Arch. Invest. Méd. (Méx.), 1987; *18:* 119

Recibido: 25 de noviembre de 1986
Received: november 25th, 1986

Aceptado: 12 de enero de 1987
Accepted: january 12th, 1987

investigación simplificada *in vitro* para correlacionar la actividad amebicida con quelación por 8-hidroxiquinolina y sus compuestos relacionados

ROY G. TAYLOR
LINDA S. O'CONNELL
LEONARD W. SHEIBEL

a simplified *in vitro* assay correlating amebicidal activity with chelation by 8-hydroxiquinoline and related compounds

Roy G. Taylor, Linda S. O'Conell and Leonard W. Scheibel. Division of Tropical Public Health, Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA.

Solicitud de sobretiros (request for reprints): *Roy G. Taylor.* Uniformed Services University of the Health Sciences. 4301 Jones Bridge Road, Bethesda, Md 20814-4799, USA.

Resumen

Se dispone de diversos métodos para someter a prueba los fármacos contra *Entamoeba histolytica* in vitro, pero abarcan el empleo de radioisótopos en microplacas o los cultivos amibianos en tubo, más engorrosos. La finalidad de este estudio consistió en desarrollar una prueba in vitro simplificada para los fármacos antiamibianos y en demostrar que los agentes quelantes matan a las amibas. Se cultivó la clona 6 de la cepa HM-1:IMSS durante 48 a 72 horas en medio TYI-S-33 de Diamond. Antes de los experimentos las amibas se incubaron durante 24 horas en placas de fondo plano de 96 pozos, estériles, dentro de una cámara incubadora modular a 35.5 °C en una atmósfera con O_2 al 2.5 por ciento. CO_2 al 1.5 por ciento y N al 96 por ciento. Se disolvieron los compuestos de prueba en dimetilsulfóxido, agua o medio TYI-S-33 antes de ponerlos en los cultivos amibianos a diferentes concentraciones. Los testigos fueron medio de cultivo sólo, dimetilsulfóxido y metronidazol. Se devolvieron a la cámara incubadora placas que contenían amibas, medio de cultivo y compuestos químicos en diluciones dobles. Tras 24, 48 y 72 horas de la introducción de los compuestos de prueba, se empleó un microscopio invertido para determinar la concentración farmacológica más baja a la que ocurrió la muerte del mayor número de amibas (concentración letal mínima). *Entamoeba histolytica* fue sensible a la 8-hidroxiquinolina y compuestos relacionados. Por primera vez se relacionó el mecanismo de acción de estos agentes antiamibianos con la quelación.

Abstract

Several methods have been developed for testing drugs against axenic amebae *in vitro*. However, these use radioisotopes or culture methods, which are difficult to implement. This study was undertaken to develop a simplified *in vitro* drug assay and to investigate the antiamebic activity of chelators *in vitro*. Clone 6 of the HM1 strain was cultured in TYI-S-33 medium, as developed by Diamond.

Drugs were added to 24-hour ameba cultures in microplates. Controles were culture medium alone, DMSO, and metronidazole. Plates were visually inspected using an inverted phase contrast microscope at 24-hour intervals until the amebae had been exposed to the drugs for 72 hours. concentration at which almost complete killing of amebae occurred. *Entamoeba histolytica* was sensitive to 8-hydroxiquinoline and related compounds. The mechanism of action of these antimebic compounds is related for the first time to its chelating properties.

Since Diamond[1,2] first cultivated *Entamoeba histolytica* axenically, several methods have been developed for testing drugs against axenic amebae *in vitro*. Diamond and Bartgis[3] pointed out the advantages of using axenically cultivated amebae instead of xenic or monoxenic cultures for drug tests, and they described a method for susceptibility testing of amebicides against *E. histolytica* in tubes containing TP-S-1 medium. Gillin and Diamond[4,5] showed that the colony-forming efficiency of axenic amebae in tubes of agar with TYI-S-33 medium provided an excellent means of determining drug activity. Finally, Cedeño and Krogstad[6] developed a quantitative method for testing drugs against axenic amebae in TYI-S-33 by measuring the uptake of ^{3}H-thymidine in microplates that were incubated under reduced oxygen tension. This study was undertaken to develop a simplified *in vitro* drug assay that did not rely on the use of radioisotopes.

