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Lipid requirements for axenic cultivation of *Entamoeba histolytica*

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Abstract. Fatty acids, cholesterol and glucose present in axenic medium are utilized by growing *Entamoeba histolytica* but the amoeba is unable to synthesize cholesterol from [U $^{-14}$ C $-$] glucose although the label is incorporated into the fatty acids and non-saponifiable fractions of the organism. Exogenously-added sonicated dispersions of cholesterol, β-sitosterol, lanosterol, lecithin and lauric, palmitic, linoleic and stearic acids are ingested by the amoebae with subsequent loss in amoeboid movement. After a few hours the movement is regained. Cholesterol, lecithin and the fatty acids stimulate amoebic multiplication but are unable to replace serum in the medium either singly or in combination.

Keywords. *Entamoeba histolytica*; axenic; cholesterol; lecithin; fatty acids; trophozoite-multiplication.

Introduction

Cholesterol has been found to convert attenuated and non-invasive strains of *Entamoeba histolytica* into invasive forms (Biagi *et al*., 1962; Bos and Van Griend, 1977; Das and Ghoshal, 1975; Das and Singh, 1965; Lwoff, 1951; Sharma, 1959; Singh, 1959; Singh *et al*., 1971; Meerovitch and Ghadirian, 1978). This observation has necessitated a detailed and systematic study of the lipid requirements of the organism.

Several workers observed the growth-promoting effect of cholesterol and dihydrocholesterol on *E. histolytica* growing in the presence of bacteria. Neutral lipid and free cholesterol fractions of horse serum (one of the constitutents of the Shaffer-Frye medium) when coated on the surfaces of culture tubes, stimulated multiplication of *E. histolytica* (NRS strain) growing in the presence of *Fusobacterium symbiosus*, while the phospholipid fraction or cholesterol esters did not promote growth (Cedillos *et al*., 1961; Lwoff, 1951; Rees *et al*., 1944). Although serum, a constitutent of the medium used for axenic cultivation of *E. histolytica*, is a source of lecithin, cholesterol and fatty acids, the effect of these lipids on trophozoite-multiplication under axenic conditions has not been studied. In earlier communications from our laboratory it was reported that a sonicated dispersion of the lipids of several human intestinal bacteria stimulated trophozoite-multiplication of *E. histolytica* 200: NIH under axenic

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Abbreviations used: TLC, thin layer chromatography; GLC, gas liquid chromatography.

conditions (Rai *et al*., 1978a) and free fatty acid, non-saponifiable and neutral lipid fractions of *Streptococcus faecalis* stimulated growth but the phospholipid fraction of the bacteria, which was mainly composed of phosphatidyl ethanolamine, phosphatidic acid and diphosphatidyl glycerol, and cephalin from egg (phosphatidyl ethanolamine+phosphatidyl serine) inhibited amoebic multiplication (Rai *et al*., 1978b). These workers also found that ^{14}C-labelled non-saponifiable fractions of lipids of the bacteria (obtained by culturing the bacteria in the presence of [U-^{14}C]-glucose) were incorporated into the amoebic cells and converted into cholesterol.

Therefore, with a view to evaluate the lipid requirements of *E. histolytica* 200:NIH, we have studied utilization by the amoeba of glucose, cholesterol and fatty acids, present in axenic medium (Singh *et al*., 1973) and the effect of these lipids, when added to the medium as sonicated dispersions, on trophozoite-multiplication.

Materials and Methods

Materials

Cholesterol was purchased from Centron Laboratories, Bombay; egg lecithin was from Biochemicals Unit, V. P. Chest Institute, New Delhi; ergosterol, lauric, palmitic, linoleic andstearic acids were from Nutritional Biochemicals Corporation, Cleveland, USA. Lanosterol was isolated from wool grease according to Downing *et al*. (1960). β -Sitosterol was a kind gift from Dr S. K. Nigam, National Botanical Research Institute, Lucknow.

