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Entamoeba histolytica: Effects of Dichlorophene and Hexachlorophene on Respiratory Activities, Growth *in Vitro* and Ultrastructure

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Introduction

Previous investigations on *Entamoeba histolytica* disclosed that bithionol, a halogenated bisphenolic derivative and the drug of choice for human paragonimiasis (Yokogawa *et al.*, 1961), potently inhibited endogenous and 2-propanol-supported respiration, and *in vitro* growth of the parasite (Takeuchi *et al.*, 1984). Although dichlorophene and hexachlorophene, both of which are structurally similar to bithionol, also inhibited 2-propanol-supported respiration of *E. histolytica* (Takeuchi *et al.*, 1984), studies on the effect of these two bisphenolic derivatives on the growth of amoebae were not attempted, because the compounds dissolved in methanol could not be solubilized in the culture media of amoebae, and consequently, it was impossible to determine concentrations of the compounds needed for killing amoebae in the cultures. Our experiences on bithionol, however, recently led us to dissolve the bisphenols in NaOH, and subsequently dilute them with appropriate buffers or distilled water. This procedure enabled us to make these compounds solubilized in the culture media, and eventually to study inhibition by the bisphenolic derivatives of the growth of *E. histolytica* in more details.

Materials and Methods

Reagents: Dichlorophene, i.e., 2, 2′-methylenebis (4-chlorophenol), and hexachlorophene, i.e., 2, 2′-methylenebis (3, 4, 6-trichlorophenol) were supplied by Tokyo Kasei Inc. (Tokyo, Japan). Other chemicals were of the highest purity commercially available.

Measurement of respiratory activities: Axenic trophozoites of *E. histolytica* (strain HM-1 : IMSS) were grown in BI-S-33 medium (Diamond *et al.*, 1978) as described previously (Takeuchi *et al.*, 1977). After cultivating for 72 hours at 35.5°C, amoebae were harvested and washed as described (Takeuchi *et al.*, 1977). Finally, amoebae were suspended in 50 mM Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose to make 20–25 mg protein/ml.

Respiratory activities were determined polarographically at 30°C with a Clark oxygen electrode (Rank Bros., Bottisham, England, UK). The assay mixture for 2-propanol-supported respiration contained 10 mM 2-propanol, the suspension of intact amoebae (2.3–4.0 mg protein) and 0.2 M Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose in a final volume of 2 ml. Endogenous respiration was determined using the same assay mixture as above except that higher amounts of trophozoites, i.e., 7–10 mg protein, were used, and no substrates were added. The reaction of 2-propanol-supported respiration was initiated by adding 2-propanol through a narrow vent of the cuvette of polarograph using a microsyringe (Hamilton Co., Reno, Nevada, USA), after the assay mixture except for the substrate was aerated for 3 min with a mechani-

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cal stirring. Endogenous respiration was also initiated after aerating as above. Dichlorophene and hexachlorophene were added before or 0.5–1 min after the reactions were started. Under the present assay conditions, the respiratory activities proceeded linearly for at least 2 min.

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Measurement of growth in the cultures: HM-1 strain was grown in BI-S-33 medium and harvested as described above. Finally, amoebae were washed once and suspended in the medium to yield 2.5×10^5 trophozoites/ml. Two tenths ml of this suspension was inoculated into each of 12 screw-capped culture tubes of 16 by 125 mm containing 15 ml of fresh BI-S-33 medium. The culture tubes were incubated for 24 hours at 35.5°C, and the growth of amoebae was confirmed with an inverted microscope. Subsequently, the number of trophozoites was counted on 0.05ml of the culture fluid, which was made homogeneous by gently inverting the tubes. The culture tubes were centrifuged at 380 g for 7 min, and 0.30 ml of the supernatint fluid was removed. Finally, 0.15 ml of dichlorophene or hexachlorophene solution was added, and the cultures were maintained at the same temperature as above. The number of live trophozoites was counted as described above at appropriate time intervals.

Because serum albumin readily binds to numerous halogenated and nitrated phenols, and inactivates their inhibitory actions as Weinbach and Garbus (1966) reported, similar experiments were done using BI-S-33 medium from which bovine serum was omitted.

