effect. It may be pointed out here that the effect of vitamin A alcohol, in damaging the lysosomal membranes and thereby releasing the lysosomal enzymes, reported by Fell and Dingle[15] as the specific physiological action of vitamin A in controlling membrane structures does not appear to be very specific either in as much as testosterone propionate, a compound structurally unrelated to vitamin A, inhibited bone growth in organ culture experiments and also released acid phosphatase and cathepsins from normal rat liver isolated lysosomes[16].

It is more possible that the oxidative destruction of vitamin A alcohol itself by red cells as was demonstrated by Pollard and Bieri[17] and Dingle and Lucy[2] initiated the damage of the cellular membranes, thereby resulting in lysis without any co-oxidation of the lipid moieties of the erythrocyte membrane. However, attention is also drawn to the recent hypothesis put forward by Lucy[18] to explain the fusion of biological membranes and suggesting that the lytic action of retinol was due to the presence of a high proportion of globular lipid micelles in membranes challenged by retinol.

We are grateful to Dr. K. Madhavan Kutty, Principal, for encouragement, and Dr. M. S. P. Nair for help in preliminary studies.

1. Dingle, J. T. and Lucy, J. A., *Biochem. J.*, 1962, 84, 611.
2. — and —, *Ibid.*, 1963, 86, 15.
3. Lucy, J. A. and Dingle, J. T., *Nature*, 1964, 204, 156.
4. Krishnamurthy, S. and Bieri, J. G., *Biochem. Biophys. Res. Comm.*, 1961, 4, 384.
5. Moore, T. and Sharman, I. M., Personal communication, quoted by Lucy, J. A. and Dingle, J. T., *Nature*, 1964, 204, 156.
6. Alsaver, *J. Lab.* and *Clin. Med.*, 1946, 31, 394.
7. Ganguly, J., Krinsky, N. I., Mehl, J. and Deuel, J. Jr., *Arch. Biochem. Biophys.*, 1952, 38, 275.
8. Beutler, E., *J. Lab. and Clin. Med.*, 1957, 49, 84.
9. Mahadevan, S., Krishnamurthy, S. and Ganguly, J., *Arch. Biochem. Biophys.*, 1957, 83, 371.
10. Seshadri Sastry, P., Krishnamurthy, S. and Ganguly, J., *Ind. Jour. Med. Res.*, 1957, 45, 263.
11. Hunter, F. E. Jr., Gebicki, J. M., Hoffsten, P. E., Weinsten, J. and Scott, A., *J. Biol. Chem.*, 1963, 238, 828.
12. — and Scott, A., Hoffsten, P. E., Guerra, F., Weinsten, J., Schneider, A., Beverby Schutz., Fink. J., Ford, L. and Smith, E., *Ibid.*, 1964, 239, 604.
13. Green, J., Diplock, A. T., Bunyan, J., Edwin, E. and McHale, D., *Nature*, 1961, 190, 318.
14. Kitabchi, A. E., McCay, P. B., Carpenter, M. D., Trucco, R. E. and Caputlo, R., *J. Biol. Chem.*, 1960, 235, 159.
15. Fell, H. B., Dingle, J. T. and Webb, M., *Biochem. J.*, 83, 63.
16. Saralamma, P. J., Krishnamurthy, S. and Thangavelu, M., *Curr. Sci.*, 1964, 83, 176.
17. Pollard, E. J. and Bieri, J. G., *Brit. J. Nutr.*, 1958, 12, 359.
18. Lucy, J. A., *Nature*, 1970, 227, 815.

IMPORTANCE OF O-R POTENTIAL IN INITIATING CULTURES OF AXENICALLY GROWN *ENTAMOEBA HISTOLYTICA* FROM SMALL INOCULA*

B. N. SINGH, S. R. DAS AND G. P. DUTTA
Central Drug Research Institute, Lucknow

ABSTRACT

It has been shown that maintenance of suitable negative O-R potential during subculturing axenically grown *Entamoeba histolytica* plays a very important role in initiating cultures from small inocula. An inoculum of 250 amoebae/ml of Diamond's TP-S-1-medium containing 0·3% cysteine without ascorbic acid *plus* 0·05% agar, given carefully at the bottom of screw-capped tubes and incubating the tubes in upright position without inverting them, yielded 200,000 amoebae/ml of the medium in 15 days. The time taken for the amoebae to reach the maximum population depended on the size of the inoculum.