Parasite

200:NIH strain of *E. histolytica*, received from Dr. L. S. Diamond, National Institute of Health, Bethesda, MD, USA was maintained regularly in TPS-2 medium (Singh *et al*.,1973). This is a modified TPS-1 medium (Diamond, 1968) containing cysteine hydrochloride but without ascorbic acid. Axenic cultivation of the amoeba was carried out in sterile screw-capped tubes (16 × 125 mm), 1 ml of the medium containing 10,000 trophozoites was inoculated into each tube containing 9 ml of fresh medium and the tubes were incubated at 37°C in upright position. Amoebic growth was followed by counting the number of trophozoites in a haemo-cytometer under an inverted microscope.

Dispersion of lipids

The lipids were dissolved in a minimum amount of alcohol, injected into deionized water under constant bubbling of N2 and the suspensions were sonicated at 20 Kc/sec, energy output 1.5 amp for 20 min at 20°C in a Mullard Ultrasonic Generator. The sonicated dispersions were filtered through Millipore membrane filters (0.22 μ). Lecithin, cholesterol and fatty acids were assayed in the filtrates as described under analytical procedures and 0.1-1.0 ml aliquots were asceptically added to culture tubes before inoculation to give the concentrations specified for each experiment.

Harvesting of trophozoites

After incubation, the culture tubes were centrifuged at 900 g for 20 min at 0°C, the pellets of amoebae were combined and washed three times with normal saline.

Glucose was estimated in fresh media and culture supernatants obtained after harvesting the cells according to Nelson (1944).

Incorporation of [$U^{-14}C$] *glucose*

Appropriate amounts of [U-^{14}C]-glucose (specific activity 2.5 mCi/mmol, Isotope Division, Bhabha Atomic Research Centre, Bombay) were added to the medium before sterilization, so that the radioactivity in each tube (10 ml) was 2.5 μCi After inoculation and incubation, the trophozoites were harvested, repeatedly washed with fresh medium (non-radioactive) till no radioactivity was detected in the washings. Aliquots of fresh media or combined culture-supernatants and washings were spotted on strips of Whatman No. 1 filter paper. Pellets of trophozoites were digested with 0.5 ml hyamine hydroxide (Packard Instruments Company, Zurich, Switzerland) at 60°C overnight. For the detection of radioactivity in the liberated CO_2, the amoebae were grown in screw-capped tubes provided with a U shaped side arm (2-3 mm diameter) just above the level of the medium in the tube and the side arm was filled with 0.5 ml hyamine hydroxide to trap the CO_2. After inoculation and incubation, the hyamine hydroxide was withdrawn with a microsyringe.

Analytical procedures

Fresh media or culture supernatants obtained after harvesting the trophozoites were first lyophilized, and then extracted with chloroform:methanol (2:1v/v) while the pellets of trophozoites were directly extracted according to Folch *et al*. (1951). Cholesterol and phosphorus in the lipids were estimated according to the method of Zlatkis *et al*. (1953) and Wagner *et al*. (1962) respectively.

The lipid samples were saponified with alcoholic sodium hydroxide (10% w/v) and the liberated fatty acids were esterified with diazomethane, according to James (1960). Gas liquid chromatography (GLC) of the methyl esters was carried out in 15% DEGS column in a Perkin-Elmer instrument through the kind courtesy of Professor S. M. Osman, Chemistry Department, Aligarh Muslim University, Aligarh, and Dr K. K. G. Menon, Hindustan Lever Research Centre, Bombay. Fatty acid composition was determined on the basis of area of individual peaks which were identified by comparison of retention time with authentic methyl esters.

Radioactive samples were taken in 15 ml of scintillation fluid (0.4% PPO, 0.1% POPOP in freshly distilled toluene diluted with equal volume of ethoxyethanol) and the radioactivity was assayed in a liquid scintillation Spectrometer (Packard Model 300, 90% counting efficiency for ^{14}C).