Our method for testing amebicides facilitated experiments designed to investigate the antiamebic activity of chelators *in vitro*. Chelators exhibit antimicrobial and antiparasitic effects against a variety of organisms,[7] but only those compounds similar to 8-hydroxyquinoline (oxine) provide compelling chemical evidence that their mechanism of action depends directly on chelation.[8,9] Although amebicides such as iodochlorhydroxyquin and diyodohydroxyquin are oxines that may liberate iodine to kill amebae,[10] a structurally related compound 5-chloro-7-(3-diethylaminopropylaminomethyl)-8-quinolinol appears to owe its antimalarial activity largely to chelation.[10,11] It follows that oxine and related compounds could have an antiamebic effect due to chelation.

Material and methods

Drugs

Drugs were obtained from the following sources: Metronidazole was obtained from the drug inventory in the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC; 8-hydroxyquinoline, 2-methyl-8-hydroxyquinoline, 5-hydroxyquinoline, and 2-mercaptopyridine-N-oxide were purchased from Aldrich Chemical Co., Milwaukee, WI; 2-mercaptoquinoline-N-oxide and the zinc salt of bis-2-mercaptoquinoline-N-oxide were gifts from Dr. D.H. Steinberg, Ciba-Geigy Co., Ardsley, NY; recrystallized tetraethylthiuram disulfide (disulfiram) was a gift from the late Dr. Ernest Bueding; sodium diethyldithiocarbamate was purchased from J.T. Baker Chemical Co., Phillipsburg, NJ; 5-chloro-7-(3-diethylaminopropylaminomethyl)-8-quinolinol dihydrochloride (KAN-322) was a gift from Dr. Leslie M. Werbel, Warner-Lambert Co., Ann Arbor, MI.

Amebae

Dr. Louis S. Diamond kindly provided a stock culture of *Entamoeba histolytica* strain HM-1:IMSS clone 6 in TYI-S-33 medium.[12] The amebae were axenically cultivated at 35.5±0.5°C in tubes of TYI-S-33 medium containing 15 per cent bovine serum according to published methods.[13] Tubes of 48 to 72 hour cultures were chilled 5 min in crushed ice and were inverted several times to obtain a uniform suspension of trophozoites for counting in a hemacytometer. A known number of amebae for experiments was then added to fresh TYI-S-33 medium in a sterile tissue culture flask.

Assay procedure

A Cetus Pro/Pette automatic pipetting system (Perkin-Elmer Corp., Norwalk, CT) was used to dispense 200 µl of the ameba suspension (6,000 or 12,000 per ml) into each well of a Costar 3596 flat bottom microplate (Costar, Cambridge, MA) under aseptic conditions. Amebae in the plates were first incubated 24 h so that they would be in logarithmic growth when drugs were added. The plates were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA), which was then purged for 45 min with 2.5 per cent O_2, 1.5 per cent CO_2, and 96 per cent N_2. The chambers were put inside an incubator at 35.5°C.

The next day, 4.0 mg of each drug was dissolved in 50-100 µl of dimethyl sulfoxide (DMSO) or water. Eight milliters of TYI-S-33 medium was added to the dissolved drug to give a starting concentration of 500 µg/ml. Four milligrams of 2-mercaptopyridine-N-oxide was dissolved directly in 8.0 ml of TYI-S-33 at 48°C because it was insoluble in DMSO. After the drug solutions were sterilized by passing them through a Gelman Acrodisc 0.22 µm or 0.45 µm filter (Gelman Sciences Inc., Ann Arbor, MI), aseptic technique was used in all subsequent steps of the assay. The Cetus Pro/Pette diluted duplicate samples of each drug in two fold steps to the desired con-

centracion in microplates, which then served, as templates for transferring 50 μl of each drug dilution to the corresponding wells of the plates containing amebae. Therefore, the drugs were further diluted five fold when they were added to 24-hour ameba cultures in microplates. Plates containing amebae, culture medium and drugs, were returned to the incubator chamber under the same conditions as before. Controls were culture medium alone, DMSO, and metronidazole. Plates were visually inspected using a Zeiss ICM 405 inverted phase contrast microscope at 24-hour intervals until the amebae had been exposed to the drugs for 72 h. The minimum lethal concentration (MLC) of a drug was considered the lowest concentration at which almost complete killing of amebae occurred.