Strain HJ-1 : KEIO was isolated from a stool of a patient with amoebic dysentery, and has been maintained using Balamuth's medium. This strain was grown in Balamuth's medium for 72 hours at 35.5°C, harvested and finally suspended in the same medium to yield 1.25×10^5 trophozoites/ml. Four tenths ml of this suspension was inoculated into each of 12 screw-capped culture tubes mentioned above, which contained 15ml of the freshly prepared medium. The culture tubes were centrifuged at 380 g for 7 min, and 0.55 ml of the supernatant fluid was removed. Subsequently, the cultures were processed and assayed as described above.

Dead trophozoites of *E. histolytica* could be readily distinguished by their loss of movement, lowered contrast and discharge of cytoplasmic components under the present assay conditions; therefore, the number of live trophozoites was counted without dyes like trypan blue unless otherwise stated.

Control experiments: Since dichlorophene and hexachlorophene were dissolved in 1 N NaOH to make appropriate concentrations, and subsequently diluted thrice with 0.1 M HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer, pH 7.4, control experiments were done as follows. Various amounts of the solvent of the bisphenols were added to the assay mixtures, and resulting changes in the respiratory activities of amoebae were recorded. Moreover, appropriate amounts of the compounds were added to the mixtures, and changes in the pH were examined. When significant changes in the pH were observed, the respiratory activities were determined at this pH, and compared with those at pH 7.4.

The cultures for assessment of the effect of the bisphenols on *in vitro* growth of *E. histolytica* were divided into four groups. One of them was set as the control, and supplemented with 0.15 ml of the solvent. Moreover, various concentrations of the bisphenols were added, and resulting changes in the pH of the culture media were recorded. When significant changes in the pH were observed, the cultures were processed in the same manner as above.

Electron microscopy: Trophozoites of HM-1 strain were observed with an electron microscope in order to confirm the effects of the bisphenols on *E. histolytica*. After incubating under various conditions at 35.5°C, the culture media were centrifuged, and the pellets were prefixed for 60 min at 4°C with 0.1 M phosphate buffer, pH 7.4 containing 2 % glutaraldehyde and 2 % tannic acid.

Table 1 Inhibition by dichlorophene and hexachlorophene of endogenous and 2-propanol-supported respiration of intact trophozoites of *Entamoeba histolytica* (strain HM-1 : IMSS)

Reaction system	Concentrations needed for 50 % inhibition	
	Dichlorophene	Heaxachlorophene
Endogenous	0.50 mM	0.85 mM
2-Propanol-supported	0.68 mM	0.63 mM

Data were averages of at least four independent determinations.

The specimens were washed once and kept stationarily overnight at 4°C in the phosphate buffer. Subsequently, the specimens were further washed once in the phosphate buffer, postfixed for 90 min at 4°C with the same buffer containing 1 % osmium tetroxide, and dehydrated in a graded series of ethanol and finally in acetone. After embedding in Epon, thin sections were cut with a Porter-Blum MT-2B ultramicrotome (Ivan Sorval, Norwalk, Connecticut, USA), stained with uranyl acetate and lead citrate, and observed with a Hitachi HU-12AS electron microscope (Hitachi Inc., Tokyo, Japan).

Results

As demonstrated in Table 1, dichlorophene and hexachlorophene potently inhibited endogenous and 2-propanol-supported respiration of trophozoites of *E. histolytica* at considerably low concentrations. There was no significant difference in the inhibitory effects on the respiratory activities between the bisphenols dissolved as described above and those in methanol. Control experiments showed that addition of up to 60 μl of the solvent did not affect the respiratory activities. Moreover, addition of 40 μl of 0.1 M solution of the bisphenols shifted the pH of the assay mixtures from 7.40 to 7.45. However, there was no difference between the respiratory activities at 7.40 and those at pH 7.45.

Although the physiological significance of the respiratory activities in *E. histolytica* is not known, these observations led us to examine if the bisphenols also inhibit the growth of amoebae in the cultures.

