range as the corresponding saturated monocarboxylic acids, although the partition coefficients of the alcohols between an organic phase and water are much higher, e.g. between polyethylene and buffer about 1,000 times higher (Sallee 1978a). Also the permeation coefficients for rat intestinal uptake are more than 1,000 times higher for saturated alcohols than for the saturated fatty acids (Sallee 1979). For the microvillus membrane of the intestine, even 1-hexanol, which does not effect the cercarial behaviour, has a higher permeation coefficient than the penetration stimulating (relatively polar) decanoic acid (Westergaard and Dietschy 1974). However Sallee (1978b) finds that the partition coefficients between buffer and biological membranes of the saturated alcohols with chain lengths C10 and C12 are only 72-fold increased compared with those of the ionized fatty acids; inexplicably at a chain length of C14 they were almost identical in Sallee's experiments. If in the stimulation of cercarial penetration a passive uptake of the molecules alone were effective, more nonpolar substances such as alcohols should work at a shorter chain length than more polar substances, such as the monocarboxylic acids. The fact that substances with different polarity are effective in the same chain length range C10–C16, speaks in favour of the structural feature chain length as a stimulating factor.

Thus most facts indicate that the main effectiveness of the stimulating molecules is based on structural properties which interact with specific receptor sites of the cercariae. The requirements molecules have to fulfil in order to stimulate penetrations are summarized in Fig. 1.

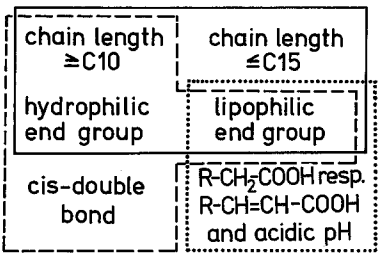

Fig. 1. Preliminary synopsis of structural requirements for a penetration stimulating effectiveness of aliphatic hydrocarbon chains. Each frame contains a complete set of required characteristics for full activity

Whether there are one or more different types of receptor sites is not known, because the results are obtained via the behaviour of the animals. For saturated hydrocarbon chains with hydrophilic end group there seems to be one type of receptor site. When a lipophilic end group and a chain length of C12 is offered to the cercariae on one molecule, and the carboxylic group on another molecule no response is obtained: For example, a mixture of 5 mM dodecane and 5 mM butanoic acid, at neutral pH, has no effect on cercarial behaviour. Also the effectiveness of short chain carboxylic acids at acidic pH seems to depend on a single type of receptor site: When carboxylic groups and lipophilic end group are offered to the cercariae on separate molecules, no effect is achieved; e.g. a mixture of 5 mM butanedioic acid and 5 mM 1-butanol, at pH 5.5, has no effect on the penetration behaviour.

Damage Caused by the Penetration Stimuli

The damage to the cercariae caused by penetration stimulating substances could be caused directly by the chemicals on the cercarial tegument; this is comparable with the antimicrobial activity of similar stubstances on microorganisms (see above). However, it is more likely that the causes are to be found in the transformation process cercaria-schistosomulum (Gazzinelli et al. 1973; Stein and Lumsden 1973; Ramalho-Pinto et al. 1974, 1975; Eveland and Morse 1975; McLaren and Hockley 1976; Brink et al. 1977; Reviews: Stirewalt 1974; Lumsden 1975). This is supported by the following arguments:

1. All tested penetration stimuli, including human skin lipids, cause this lethality and the intensity of the effect on the penetration behaviour is closely correlated with the degree of damage.

2. The damage caused by penetration stimulating substances was much reduced by transfer of the parasites into isotonic media. This indicates that the damage, in our experiments, is caused by the osmotic conditions. Indeed, cercariae lose their osmotic protection during the transformation. Important is the fact that not only electrolyte solutions, but also isotonic sugar solutions are able to protect against the damage of penetration stimuli. This proves that the protection is neither achieved by a stabilization of the glycocalyx by binding cations (Kusel 1971; Howells et al. 1974; Asch 1975), nor by the preventation of loss of ions, which was incriminated by Becker (1971) to be the main cause of natural death of cercariae. The crucial point is therefore the inflow of water through the tegument which obviously has already been transformed.

3. The chemical properties of the penetration stimulating substances indicate that they interact with specific receptor sites (see above). A modification of the cercarial tegument by an exclusively passive permeation of these substances is less likely.

4. Cercariae that have been pretreated with penetration stimulating substances show a weaker Cercarienhüllen-Reaktion (Vogel and Minning 1949a, b) in the serum of infected patients than do control animals. It is known that only cercariae, not the transformed schistosomulae, cause this reaction (Stirewalt 1963).

Coupling of the Trigger for Penetration and Transformation of the Tegument

Penetration stimuli also start the transformation of the tegument when the parasites have no opportunity to penetrate. This coupling of the trigger for the penetration behaviour with that for the transformation of the tegument seems to be dangerous for the cercaria. However a fast transformation of the tegument is indispensable for the survival of the parasite in the host. The antigen containing glycocalyx must be shed as fast as possible (by casting off of microvilli? McLaren and Hockley 1976) and replaced by the new surface with its protection against the immune system of the host and increased permeability (Reviews: Clegg 1972; Stirewalt 1974; Lumsden 1975; Brown 1976a, b; Smithers and Terry 1976). As the penetration process is very fast, it is imperative for the parasite to start the transformation as early as possible because it takes longer. In natural conditions, the transformation will be started anyway, when a cercaria can really penetrate: The stimulating lipid soluble substances adhere usually to the skin of the host and thus are relevant for the cercariae just after the attachment.

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