Results

Preliminary experiments showed that axenic amebae (12,000 per ml) grew and remained healthy in microplates under reduced oxygen tension for 96 h (data not shown). This meant that drugs could be tested for 72 h after exposure to 24-hour ameba cultures that were already in logarithmic growth. Seventy-two hours was considered sufficient time for both amebicidal and amebistatic drugs to be found active in the test system.

Endpoints were determined visually by examining the plates at 24-hour intervals with an inverted microscope, and the MLC of drugs was easily determined (figures 1-4).

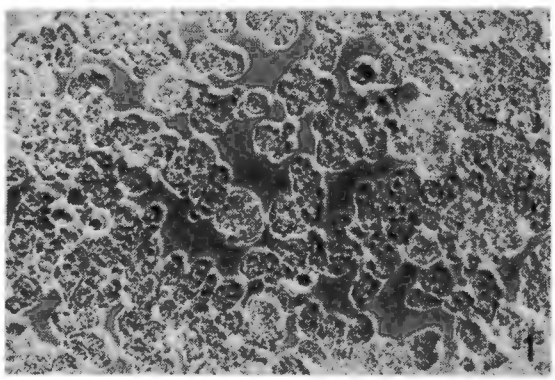

Figure 1. Normal control.

Figure 2. Miminum lethal concentration of 8-hydroxyquinoline at 25 μg/ml.
Note the complete absence of normal appearing trophozoites.

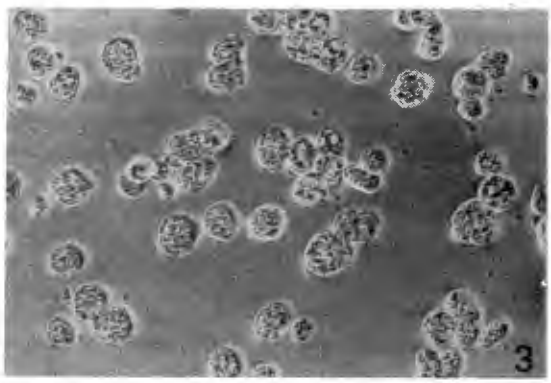

Figure 3. Metronidazole control at a concentration of 0.39 μg/ml. The presence of many motile amebae indicated that the MLC was at a higher concentration of the drug.

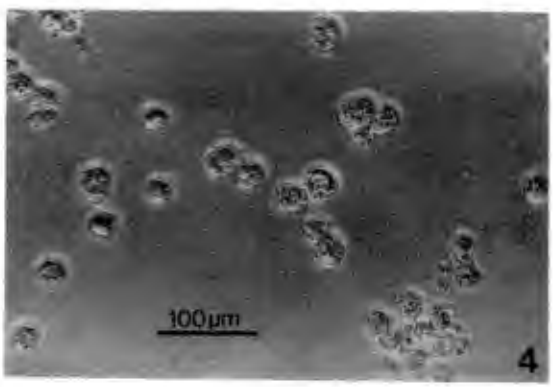

Figure 4. MLC of metronidazole at 0.78 μg/ml. None of the organisms appeared normal and none were motile.

Structures for the drugs used in this study are shown in figure 5. All of them are aromatic chelators related to oxine except for the aliphatic chelators, tetraethylthiuram disulfide and diethyldithiocarbamate.

Table I gives the MLC for each drug at 24, 48, and 72 h. Each drug was tested three times and gave consistent results. DMSO had no effect on the amebae when they were exposed to it at the same concentration used to dissolve the drugs in TYI-S-33: Metronidazole had an MLC of 0.78 μg/ml when exposed to amebae for at least 48 h. As expected, more 2-methyl-8-hydroxyquinoline was required to achieve an MLC than for the unsubstituted parent compound 8-hydroxyquinoline due to steric hidrance caused by a methyl group in the 2 position (figure 5).

5-Hydroxyquinoline was inactive at 100 μg/ml because the hydroxyl group of a monohydroxyquinoline must be in the 8 position to form a ligand-metal complex. *E. histolytica* was sensitive to 2-mercaptopyridine-N-oxide, 2-mercaptoquinoline-N-oxide, and the 2:1 (chelator:metal) complex bis-2-mercaptoquinoline-N-oxide at levels comparable to metronidazole.