Effects of dichlorophene and hexachlorophene on the growth of MH-1 strain in complete BI-S-33 medium were demonstrated in Figs. 1 and 2 respectively. Addition of 100 and 150 μg/ml dichlorophene killed and disrupted virtually all amoebae in shorter than

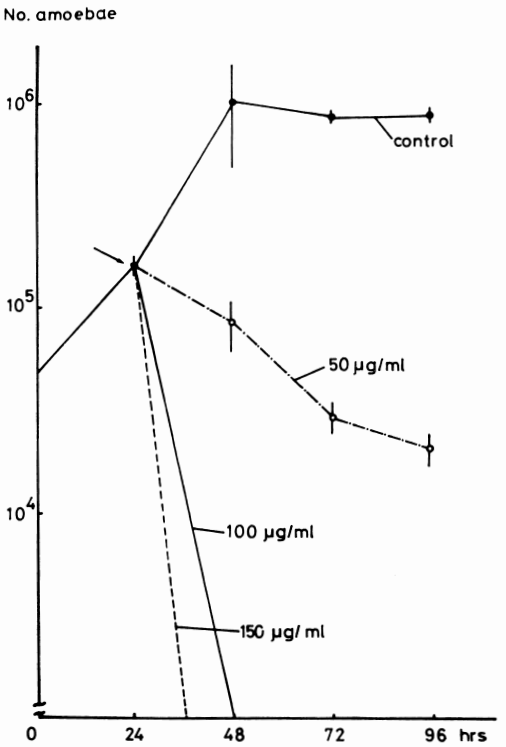


Fig. 1 Effects of dichlorophene on the growth of *Entamoeba histolytica* (strain HM-1 : IMSS) in BI-S-33 medium. The number of live trophozoites was counted in triplicate throughout the observation, because each experimental group consisted of three culture tubes. The ordinate shows the total number of live amoebae in the culture. The arrow indicates addition of dichlorophene.

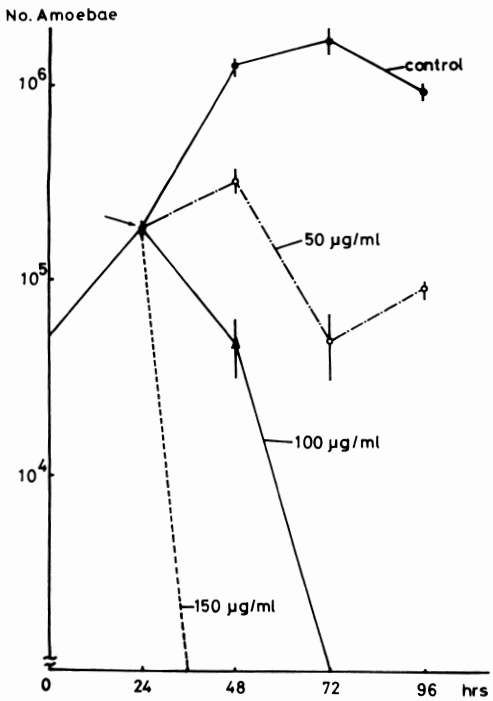


Fig. 2 Effects of hexachlorophene on the growth of *E. histolytica* (HM-1 strain) in BI-S-33 medium. Other details as in the legend to Fig. 1.

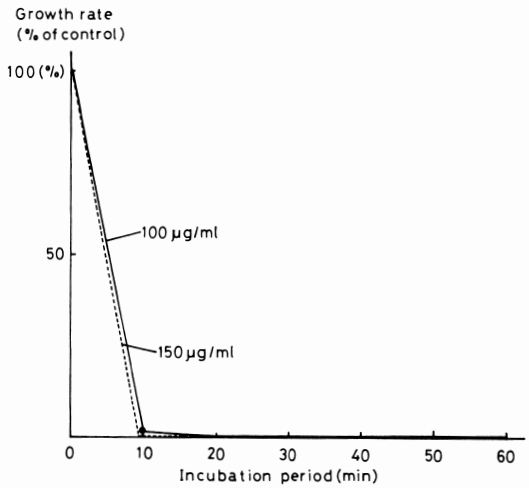


Fig. 3 Effects ot hexachlorophene on *E. histolytica* (HM-1 strain) in the serum-free BI-S-33 medium. The ordinate is the ratio of the number of live trophozoites in the experimentals to that in the controls, which was represented as percent of the controls. The abscissa shows the period of incubation in the serum-free medium containing hexachlorophene. Other details as in the text.