Results and discussion

Axenized *E. histolytica* 200:NIH contained about 24 mg lipid/100 million trophozoites, 30% of the lipid was phospholipid and 10% was sterol. Cholesterol was the major sterol but traces of another spot giving blue colour with orthophosphoric spray and of lower mobility than cholesterol on TLC was also present. Fatty acid composition (%) of the lipid was: lauric, 9.5; myristic, 13.9; palmitic, 11.8; stearic, 10.5; oleic, 5.0; linoleic 1.7 and unidentified fatty acids equivalent to chain length C20:4,14.8 and C27:5, 39.6. The axenic culture medium used in this study contains 5.6 mg cholesterol/ 100 ml and the % composition of constituent fatty acids of the medium were:

palmitic, 30; oleic, 20 and linoleic, 50. The culture supernatant obtained after 48 h of amoebic growth contained 5.5 mg cholesterol per 100 ml and gave the following % fatty acid composition: palmitic, 20; oleic, 5 and linoleic, 75. Thus 100 μg cholesterol/100 ml medium was utilized during amoebic growth and palmitic and oleic acids were preferentially utilized.

During growth, the amoeba consumed about 300 mg glucose per 100 ml medium. Fifty per cent of the radioactivity added as [U^{-14}C]-glucose to the medium was recovered in the culture supernatant obtained after harvesting the trophozoites, only 0.1% of the radioactivity added to the culture was incorporated into the trophozoites and appreciable amount was detected in CO_2 liberated during amoebic growth. Only 12% of the total radioactivity incorporated in the amoebae was in the lipids, but there was no incorporation in the cholesterol fraction.

TPS-2 medium supplemented with lecithin and cholesterol separately upto 40 μg/ml stimulated amoebic multiplication to 2 and 2.45 times that of control respectively, while the addition of both lecithin and cholesterol at this concentration stimulated the amoebic multiplication to 2.5 times that of the control (figure 1).

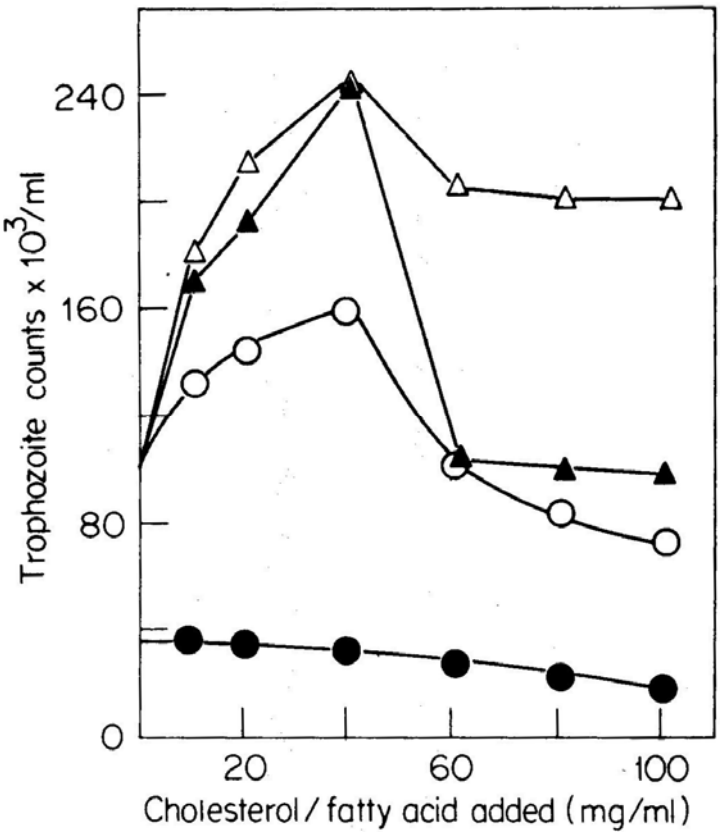

Figure 1. Effect of exogenous cholesterol and/or lecithin on multiplication (96 h) *E. histolytica*, Serum omitted+cholesterol and/or lecithin, ●;
Serum+cholesterol, O;
Serum+lecithin, ▲
Serum+lecithin +cholesterol, Δ
(10,000 trophozoites inoculated, 96 h incubation).