The greater lipid solubility of 2-mercaptoquinoline-N-oxide and bis-2 mercaptoquinoline-N-oxide did not enhance their antiamebic activity over 2-mercaptopyridine-N-oxide, an observation previously made in the malaria parasite.[11,14] Tetraethylthiuram disulfide and its reduction product diethyldithiocarbamate are aliphatic chelators, which have been shown to kill *P. falciparum* presumably by chelation.[15] They were at least as active as 8-hydroxyquinoline against the ameba (table I). Finally, KAN-322 was active against axenic trophozoites, as expected.

Discussion

We have developed a simplified *in vitro* assay for testing drugs against axenic *Entamoeba histolytica* in microplates. Minimun lethal concentrations of drugs were determined by looking at the morphology of

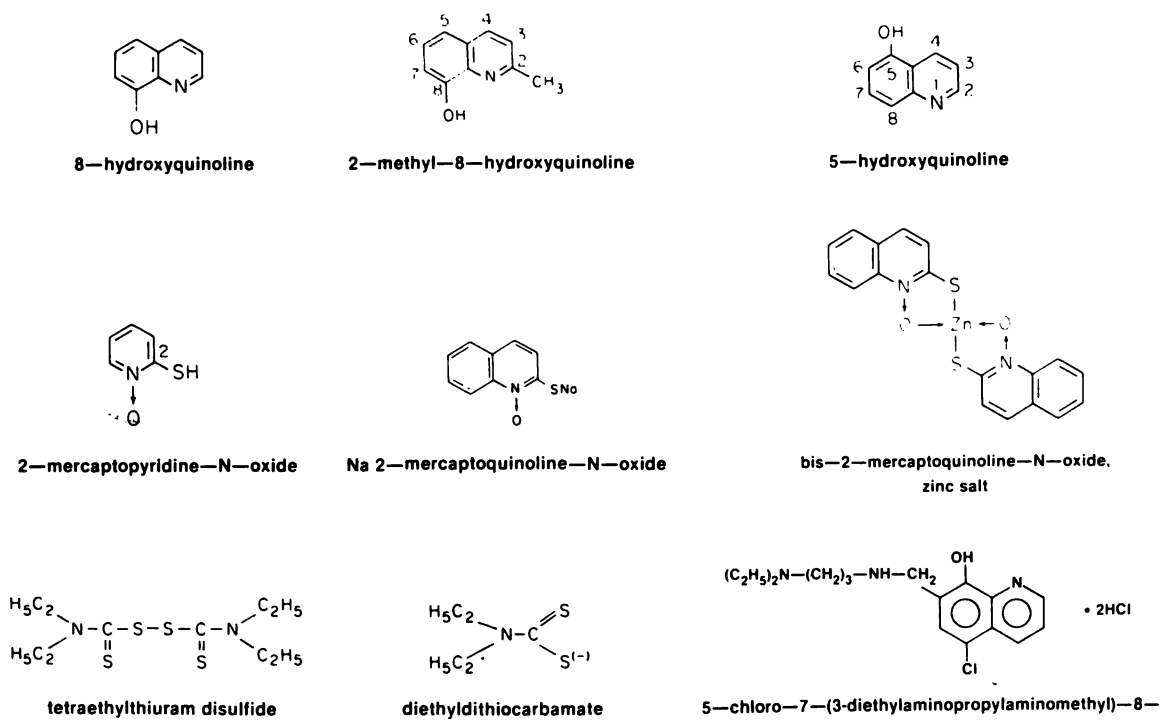

Figure 5. Structures of chelators used in this study.

TABLE I

MIMIMUM LETHAL CONCENTRATION (μg/ml) OF ANTIAMEBIC DRUGS

TESTED AGAINST AXENICALLY CULTIVATED *Entamoeba histolytica* STRAIN HM-1-IMSS CLONE 6 IN MICROPLATES INCUBATED FOR VARIOUS TIME PERIODS

AT 35.5°C IN 2.5% O_2, 1.5% CO_2, AND 96% N_2.