IT is well known that maintenance of strict anaerobic condition is necessary for the growth of *Entamoeba histolytica* in association with bacteria (Chang[1]). In spite of this finding, no attention has been paid to study the effect of O-R potential on the growth of axenic *E. histolytica*. Diamond[2] in 1961 used 0·1% L-cysteine HCl and 0·02% l-ascorbic acid in a diphasic medium to grow *E. histolytica* (strain 200 : NIH) axenically. The presence of 0·05% agar in the overlay was found to be necessary for the growth of amoebae. In a well-established culture, an inoculum of 50,000 amoebae

* Communication No. 1828 from Central Drug Research Institute, Lucknow.

yielded 150,000 to 200,000 organisms in 72 hr. Since the work of Diamond[2], it has become customary to use the same concentration of cysteine and ascorbic acid in axenic culture media. Diamond and Bartgis[3] in 1965 reported growth of axenic strains of *E. histolytica* in a liquid monophasic medium without agar. Wittner[4] in 1968 found only limited growth of *E. histolytica* when agar from the overlay was removed from axenic diphasic media. He regards agar as an important part of the medium because it provides the particulate matter necessary to stimulate feeding of amoebae. An inoculum of 50,000 amoebae yielded about 250,000 organisms in about 4 days. Using polarographic methods, Wittner[4] has claimed that amoebae growing axenically consume oxygen (*see also* Montalvo, Reeves and Warren[5]). Diamond[6] in 1968 has developed two new media, TTP-S-diphasic and TP-S-1-monophasic, for axenic cultivation of *E. histolytica*. He found that in setting up initial cultures of *E. histolytica* and *E. histolytica*-like amoebae (Huff and Laredo strains) in TP-S-1-medium, addition of 0·01% agar is very beneficial. Diamond advocates that for sub-culturing amoebae in TP-S-1-medium in screw-capped tubes, the tubes should be inverted gently 4-5 times after the inoculum and incubated at about 36° C in slanted position (5° to 10° horizontal). He[6] found that with strain 200 : NIH, an inoculum of 5,000 or 10,000 amoebae/ml of the medium gave best results. An inoculum of 10,000 amoebae/ml of the medium gave 10 to 15-fold increase in 72 or 84 hr. Diamond failed to initiate successful cultures with less than 5,000 amoebae/ml of the medium.

The present communication deals with importance of O-R potential in initiating cultures of axenically grown *E. histolytica* from small inocula.

Materials, Methods and Results

Source of inoculum.—For sub-culturing *E. histolytica* (strain 200 : NIH), 9 ml of TP-S-1-medium was added to a screw-capped tube (16 mm in diameter and 125 mm in length) and 10,000 amoebae/ml of the medium were inoculated at the top of the medium. The tube was inverted gently 4–5 times and incubated in upright position at 37° C. There was always good growth of amoebae in 3–4 days. The amoebae after this period were chilled in ice-cold water for 5 min., gently shaken and their number/ml of the medium was determined by a haemocytometer. Freshly prepared TP-S-1-medium was used only upto 3-4 days.

Carrel flask.—Flasks with flat bottom of 15 ml capacity, made in this Institute, were plugged with cotton and sterilized by dry heat. One ml of the suspension of amoebae, grown in TP-S-1-medium in tube, was added to a flask and then it was filled with fresh TP-S-1-medium to the top. The flask was gently rotated to mix the amoebae. The cotton plug outside the flask was sealed with plastocine to prevent air entering the flask. With an inoculum of 10,000 amoebae/ml of the medium, there was profuse growth of amoebae after a lag phase. When 5,000 amoebae/ml of the medium were used, sometimes amoebae survived upto 4–5 days without multiplying and then died out and at other times they started multiplying after 3–4 days till maximum population was reached. It has been repeatedly observed that amoebae survived for a few days and then died out when 2,000 or 1,000 amoebae/ml of the medium were used.

Screw-capped tube.—As in Carrel flasks, efforts to sub-culture amoebae in TP-S-1-medium in tubes with an inoculum of 2,000 or 1,000 amoebae/ml of the medium have failed. Amoebae died out in 24–72 hr without multiplying.