24 and 12 hours respectively. Hexachlorophene killed amoebae within 48 and 12 hours at 100 and 150 μg/ml respectively.

To evaluate the possibility that bovine serum affects the inhibitory effects of the bisphenols, HM-1 strain was incubated in serum-free BI-S-33 medium containing various concentrations of the bisphenols for appropriate peridos at 35.5°C, and the number of live amoebae was counted as described above in the presence of trypan blue. Subsequently, the culture tubes were centrifuged at 450 g for 5 min. The pellets were gently resuspended in the same volume of freshly prepared complete BI-S-33 medium free from the bisphenols, and further incubated for 24 hours at the same temperature. The controls were processed in the same manner as above in the presence of the solvent of the bisphenols. The number of live amoebae in each culture was counted, and the ratio of the average number of live trophozoites in the experimentals to that in the controls was calculated.

Under such experimental conditions, omission of bovine serum resulted in a slight increment in the number of trophozoites, which looked undisrupted, even if the amoebae were incubated with 150 μg/ml of the bisphenols for 12 hours. However, determination of the number of live amoebae in the presence of trypan blue showed that virtually all amoebae, which looked undisrupted, were stained with the dye. Moreover, no actively motile trophozoites could be found, and the number of amoebae did not increase at all even 24 hours after replacing the experimental culture media with freshly prepared complete BI-S-33 medium, which was free from the bisphenols, if the amoebae had been incubated in the serum-free medium containing 100 μg/ml hexachlorophene for 10 min (Fig. 3). Dichlorophene also killed trophozoites of HM-1 strain in the serum-free medium at 100 μg/ml, if the incubation with this compound is conducted for at least 20 min at the same temperature

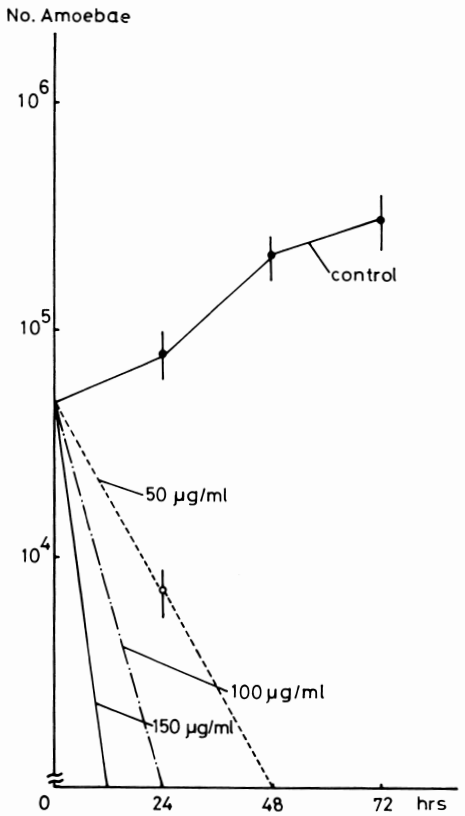


Fig. 4 Effects of dichlorophene on the growth of *E. histolytica* (strain HJ-1: KEIO) in Balamuth's medium. Details as in the text and in the legend to Fig. 1.