Drug	24 h	48 h	72 h
Metronidazole	1.56	0.78	0.78
8-Hydroxyquinoline	25	25	25
2-Methyl-8-hydroxyqiunoline	50	50	50
5-Hydroxyquinoline	>100	>100	>100
2-Mercaptopyridine-N-oxide	0.78	0.39	0.39
2-Mercaptopyridine-N-oxide	1.56	1.56	0.78
Bis-2-mercaptoquinoline-N-oxide, zinc salt	1.56	1.56	1.56
Tetraethylthiuram disulfide	25	12.5	12.5
Diethyldithiocarbamate	12.5	12.5	12.5
5-Chloro-7-(3-diethylaminopropylaminomethyl)-8-quinolinol, dihydrochloride	50	50	50

trophozoites by means of an inverted microscope thereby enabling one to observe the effects of a drug on the parasite. The method was relatively inexpensive because radioisotopes and a scintillation counter were unnecessary. Although we used a Cetus Pro/-Pette to dilute drugs in the plates, any of several microdiluters would suffice. It would not be unrealistic to screen 50 compounds at once using this assay procedure. Precise quantitative data (e.g. 50 per cent effective dose or ED_{50}) was not obtained by this method, but the assay was reliable enough to ascertain the antiamebic activity of compounds relative to the control drug metronidazole.

The MLC for metronidazole in this study (0.78 μg/ml at 48-72 h) was almost identical to the 72-hour amebicidal endpoint for strains HK-9 and F-22 in TP-S-1 or TYI-S-33 medium (0.75 μg/ml)[3,5] and for strain HM-1:IMSS in agar (0.6 μg/ml).[4,5] Less drug is required to achieve the ED_{50} after 48 h exposure of strain HK-9 to metronidazole in TYI-S-33 (ED_{50} = 0.35 μg/ml by radiometric means; ED_{50} = 0.17 μg/ml by counting parasites).[6]

The use of microplates required the modular incubator chamber to be purged with a compressed gas mixture that met the growth requirements of the ameba.[6] Diamond[13] and Reeves[16] reported on the need to reduce oxygen tenison for axenic cultivation of *E. histolytica*. We found that 2.5 per cent O_2, 1.5 per cent CO_2, and 96 per cent N_2 was a suitable gaseous environment fo the ameba whereas Cedeño and Krogstad[6] used 3 per cent O_2, 3 per cent CO_2, and 94 per cent N_2. The difference was probably inconsequential. *Plasmodium*

falciparum, which is sensitive to the same chelators used in this study, also has microaerophilic requirements when cultured *in vitro*.[17] The optimum gaseous mixture for the malaria parasite is 3 per cent O_2, 2 per cent CO_2, and 95 per cent N_2.[17,18]

Scheibel and Stanton[10] reviewed proposed mechanisms of action to explain the antimicrobial and antiparasitic action of chelators. Two hypotheses prevail. Some chelators form complexes with metal ions and may deprive the microbial organism of an essential nutrient. On the other hand, chelators such as the dithiocarbamates and 8-hydroxyquinolines may owe their antimicrobial and antiparasitic activity to the toxicity of the chelator: metal complex. For the malaria parasite, the evidence weighs in favor of extracellular chelator: metal complexes that rapidly penetrate the parasite to exert a lethal effect.[10] Scheibel[18] has postulated further that the toxic, free chelator may be liberated after a 2:1 (chelator:metal) complex enters the parasite and dissociates into a 1:1 complex plus free chelator. Siderochromes, but not oxines, apparently kill *Plasmodium* by iron deprivation.[18]

Several halogenated derivatives of 8-hydroxyquinoline have been used as intestinal amebicides: 5-chloro-7-iodo-8-quinolinol (iodochlorhydroxyquin or Vioform), 5,7-diyodo-8-quinolinol (iodoquinol or Diodoquin), and 7-iodo-8-hydroxyquinoline-5-sulfonic acid (Chiniofon).[10,19] Release of iodine from the 7 position of these molecules has been thought to explain their amebicidal effect.[10,20] However, KAN-322 (figure 5) is an analog of 8-hydroxyquinoline that lacks an iodine atom but still retains excellent amebicidal properties.[21] Thompson et al.[22] and Burckhalter et al.[21] postulated that chelation is the mechanism of action for KAN-322 and structurally related compounds.

