Inhibition of Excystment of *Schizopyrenus russelli* Cysts in the Presence of Emetine and its Cysticidal Effect in Conjunction with Sodium Lauryl Sulphate

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**SUMMARY**

Excystment agents (extract of *Escherichia coli*, living *E. coli* and glutamic acid) failed to cause excystment of *Schizopyrenus russelli* cysts in the presence of either emetine or compounds I and II (structurally based on emetine). A very high percentage of the cysts excysted in the presence of the excystment agents after the removal of emetine, showing that the treated cysts were viable. Ninhydrin reaction, using a fixed amount of glutamic acid and increasing concentrations of emetine, showed progressive inhibition of colour development. This suggests the possible binding of excystment factor to emetine, thus preventing excystment. Excystment inducing property of the excystment agents could not be prevented in the presence of carbarsone. When the cysts were treated with sodium lauryl sulphate and then with emetine or with sodium lauryl sulphate and emetine together, there was hardly any excystment. Sodium lauryl sulphate rendered the cyst wall permeable to emetine and the latter killed the cysts.

**INTRODUCTION**

Relapses encountered commonly in the treated cases of human intestinal amoebiasis seem to be due to the *in vivo* persistence of cysts of *Entamoeba histolytica* which escape the action of drugs. Known amoebicides appear to have little or no effect on the cystic stage of *E. histolytica*. 5% emetine HCl and yatren were found to have no effect on the cysts of *E. histolytica* treated for 30 min. *in vitro* (Yorke & Adams, 1926). It is, therefore, important to discover drugs that are, in addition to having amoebicidal property, cysticidal or prevent amoebae from forming cysts or make the amoebae come out of the cysts. This question has not, so far, seriously attracted the attention of biologists and chemists engaged on the chemotherapy of intestinal amoebiasis.

Excystment of cysts of free-living amoebae and *Entamoeba histolytica* in the presence of certain living bacteria has been noted by several workers (Crump, 1950; Drozanski, 1961; Dudziak, 1955; Kunicki-Goldfinger et al., 1957; Singh, and his co-workers, 1956, 1958, 1963, 1965.). Singh, Mathew & Anand (1958) and Singh, Saxena & Iyer (1965) found that an aqueous extract of an *Aerobacter* sp. and *Escherichia coli* caused excystment of five species of free-living amoebae. It was further shown by them that certain amino acids present in the aqueous extract were responsible for the excystment of *Schizopyrenus russelli* cysts. Certain chemically pure amino acids, at suitable pH range, were also found to cause excystment of...
alter the pH of the bacterial extract from its original value of 6.5. Once emetine was washed off, a very high percentage of the cysts excysted in the presence of *E. coli* extract (Table 1).

Living *Escherichia coli* cells also failed to cause excystment in the presence of emetine-HCl (1000 μg./ml.). The cysts appeared normal (Pl. 1, fig. 5) and nearly all the cysts excysted readily in the presence of living *E. coli* when emetine was removed.

### Table 1. Inability of excystment inducing agents to cause excystment of *Schizopyrenus russelli* cysts in the presence of emetine

<table>
<thead>
<tr>
<th>Cysts treated with</th>
<th>No. of cysts</th>
<th>No. of cysts excysted</th>
<th>% excystment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> extract</td>
<td>166</td>
<td>148</td>
<td>89</td>
</tr>
<tr>
<td>Emetine (1000 μg./ml.); excystment with <em>E. coli</em> extract after removal of emetine</td>
<td>183</td>
<td>180</td>
<td>98</td>
</tr>
<tr>
<td>E. coli extract + emetine (1000 μg./ml.)</td>
<td>443</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Excystment with <em>E. coli</em> extract after the removal of emetine</td>
<td>451</td>
<td>410</td>
<td>91</td>
</tr>
<tr>
<td><em>E. coli</em> extract + emetine (125 μg./ml.)</td>
<td>254</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>Excystment with <em>E. coli</em> extract after the removal of emetine</td>
<td>157</td>
<td>140</td>
<td>89</td>
</tr>
<tr>
<td>Live <em>E. coli</em></td>
<td>300</td>
<td>294</td>
<td>98</td>
</tr>
<tr>
<td>Live <em>E. coli</em> + emetine (1000 μg./ml.)</td>
<td>599</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Excystment with live <em>E. coli</em> after the removal of emetine</td>
<td>494</td>
<td>494</td>
<td>100</td>
</tr>
<tr>
<td>Glutamic acid (2%, pH 6.0)</td>
<td>150</td>
<td>128</td>
<td>85</td>
</tr>
<tr>
<td>Glutamic acid (2%, pH 6.0)+ emetine (1000 μg./ml.)</td>
<td>590</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Excystment with glutamic acid after the removal of emetine</td>
<td>418</td>
<td>384</td>
<td>92</td>
</tr>
</tbody>
</table>

* The cysts were treated for 24 hr with emetine alone or with emetine and excystment agents together.