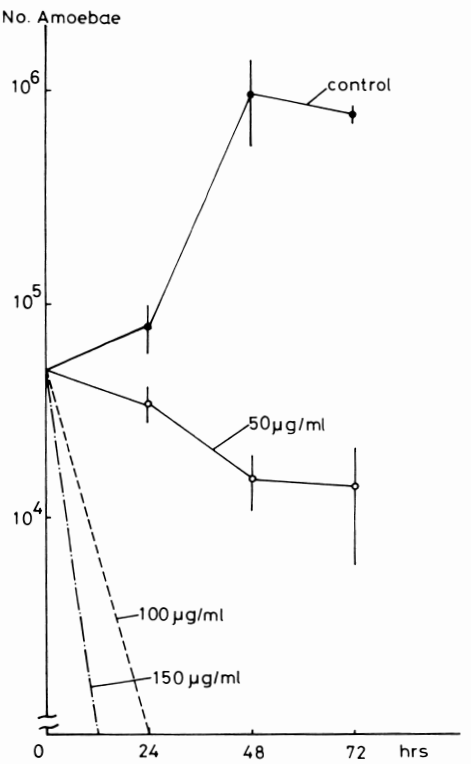


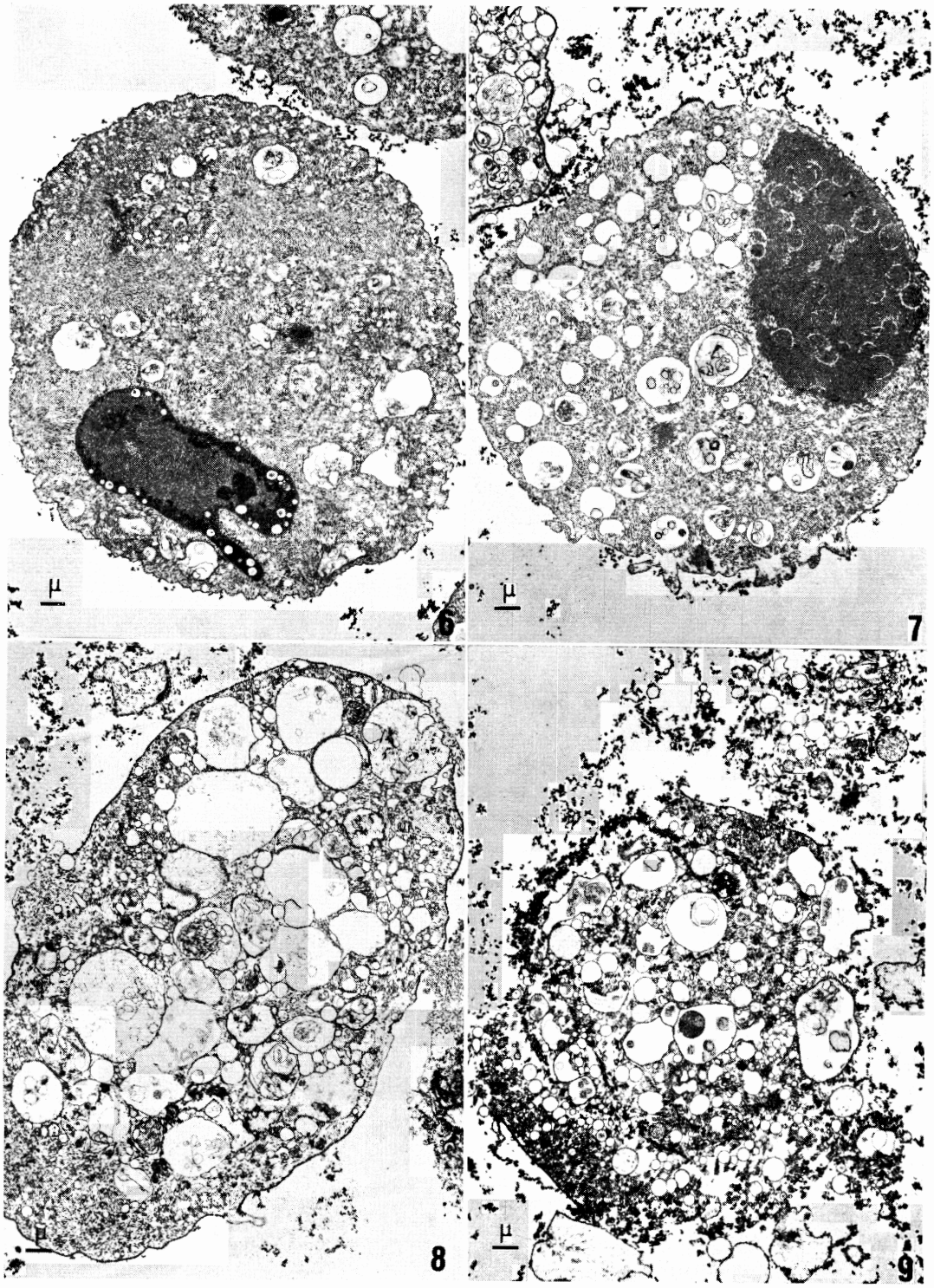
Fig. 5 Effects of hexachlorophene on the growth of *E. histolytica* (HJ-1 strain) in Balamuth's medium.

as above (data not shown).