8-Hydroxyquinoline is the only monohydroxyquinoline capable of chelation. By sterically hindering chelation with a methyl group in the 2 position (figure 5), 2-methyl-8-hydroxyquinoline was less active than oxine against *E. histolytica* (tables I). Frustrating chelation by moving the hydroxyl group to the 5 position (figure 5) resulted in 5-hydroxyquinoline being inactive at 100 μg/ml, thereby suggesting that the antiamebic activity of these compounds was a result of chelation. The substituted oxine KAN-322 also exhibited marked antiamebic activity (table I). Presumably, the amebicidal activity of KAN-322 was in large part due to chelation because the molecule does not possess iodine in the 7 position. Comparable results using these compounds have been reported for *P. falciparum*.[10]

Entamoeba histolytica was also exposed to other lipophilic chelators that have high metal-binding constants and lipid/water partition coefficients favoring penetration into the parasite. 2-Mercaptopyridine-N--oxide, 2-mercaptoquinoline-N-oxide, and bis-2-mercaptoquinoline-N-oxide are aromatic chelators with these properties; and they were potent amebicides (table I). The aliphatic chelators disulfiram and diethyldithiocarbamate also killed the ameba. These findings further substantiated the hypothesis that chelation is the mechanism of action for this class of compounds.

The case may not be closed, however. Some chelators may deprive the ameba of an essential nutrient like iron.[23-26] Others may exert their lethal effect due to the toxicity of the chelator: metal complex or due to direct inactivation of metallo-enzymes by the free chelator. Latour and Reeves[19] suggested that the antiamebic effect of 7-iodo-8-hydroxyquinoline-5-sulfonic acid is due to deprivation of iron from the ameba, but this remains to be proved. Scheibel and Stanton[10] have demonstrated by 59 Fe uptake experiments that toxic chelator: metal complexes may explain the antimalatial activity of 8-hydroxyquinoline and structurally related drugs. Whether it is the 1:1 complex, the free chelator, or both that kill the malaria parasite remains to be explored. Similar experiments are planed for *E. histolytica.*

Acknowledgments

This work was supported by project 85MM5517 from the U.S. Army Medical Research and Development Command. The authors wish to thank the following people for their assitance: TSGT James Coker, Ellen Goldman, Cynthia Holdin, Gregory Holmes, Hortense Koller, Victor Lagrange, Frances Langley, Mark Pape, and Jeffrey Swope.

Referencias

1. DIAMOND, L.S.: *Axenic cultivation of* Entamoeba histolytica. Science, 1961; *134:*336.

2. DIAMOND, L.S.: *Techniques of axenic cultivation of* Entamoeba histolytica *Schaudinn, 1903 and* E. histolytica*-like amebae.* J. Parasitol., 1968: *54:* 1047.

3. DIAMOND, L.S.; BARTGIS, I.L.: *Axenic cultures for in vitro testing of drugs against* Entamoeba histolytica. Arch. Invest. Méd. (Méxc.), 1971; *2*(suppl.):339.

4. GILLIN, F.D.; DIAMOND, L.S.: *Clonal growth of Entamoeba histolytica and other species of* Entamoeba *in agar.* J. Protozool., 1978; *25:*539.

5. GILLIN, F.D.; DIAMOND, L.S.: *Clonal growth of* Entamoeba *in agar: some applications of this technique to the study of their cell biology.* Arch. Invest. Méd. (Méx.), 1978; *9* (suppl. 1):237.

6. CEDEÑO, J.R.; KROGSTAD, D.J.: *Susceptibility testing of* Entamoeba histolytica. J. Infect. Dis., 1983; *148:* 1090:

7. SCHEIBEL, L.W.; STANTON, G.G.: *New approach in design of selectively toxit antimalarials.* In Proceedings of the IXth International Congress of Pharmacology. Paton, W.; Mitchell, J.F.; Turner, P. (eds.), Macmillan Press, London; 1984; p. 385.

8. SCHEIBEL, L.W.; ADLER, A.: *Antimalarial activity of selected aromatic chelators.* Mol. Pharmacol., 1980; *18:*320.

9. ALBERT, A.: *Selective toxicity: the physico-chemical basis of therapy, 7th ed.,* Chapman & Hall, London; 1985.

10. SCHEIBEL, L.W.; STANTON, G.G.: *Antimalarial activity of selected aromatic chelators. IV. Cation uptake by* Plasmodium falciparum *in the presence of oxines and siderochromes.* Mol. Pharmacol. In press.