Glutamic acid gave an excystment of 85 %, whereas glutamic acid containing emetine-HCl (1000 μg./ml.) showed no excystment during a period of 72 hr. After the removal of emetine, these cysts could excyst normally (92 %) with fresh glutamic acid (Table 1). Lower concentration of emetine-HCl (125 μg./ml.) in glutamic acid, however, did not inhibit excystment but only delayed the excystment process. Nearly all the cysts excysted within 48 hr. Still lower concentration of emetine-HCl (62.5 μg./ml.) did not affect the excystment in the presence of glutamic acid.

Ninhydrin reaction was run in a parallel experiment and a progressive inhibition of colour development was noticed when increasing amount of emetine were added to a fixed amount of glutamic acid. When this *in vitro* system was run chromatographically (butanol + acetic acid + water: 4 + 1 + 5) emetine and glutamic acid could be completely separated.

Compounds I and II (1000 μg./ml.) also completely inhibited the excystment of *Schizopyrenus russelli* in the presence of *Escherichia coli* extract up to 72 hr. When they were removed, nearly all the cysts excysted in the presence of *E. coli* extract. In this respect the action of these compounds was like that of emetine-HCl, although they have...
Emetine and excystment in amoebae

DISCUSSION

It has been shown in the present investigation that excystment-inducing agents failed to cause excystment of Schizopyrenus russelli cysts in the presence of emetine hydrochloride and compounds I and II. The results of the ninhydrin reaction suggest a possible binding of excystment agent to emetine, whereas, by paper chromatography, the excystment agent and emetine could easily be separated. This binding may thus be mediated by weak electrostatic and van der Waal’s forces, as suggested by Dhar (1959). When the cysts are treated with sodium lauryl sulphate and then with emetine or with sodium lauryl sulphate and emetine together, there is practically no excystment. This suggests that sodium lauryl sulphate renders the cyst wall permeable to emetine, the latter killing the cysts. The action of surfactant is not interfered with by the presence of emetine. These findings may have application in eliminating the cysts of Entamoeba histolytica from human carrier cases.

It is a great pleasure to express our sincere thanks to Dr M. L. Dhar, Director, Central Drug Research Institute, for his keen interest in this work. Grateful thanks are also due to Dr B. N. Singh, Scientist-in-Charge, Microbiology Division, for helpful suggestions during the course of this work and in the preparation of the manuscript. Authors are thankful to Mr L. M. P. Singh for his technical assistance throughout this investigation.

REFERENCES

Plate 1

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I. Excystment and permeability of cyst wall of amoebae.

Amoebicidal drugs discovered so far have little or no effect on the cystic stage of Entamoeba histolytica. This explains the frequent relapses encountered in treated cases of chronic human intestinal amoebiasis. When the effect of drugs disappears, the cysts excyst in the large intestine, because of the presence of excystment factors, the amoebae feed on suitable bacteria, multiply and cause relapse. It has been shown that cysts of several species of small free-living amoebae, free from micro-organisms, excysted in the presence of aqueous extract of Escherichia coli and amino acids at a suitable pH. A mixture of amino acids gave much higher percentage excystment than that obtained by individual amino acids. Excystment agents in the presence of emetine, pH 6.5, failed to cause excystment of cysts because of the possible binding of the excystment agents with emetine. The cysts excysted normally after the removal of emetine, showing that the treated cysts were viable. Trypsin at 2% concentration, pH 6.5 or 8.5, caused excystment, but it, in the presence of emetine, failed to cause excystment. When emetine was removed, the cysts excysted readily. When the cysts were treated with sodium lauryl sulphate, at concentrations not lethal to them, and then with emetine or with sodium lauryl sulphate and emetine together (pH 7.0), there was hardly any excystment. Sodium lauryl sulphate rendered the cyst wall permeable to emetine, the latter killed the
cysts. Similar results have been produced when cysts were treated with lysozyme in conjunction with emetine. Alkaline pH 8-10 has also been found to render the cyst wall permeable to emetine.

The above findings may lead to rational design and synthesis of chemotherapeutic agents to deal with cysts.