Effects of dichlorophene and hexachlorophene on the growth of polyxenic HJ-1 strain in Balamuth's medium were demonstrated in Figs. 4 and 5 respectively. Addition of 50, 100 and 150 μg/ml dichlorophene killed and disrupted virtually all trophozoites of HJ-1 strain in shorter than 48, 24 and 12 hours respectively. Hexachlorophene could not kill all trophozoites of this strain at 50 μg/ml, whereas higher concentrations of this compound killed amoebae within 24 hours. It was also tested whether trophozoites of HJ-1 strain remained undistrupted after incubating as above. However, there was no evidence that the trophozoites, which looked undisrupted, increased in number.

Control experiments indicated that addition of 150 μg/ml of the bisphenols, the highest concentration employed in the present experiment, did not significantly change the pH of the culture media. Moreover, our preliminary study suggested that the growth rates of HM-1 and HJ-1 strains were almost identical irrespective of the presence of 0.15 ml of the solvent.

Ultrastructural studies on HM-1 strain incubated in complete BI-S-33 medium containing dichlorophene were illustrated in Figs. 7–11. Fig. 6 indicates the control, which was processed as described above in the presence of the solvent of the compound. Dichlorophene appeared to damage the plasma membrane of *E. histolytica*. As shown in Figs. 7 and 8, portions of the plasma membrane became thinner. Eventually, the amoebae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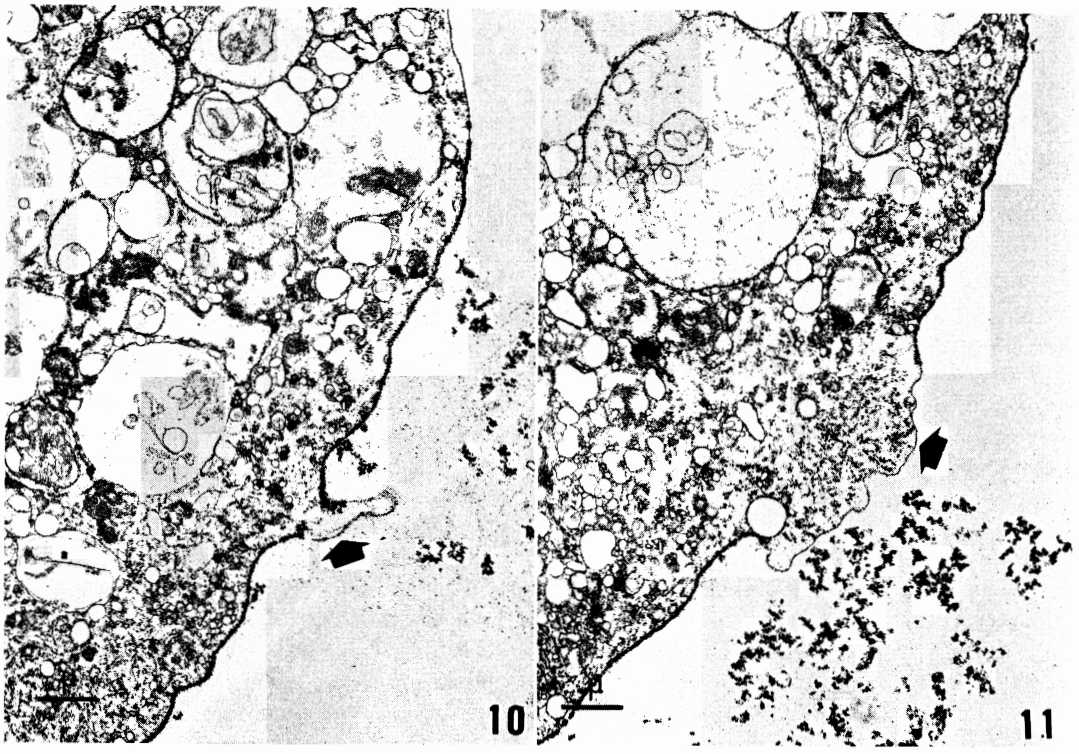

Fig. 6 An electron micrograph of HM-1 strain incubated in BI-S-33 medium containing the solvent of dichlorophene for 12 hours as the control for Figs. 7–11. No appreciable changes of the parasite can be observed.