11. SCHEIBEL, L.W.; ADLER, A.: *Antimalarial activity of selected aromatic chelators. III. 8-Hydroxyquinolines (oxines) substituted in positions 5 and 7, and oxines annelated in position 5, 6 by an aromatic ring.* Mol. Pharmacol., 1982; *22:*140.

12. DIAMOND, L.S.; HARLOW, D.R.; CUNNICK, C.C.: *A new medium of the axenic cultivation of* Entamoeba histolytica *and other* Entamoeba. Trans. R. Soc. Trop. Med. Hyg., 1978; *72:*431.

13. DIAMOND, L.S.: *Lumen dwelling protozoa:* Entamoeba, *trichomonads, and* Giardia. In vitro cultivation of protozoan parasites. Jensen, J.B. (ed.), CRC Press, Inc., Boca Raton, FL, 1983; p. 65.

14. SCHEIBEL, L.W.; ADLER, A.: *Antimalarial activity of selected aromatic chelators. II. Substituted quinolines and quinoline-*N*-oxides.* Mol. Pharmacol., 1981; 20:218.

15. SCHEIBEL, L.W.; ADLER, A.; TRAGER, W.: *Tetraethylthiuram disulfide (Antabuse) inhibits the human malaria parasite* Plasmodium falciparum. Proc. Natl. Acad. Sci. USA, 1979; *76:*5303.

16. REEVES, R.E.: *Metabolism of* Entamoeba histolytica *Schaudinn, 1903.* Adv. Parasitol., 1984; *23:*105.

17. SCHEIBEL, L.W.; ASHTON, S.H.; TRAGER, W.: Plasmodium falciparum: *microaerophilic requirements in human red blood cells.* Exp. Parasitol., 1979; *47:*410.

18. SCHEIBEL, L.W.: *In vitro inhibition of the human malaria parasite by selected lipophilic chelators.* In the red cell: Sixth Ann Arbor Conference. Brewer, G.J. (ed.), Alan R. Liss, Inc., New York, 1984; p. 377.

19. LATOUR, N.G.; REEVES, R.E.: *An iron-requirment for growth of* Entamoeba histolytica *in culture, and the antiamebal activity of 7-iodo-8-hydroxy-quinoline-5-sulfonic acid.* Exp. Parasitol., 1965; *17:*203.

20. ALBERT, A.; RUBBO, S.D.; GOLDACRE, R.J.; et al.: *The influence of chemical constitution on antibacterial activity. Part III. A study of 8-hydroxyquinoline (oxine) and related compounds.* Br. J. Exp. Pathol., 1947; *28:*69.

21. BURCKHALTER, J.H.; BRINIGAR, W.S.; THOMPSON, P.E.: *Antiamebic agents. V. Promising basic amebicides derived from 5-chloro-8-quinolinol.* J. Org. Chem., 1961; *26:*4070.

22. THOMPSON, P.E.; REINERTSON, J.W.; BOYLES, A.; et al.: *Antiamebic action of 5-chloro-7-diethylaminomethyl-8-quinolinol and of other substituted 8-quinolinols in vitro and in experimental animals.* Am. J. Trop Med. Hyg., 1955; *4:*224.

23. DIAMOND, L.S.; HARLOW, D.R.; PHILLIPS, B.P.: *et al.: Entamoeba histolytica: iron and nutritional immunity.* Arch. Invest. Méd. (Méx.), 1978; *9*(suppl. 1):329.

24. WEINBACH, E.C.; TAKEUCHI, T.; CLAGGETT, C.E.; et. al.: *Role of iron-sulfur proteins in the electron transport system of Entamoeba histolytica.* Arch. Invest. Méd. (Méx.), 1980; *11* (suppl. 1): 75.

25. DIAMOND, L.S.: *Amebiasis: nutritional implications.* Rev. Infect. Dis., 1982; *4:* 843.

26. SMITH, J.M.; MEEROVITCH, E.: *Specificity of iron requirments of* Entamoeba histolytica *in vitro.* Arch. Invest. Méd. (Méx.), 1982; *13*(suppl. 3): 63.