II. Importance of O-R potential in initiating cultures of axenically grown E. histolytica from small inocula.

L.S. Diamond and others have failed to initiate cultures of axenically grown E. histolytica with less than 5,000 amoebae/ml of the medium. There was only 10 to 15 fold increase in amoebae population in 72 to 84 hr. By maintaining suitable O-R potential during subculturing, it has been found that successful subcultures can be obtained with an inoculum of 250 amoebae/ml of the medium. The time taken for the amoebae to reach the maximum population depended on the size of the inoculum.

III. Effect of antiamoebic agents on axenic culture of E. histolytica. Out of the known antiamoebic drugs tested, ambilhar and metronidazole showed amoebicidal activity at 3.9 µg/ml, emetine and dehydroemetine at 11.7/µg/ml, Intestopen, Enterovioform and Mepacrine at 31.2/µg/ml and camoform, emetine-bismuth iodide, vioform and furamide at 62.5/µg/ml. Amongst the antibiotics tested, actidione
showed amoebicidal activity at 0.98 µg/ml, paramomycin sulphate at 3.9 µg/ml, demeclocycline HCl, Oxytetracycline HCl, tetracycline HCl, and Chlortetracycline HCl at 135 µg./ml. and chloramphenicol at 250 µg./ml. Numerous combinations of antiamoebic drugs and antibiotics are now available for treatment of intestinal and extra-intestinal amebiasis. It is not known whether the different combinations of drugs and antibiotics have any direct synergistic action on E. histolytica. Tests on 17 such combinations have not revealed any synergistic action. Emetine has been shown to be highly active at alkaline pH than at acid pH. Sodium taurocholate, sodium tauroglycocholate which are present in human bile, have been found to be amoebicidal.

IV. Hepatic amoebiasis. A simple method of producing hepatic amoebiasis in hamsters by intraperitoneal inoculation of trophozoites of E. histolytica without laparotomy, has been developed. A satisfactory system of scoring hepatic lesions has also been described. This method has been successfully used for screening of drugs.

V. Virulence of strains of E. histolytica of the large race. It has been found that strains of E. histolytica from human acute cases of amoebic dysentery are invasive or virulent to rats. Strains from carrier cases may range from non-invasive to invasive ones. Cholesterol has been shown to render non-invasive strains into virulent form. The acquired virulence of the strains could be maintained by feeding the amoebae with cholesterol or by rat caecal passage. One
non-invasive strain became virulent to rat by hamster liver passage. These findings do not support the views expressed by some workers that the large race of \textit{E. histolytica} consists of two stable races differing only in virulence. The above findings support the view that virulent amoebae arise by some kind of adaptation from avirulent amoebae. This is in agreement with the epidemiological data.

VI. Enzymes of \textit{E. histolytica}. Detailed studies on the biochemical characteristics of some of enzymes of a virulent strain of \textit{E. histolytica} have been carried out. Transaminase, succinate dehydrogenase, aldolase and proteolytic activity of \textit{E. histolytica} have been studied with particular reference to the action of metabolic activators, inhibitors, drugs and antibiotics on these enzyme systems.

VII. Biochemical studies on host-parasite relationship in experimental amoebiasis of rat. Studies were initiated on gut wall of rats infected with a virulent strain of \textit{E. histolytica} with a view to follow the biochemical changes involved in the necrosis and tissue damage caused by amoebae. It has been found that the caecum which is the site of infection or attack by \textit{E. histolytica} showed highly significant increase in aldolase, \textit{a}-amylase, alkaline phosphatase, ribonuclease and deoxyribonuclease, while the acid phosphatase showed nearly 15\% increase and the levels of transaminase, succinate dehydrogenase had slightly decreased whereas the proteolytic activity of the caecum was not altered even in caeca showing high grade ulceration. In the rats which
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were treated with enterovioform, emetine or metronidazole after infection with *E. histolytica*, the enhanced enzyme activities were lowered to the level observed in normal rats. It is clear from the above studies that the amoebic invasion of caecum which leads first to host inflammatory response and finally necrosis, is accompanied by significant increase of alkaline phosphatase, nucleases, aldolase and *L*-amylase. Increased activities of these enzymes appear to cause tissue necrosis.

VIII. Metacystic divisions of *E. histolytica*. In the present study metacystic division from man have been reported.

IX. Bovine serum for growing *E. histolytica*. Bovine blood can be collected in large bulk from slaughter house, and serum separated and stored at 4°C. Since 1965 this serum has been used in this Institute for growing amoebae and it has greatly cut down the expenditure which was incurred earlier on purchasing horse serum in sealed 10 ml. ampules.