Fig. 7 An electron micrograph of HM-1 strain incubated in the medium containing 150 μg/ml dichlorophene for 6 hours. Some extrusions of the plasma membrane appeared.

Fig. 8 HM-1 strain incubated as in Fig. 7. Note thinner portions of the plasma membrane as well as the extrusions.

Fig. 9 HM-1 strain incubated in the same experimental medium as in Fig. 7 for 12 hours. The parasite is disrupted.

Figs. 10, 11 Enlarged views of Fig. 8. Arrows indicate the portion of extrusion where the plasma membrane of *E. histolytica* looks thinner.

seemed to be swollen and disrupted (Fig. 9). Of noteworthy was that some irregular extrusions, probably due to swelling of the amoebae, came out on the plasma membrane (Figs. 7, 8). Enlarged veiws of Fig. 8 (Figs. 10, 11) suggest that the extrusions appeared from the thinner portions of the plasma membrane. Other degenerative changes like condensation of the cytoplasmic organelles of *E. histolytica* were also observed. Degenerative processes of *E. histolytica* in BI-S-33 medium containing hexachlorophene seemed essentially the same as those in Figs. 7–11 (data not shown).

Discussion

Our present investigations disclosed that dichlorophene and hexachlorophene, the halogenated bisphenols which were structurally similar to bithionol, i.e., 2, 2′-thiobis (4, 6-dichlorophenol), inhibited endogenous and 2-propanol-supported respiration of *E. histolytica*. It was also observed that these bisphenols were able to kill and disrupt both axenic and polyxenic *E. histolytica* in the cultures. Ultrastructural studies on HM-1 strain incubated with dichlorophene suggest that one of the primary targets of the action of the

compound may be the plasma membrane of the parasite. It is not known, however, whether inhibition of the respiratory activities bears relation to the degenerative changes in the plasma membrane illustrated in Figs. 7–11. The control experiments indicated that the inhibitory actions of the bisphenols were not attributable to nonspecific changes in the assay mixtures and the culture media.

As regards influences of bovine serum on the effects of the bisphenols, our studies suggest that it diminishes their anti-amoebic actions in the cultures. However, comparisons with our previous data on bithionol (Takeuchi *et al.*, 1984) show that the rate of enhancement of the anti-amoebic actions of bithionol by removing bovine serum from the medium was a little greater than that of dichlorophene and hexachlorophene, because bithionol was able to kill virtually all *E. histolytica* in 10 min at 50 μg/ml under the same assay conditions.

Since axenic and polyxenic amoebae have been reported to be different in some of their biological properties (see Albach and Booden, 1978), both types of *E. histolytica* were tested in the present study. Our findings suggest that axenic HM-1 strain is different from HJ-1 strain in regard to the susceptibility to the bisphenols, and appear to be compatible with the view mentioned above. These studies also suggest that bisphenols, which are highly substituted with chlorine like hexachlorophene, do not necessarily have much higher inhibitory actions against the growth of *E. histolytica*. However, because 2-propanol-supported respiration as well as the growth of *E. histolytica* in the cultures appeared to be a little more markedly inhibited by hexachlorophene, we speculate that 2-propanol-supported respiration may be more relevant to viability of this parasite, although the physiolgical meanings of the aerobic metabolism are not known. Further studies are apparently needed for correlating structural characteristics of the halogenated bisphenols with their inhibitory actions against *E. histolytica*.