However, when lecithin or cholesterol or both were added to the medium which did not contain serum, no multiplication of trophozoites was observed. Addition of lauric, palmitic, linoleic and stearic acids to the medium stimulated amoebic growth by 4.7, 7.0, 5.0, 4.6 fold, respectively while the corresponding fold multiplication in control sets where no fatty acid,was added was 3.4. With the exception of stearic acid, amoebae grown in the presence of other fatty acids were more active. The addition of cholesterol in conjunction with palmitic or linoleic or stearic acids markedly stimulated amoebic multiplication by 22, 18 and 14 fold, respectively. The amoebae were more active, bigger in size and ingested lipid micelles were seen (table 1). However, addition of lauric acid and cholesterol to the axenic medium inhibited multiplication and caused reduction in the size and activity of trophozoites.

Table 1. Effect of exogenous fatty acids and cholesterol

Fatty acid	Cholesterol	× Fold multiplication	Morphology/motility
Lauric	−	4.7	Active movement
	+	2.0	Dead amoebae; few are round; smaller size
Palmitic	−	7.0	Active movement
	+	20.0	Very active movement; bigger size; lipid micelles ingested.
Linoleic	−	5.0	Active movement
	+	18.0	Very active movement; medium size; lipid micelles ingested
Stearic	−	4.6	Sluggish
	+	14.0	Active movement
None	−	3.4	Sluggish movement
	+	11.0	Active movement

– Cholesterol not added; + 100 μgcholesterol/ml medium; 100 μg. fatty acid/ml medium 72h incubation.

When the sonicated dispersion of cholesterol or **ß**-sitosterol or lecithin was added aseptically to the trophozoite suspension the amoeboid movement increased in the first 1-3 h after which the amoebae became rounded. Active movement of trophozoites was restored after 18 h. Lanosterol did not exhibit this phenomenon while ergosterol caused lysis of the amoebae.

Utilization of glucose in the medium by *E. histolytica* (strains DKB and K9) and the incorporation of the carbon fragments of the sugar into fatty acids of the amoeba has been demonstrated by Sawyer *et al* (1967), but this work was carried out in the presence of bacterial cells and antibiotics.

On the other hand Van Vliet *et al*. (1975) have shown that under axenic conditions *E. invadens* exhibits absolute auxotrophy for cholesterol and fatty acids. Our results show that *E. histolytica* NIH:200utilizes [U-^{14}C]-glucose present in the axenic medium;

carbon fragments of the sugar are incorporated into fatty acids of the amoeba but there is no incorporation into cholesterol. Palmitic and oleic acids of the medium are utilized by the amoeba and converted into its constituent fatty acids. Exogenous fatty acids, when added to the medium, are ingested by the amoeba and stimulate multiplication of the trophozoites, Similarly, cholesterol of the medium is utilized by the amoeba during growth and micelles of cholesterol added to the medium are ingested by the amoeba and stimulate growth and multiplication. Thus it is evident that *E histolytica* 200:NIH exhibits, during axenic cultivation, absolute auxotrophy for cholesterol and partial auxotrophy for fatty acids.

Latour and co-workers (1965) have shown that the phospholipid fraction of horse serum is unable to stimulate growth in *E. histolytica* (NRS strain) in the presence of *Bacteroides symbiosus*. Phospholipases are known to be present in bacteria (Van Deenen and De Hass, 1966). It is quite likely that the phospholipids added by these workers to amoeba-bacteria cultures were hydrolyzed during growth. Our results show that a sonicated dispersion of lecithin, when added to the medium, stimulated trophozoite-multiplication under axenic cultivation. Since lecithin cannot be removed from the medium without extracting other lipids and denaturing proteins and lipo-proteins, the absolute requirement of lecithin in axenic cultivation of the amoeba cannot be demonstrated by reconstitution. This phospholipid is present in *E. histolytica* (Sawyer *et al*., 1967) but it is not known if the amoeba can synthezise it from precursor molecules such as the fatty acids, glycerol, phosphate and choline.

Although, the present study has demonstrated the requirement of cholesterol, fatty acids and lecithin in axenic cultivation of *E. histolytica* 200:NIH, these lipids, either singly or in combination, cannot replace serum, which is an essential component of the axenic medium (Diamond, 1968) and is a source of these lipids. It is likely, that in addition to cholesterol, fatty acids and lecithin, carrier-proteins or lipoproteins of serum are essential for transport of lipids to amoeba and for its growth and multiplication.

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