X. New method of growing anaerobic amoebae on non-nutrient agar in Brewer's anaerobic Petri-dish. Anaerobic amoebae can be grown on non-nutrient agar containing reducing agent, supplied with rice starch and suitable edible bacteria, and sealed under an atmosphere of N₂. *E. invadens*, *E. moskowskii* and *Balamuthia* have been maintained for several years in Petri-dishes. They form cysts in these plates. Primary cultures of *E. histolytica*, *E. coli* and *Iodamoeba butschlii* from stool samples have been obtained.
XI. Electron microscopy, fluorescence microscopy and cytochemistry of Protozoa.

A. Electron microscopy. Studies on the cyst wall of amoebae have been initiated and structure of the cyst wall of Schizopyrenus russelli has been studied.

Action of amoebicidal drugs on the axenic culture of E. histolytica has been studied. Metronidazole (1/100,000) induces the formation of large chromatoid bodies in the cytoplasm within 8 hours. In contrast to this emetine (1/35000) did not produce chromatoids.

B. Fluorescence microscopy of Protozoa. In continuation to the earlier cytochemical studies, further work has been carried out with fluorescence microscopy. New techniques have been developed for the demonstration of neutral polysaccharides with acridine orange, staining of nucleic acid with acridine orange by three methods (in vivo staining differential fluorescence and staining and staining after acid hydrolysis), simultaneous demonstration of nucleic acids (DNA and RNA) and lipids by staining with a combination of 3,4- benzpyrene and acridine orange. Fluorescence microscopy methods to study the action of drugs and chemicals on the nucleic acids of Protozoa have been developed. Observations on a colourless euglenoid Cyclidopsis acus, Plasmodium gallinaceum, P. falciparum, Chilomastix and Giardia, Stylonychia have been recorded.

C. Cytochemistry of Protozoa. In order to study the
7.

Cytochemistry of Protozoa, a satisfactory method was developed to cut frozen and paraffin sections of Protozoa. Cytochemical studies conducted on Opalina, Nyctotherus, Balantidium, Herpetomonas, Stomatophora, Paramecium, E Colpoda, Eimeria, Vorticella, Amoeba, Giardia, Lophomonas and Khawkinga, formed the basis of review published in 1962.

Most of the work which has appeared on the Cytochemistry and ultrastructure of flagellates, opalimids and ciliates from 1962 to 1972, has been compiled and presented here in the form of two reviews. An integrated account of recent developments in the fields of cytochemistry, electron microscopy, fluorescence microscopy and autoradiography, of the cytoplasmic inclusion of Mastigophora, Opalinata and Cliophora, has been presented in the review articles. Attempt has also been made in these reviews to correlate the cytochemical and ultrastructural findings with the available biochemical data with a view to clearly define the functions of various cytoplasmic organelles.

C.P.D.
17-3-1973
List of Publications

I. Excystment and permeability of cyst wall of amoebae

1. Factors inducing excystation in free-living amoebae. 
   *Indian J. Exp. Biol.* 9, 350-357; (1971).

II. Chemotherapy of amoebiasis


B. Screening of Indian plants for antiamoebic activity against *Entamoeba histolytica*.


C. Loss of amoebicidal activity

IV. Action of metabolic inhibitors.


V. *Hepatic amoebiasis*


15. Chemotherapy of hepatic amoebiasis; screening of potential antiamoebic compounds. (Unpublished).

III. *Virulence of E. histolytica*


IV. *Enzymes of Entamoeba histolytica*


V. Host-parasite studies in experimental intestinal and hepatic amoebiasis.


VI. Metacystic divisions


VII. Bovine serum for culturing *E. histolytica*


VIII. New method of growing anaerobic amoebae

II. Electron microscopy


34. Electron microscopic observations on the action of some amoebicidal drugs on Entamoeba histolytica. Proc. of "Workshop on amoebiasis" All India Institute of Medical Sciences, New Delhi, p. 14 (1972).

X. Fluorescence microscopy of Protozoa


42. Comments on simultaneous demonstration of deoxyribonucleic acid, ribonucleic acid and lipids. *J. Histochem. Cytochem.*, 19, 252.


XI. *Cytochemistry of Protozoa.*


46. Histochemical studies of Protozoa. III. Studies on the
47. Histochemical studies on Herpetomonas muscarum. Quart.
(1954).
49. Studies on the nucleic acids in Colpoda cucullus. Proc. 47th
50. Origin and cytochemistry of Paramylum bodies in Khudkinga
53. Observations on the macrogametocyte leading to the forma-
54. Cytochemistry of Paramocium caudatum, Colpoda cucullus
and Vorticella sp. Res. Bull. Panjab. Univ. N.S., 12,
55. The contractile vacuole of Protozoa and the Golgi appara-


XII. Reviews on Protozoa