The above findings suggested that smaller numbers of amoebae exposed to dissolved atmospheric oxygen in the medium, during sub-culturing and rotating the flask or inverting the tube, are damaged in such a way that they die out without multiplying. Therefore, it was considered of interest to inoculate *2,000 or 1,000 amoebae/ml of the TP-S-1-medium* in tubes at the bottom and incubate the tubes in upright position without inverting them. There was always profuse growth of amoebae, after a lag phase, with these inocula. The air entering at the top of the medium in tubes was not lethal to amoebae inoculated at the bottom of the tubes.

Since rigid negative O-R potential is necessary to grow *E. histolytica* with bacteria, it was considered of interest to find out roughly the O-R potential created by cysteine + ascorbic acid, as used by Diamond[2,6] and cysteine alone, using indigo carmine which decolourizes at about −200 m V. Phosphate buffer in 0·5% NaCl, containing reducing agents, pH 7·0, was distributed in 9 ml quantity in screw-capped tubes and sterilized by autoclaving. Sterile solution of indigo carmine in buffer was then added to the tubes to make its concentration 0·002%. Proper controls were kept. Cysteine at 0·1% concentration did not decolourize the colour of indigo carmine completely. At 0·2 or 0·3% the colour of indigo carmine was completely decolourized in a few hr. When the oxygen from the air above the surface of the liquid was absorbed by shaking the tubes occasionally, the colour did not appear again during 10 days by shaking the tubes. With 0·1% cysteine + 0·02% ascorbic acid, the result was similar to that with 0·1% cysteine alone. When

the tubes were shaken after 4–5 days, the indigo carmine colour became more or less similar to that of the control. The intensity of the colour remained the same when the tubes were kept for 10 days without shaking. In the case of 0·2% cysteine + 0·02% ascorbic acid, there was partial appearance of the colour on shaking the tubes after 5 days. It did not disappear on keeping the tubes without shaking. Similar results have been obtained in TP-S-1-medium containing cysteine alone or cysteine + ascorbic acid. In these experiments panmede, giving clear solution, prepared in this Institute, was used. These findings suggest some kind of interaction between cysteine and ascorbic acid leading to a shift in O-R potential towards positive side.

TABLE I

Multiplication of E. histolytica *in TP-S-1-medium containing cysteine alone* plus *agar (0·05%) Inoculum was given at the bottom of the tube and the tubes were incubated without inverting them*

Medium (TP-S-1) containing	Initial number of amoebae/ml of the medium	Number of amoebae/ml of the medium after days*	
		11	15
0·2% cysteine alone	2,000	240,000	..
	1,000	..	240,000
	500	..	220,000
	250	..	200,000
0·3% cysteine alone	2,000	260,000	..
	1,000	..	250,000
	500	..	230,000
	250	..	200,000

* Mean count from duplicate tubes.

The above experiments led to the use of cysteine alone in TP-S-1-medium. With 0·2 or 0·3% cysteine no growth of amoebae could be obtained either in Carrel flasks or in tubes when the inoculum was 1,000 amoebae/ml of the medium and the flasks were rotated and the tubes were inverted after the inoculum. With 2,000 amoebae/ml of the medium, there was growth of amoebae on rare occasions. There was always profuse growth of amoebae after 24–48 hr when 2,000, 1,000, 500 or 250 amoebae/ml of the medium were inoculated at the bottom of the tubes and the tubes were incubated without inverting them. Inoculum lower than 250 amoebae/ml of the medium has not yet been tried.

In order to find out whether the interaction between cysteine and ascrobic acid leading to a shift in O-R potential towards the positive side is suitable for the growth of amoebae, TP-S-1-medium containing 0·1% cysteine + 0·02% ascorbic acid or 0·2 or 0·3% cysteine alone was stored in tubes at room temperature. After 10–15 days an inoculum of 10.000 or 5,000 amoebae/ml of the medium was given and the tubes were inverted and incubated in upright position. In the medium containing cysteine + ascorbic acid all the amoebae died out in 3–5 days without multiplication. Medium containing cysteine alone gave luxuriant growth of amoebae after a lag phase of 24–48 hr.

Reed and Orr[7] in 1943, in their work on anaerobic bacteria, have shown that a combination of a reducing agent and small quantity of agar in the liquid medium provides a well-poised system which maintains a negative potential level even in the face of some mixing with air. The free diffusion of the atmospheric oxygen is prevented in the presence of agar. These findings led us to the use of agar in the axenic medium. The results presented in Table I show that 0·3% cysteine is slightly better than 0·2% in order to initiate cultures from small inocula. This is due to the shortening in the time of the lag phase. Media containing agar were kept at 37° C to prevent agar settling down.