It is well known that dichlorophene has been an effective chemotherapeutic agent for some cestode infections in man (Rollo, 1975; Yoshimura, 1975). In particular, this compound has been used for treatment of taeniasis and diphyllobothriasis (Standen, 1963; Rollo, 1975). Recently, moreover, Idris *et al.* (1980) reported that dichlorophene was also effective for treatment of fasciolopsiasis. Hexachlorophene, in contrast, has not been employed for treatment of human parasitic diseases, although it has been used for veterianry purposes and for disinfection in various parts of clinical medicine because of its effectiveness against gram-positive bacteria (Harvey, 1975). To our knowledge, however, these halogenated bisphenols have not been tested against protozoa like *E. histolytica* lacking aerobic metabolic systems (Weinbach *et al.*, 1976).

The pharmacological mechanism of action of dichlorophene against cestodes is not known (Rollo, 1975), whereas bithionol was reported to have an uncoupling effect on mitochondrial oxidative phosphorylation at 10^{-8}M, and inhibit some enzymes such as succinate dehydrogenase and fumarate reductase widely distributed among helminths at 10^{-3} to 10^{-4} M (Murakoshi and Moriya, 1968; Murakoshi *et al.*, 1969).

Since metronidazole, the drug of choice for human amoebiasis, has mutagenicity in bacteria and carcinogenicity in rodents (see Hunter, III *et al.*, 1976), a large number of investigations have been done to evaluate adverse effects of metronidazole in man. Recent studies (Roe, 1979; Goldman, 1980) agreed that there was no evidence of cancers or birth defects caused by metronidazole in patients. We suggest, however, that these bisphenols are worth further evaluations for treatment of human amoebiasis, because the compounds like bithionol and dichlorophene also have no serious side effects.

Summary

Endogenous and 2-propanol-supported respiration of intact trophozoites of *Entamoeba histolytica* (strain HM-1 : IMSS) were inhibited by dichlorophene and hexachlorophene, the halogenated bisphenols which are structurally similar to bithionol. These bisphe-

nolic derivatives were also able to kill and disrupt both axenic (HM-1 strain) and polyxenic (strain HJ-1 : KEIO) amoebae in BI-S-33 and Balamuth's medium respectively. It took shorter than 12 hours to kill and disrupt virtually all amoebae of the two strains at a concentration of 150 μg/ml of the bisphenols at 35.5°C in the cultures. Omission of bovine serum from BI-S-33 medium resulted in a considerable enhancement of the inhibitory action of the compounds against *in vitro* growth of HM-1 strain. It took less than 20 min at the same temperature to kill practically all trophozoites of HM-1 strain with 100 μg/ml of the bisphenols in the serum-free medium. Electron microscopically, incubation of HM-1 strain in BI-S-33 medium containing the bisphenols demonstrated appearance of the thinner portions of the plasma membrane of amoebae, where the fine extrusions came out. Our present investigations suggest that these bisphenols, in particular dichlorophene, are worth further evaluations to ascertain whether they are useful for treatment of amoebiasis, although serum appears to diminish their anti-amoebic actions.

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ジクロロフェンおよびヘキサクロロフェンの赤痢アメーバの呼吸活性，培地内増殖，形態に対する効果

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ジクロロフェン，ヘキサクロロフェンというビチオノールに構造の類似しているハロゲン化ビスフェノールの赤痢アメーバに対する効果を調べた結果，これら二種の化合物は無菌株（HM-1：IMSS株）の内因性，および2-プロパノール依存性の呼吸を強く阻害することが判明した．これらの化合物はさらに試験管内における赤痢アメーバの増殖を強く抑制し，無菌株（HM-1株），および細菌共棲株（HJ-1：KEIO株）を150μg/mlで12時間以内に35.5°Cにて全て殺滅した．HM-1株に関しては培地中の血清を除去するとさらに増殖抑制効果は高まり，100μg/mlで20分以内に全てのHM-1株栄養型を殺滅した．電顕的にはこれらの化合物とインキュベートしたHM-1株の細胞膜に薄化がみられ，その同一個所より膨化を思わせる突出した構造がみられた．

以上の所見は，血清によつてやや抑制されるものの，これらの化合物は抗アメーバ剤として更に調査する価値あるものであることを示唆している．