DISCUSSION

The results presented in this communication clearly show that it is very important to maintain suitable negative O-R potential during sub-culturing axenically grown *E. histolytica*. This is the reason why Diamond[6] and Wittner[4] failed to get cultures from small inocula. It is dangerous to use cysteine + ascorbic acid in the axenic medium, as suggested by Diamond[2,6], because this combination leads to a shift in O-R potential towards positive side which is lethal to amoebae when the medium is stored for 10 days or more. This seems to be the reason why very few people are able to maintain cultures of axenic strains of *E. histolytica* supplied by Diamond. Moreover, 0·1% cysteine + 0·02% ascorbic acid do not give sufficiently low negative O-R potential for rapid growth of amoebae. Although the importance of adding small quantity of agar in the axenic medium has been emphasized by Diamond[2,6] and Wittner[4], they have not realized that its role is to maintain

well-poised negative O-R potential in combination with a reducing agent.

The authors are grateful to Dr. L. S. Diamond for taking the trouble of sending axenic culture of *E. histolytica* (strain 200 : NIH) by air and for providing instructions for maintaining the amoebae.

1. Chang, S. L., *Parasitology*, 1946, 37, 101.
2. Diamond, L. S., *Science*, 1961, 134, 336.
3. — and Bartgis, I. L., In *Progress in Protozoology*, Excerpta Med., Int. Congr. Ser., No. 91, 1965, p. 102.
4. Wittner, M., *J. Protozool.*, 1968, 15, 403.
5. Montalvo, F. E., Reeves, R. E. and Warren, L. G., *Exp. Parasit.*, 1971, 30, 249.
6. Diamond, L. S., *J. Parasitology*, 1968, 54, 1047.
7. Reed, G. B. and Orr, J. H., *J. Bact.*, 1943, 45, 309.

MECHANISM OF VENTILATORY MOVEMENTS IN *TESSARATOMA JAVANICA* AND THEIR RECORDING TECHNIQUES

P. G. PURANIK, N. CHARI AND R. D. SUKHDEV

Biophysics Laboratory, Department of Physics, Nizam College, Hyderabad (A.P.)

ABSTRACT

The mechanism of ventilation has been studied in *Tessaratoma Javanica* (Thunberg). The expiration is due to contraction of the tergosternal muscles and inspiration is due to elasticity of the pleuron and dorsal membrane. For recording and analysing these movements, optical device is the most suitable one as compared to mechanical or oscilloscopic devices, used. The expiration time is greater than the inspiration time and the tetanic fusion frequency of the tergosternal muscles is in the range of 100-120 c/s.

INTRODUCTION

THE respiratory movements in insects are being studied in order to understand the relevant physiological factors. For recording these movements, different methods such as those of mechanical levers, strain-guage transducers and the pressure changes in the enclosure surrounding the abdomen have been used (Miller, 1966). In the present investigations the ventilatory mechanism of *Tessaratoma javanica* (Thunberg), a pentatomid bug, has been described and the ventilatory movements have been studied by using different techniques. *T. javanica* being a large terrestrial bug appears to be a suitable model for the analysis of various ventilatory parameters, such as expiration, inspiration, twitch interval and the different factors that effect the ventilation.

MATERIALS AND METHODS

Adult males and females of *T. javanica*, the soapnut bugs were collected from the soapnut trees in and around Hyderabad. Insects were maintained in the rearing cages at room temperature (27° C) and they survive for several weeks. For morphological studies, a piece of abdominal sternum with segmental mixed nerve, spiracular muscle and tergosternal muscle was dissected and fixed. These preparations were stained with borax carmine or haematoxylin, dehydrated as usual, cleared and mounted in Canada balsam.

In the present study the ventilatory movements have been recorded by using mechanical, oscillographic and optical devices. Although each of the three recording techniques mentioned suits a particular experimental set up it has its inherent limitations which are likely to impair the accuracy of the experimental results. Therefore in the present studies, the results obtained by the three techniques are analysed and compared with a view to fix the most accurate technique for recording the ventilatory movements.

PROPOSED MECHANISM FOR ABDOMINAL MOVEMENTS

In *T. javanica* the ventilatory movements are due to the down and up movements of the abdominal tergum. There are six pairs of expiratory tergosternal muscles in the abdomen and inspiratory muscles are absent. The transverse section of the abdomen (Fig. 1) shows that each bundle