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Axenic Cultivation of Entamoeba histolytica

Abstract. *Entamoeba histolytica* (National Institutes of Health strain 200) has been maintained in axenic culture through 50 transfers over a period of 6 months. The medium used is diphasic and contains a cell-free extract of chick embryo which is essential for growth. Inocula of 50,000 amoebas yield 150,000 to 200,000 organisms in 72 hours of incubation at 35°C. An extract prepared from boiled embryos supports growth equally well.

Cultures of *Entamoeba histolytica* are readily established in association with certain species of bacteria or trypanosomatids. In the absence of these microbial associates, cultures are difficult to initiate and thus far have been established only in the presence of intact metazoan cells, namely, chick embryo cells (*1, 2*). To my knowledge there is no published report presenting unequivocal evidence of the axenic cultivation of this amoeba—that is, the indefinite subcultivation of amoebas in an environment free of metabolizing bacteria, fungi, protozoa, or metazoan cells. Evidence that axenic cultivation of a strain of *E. histolytica* has been achieved is presented in the present report.

The medium used is diphasic. The ingredients of the nutrient broth employed in both phases are listed in Table 1. To prepare the base, 1.6 g of agar are added to 80 ml of nutrient broth and the mixture is boiled, distributed in 4-ml portions to screw-capped tubes (16 by 125 mm), autoclaved for 10 min (15 lb, 121°C), cooled, and stored in the refrigerator. One week before use the base is melted, 1 ml of horse serum (inactivated 30 min, 56°C) is added per tube, and a slant with a short butt is made. After the slant hardens it is overlaid with 3.25 ml of sterile diluted nutrient broth (1:4, with distilled water) containing 0.05 percent agar and is then replaced in the refrigerator for 1 wk. Immediately before use the medium is brought to room temperature, and to the overlay are added 1.5 ml of chick embryo extract and 0.25 ml of vitamin supplement No. 109 (*3*).

Table 1. Ingredients of nutrient broth used in preparation of the diphasic medium for axenic cultivation of *Entamoeba histolytica* [*p*H adjusted to 7.2 with 1*N* NaOH; autoclaved for 10 min (15 lb, 121°C)].

Ingredient	Amount
Tryptose (Difco)	1.00 g
Trypticase (BBL)	1.00 g
Yeast extract (BBL)	1.00 g
Glucose	0.50 g
L-Cysteine hydrochloride	.10 g
Ascorbic acid	.02 g
NaCl	.50 g
KH_2PO_4	.08 g
K_2HPO_4 (anhydrous)	.08 g
Water (distilled)	80.00 ml*
Resazurin (0.05-percent aqueous solution)	0.02 ml

* Or as much as is needed.

Extracts are prepared from 11- or 12-day chick embryos. These are harvested aseptically and, after removal of the eyes and beak, are cut into two or three pieces, weighed, and processed in one of two ways. In one process the extract is prepared by a modification of the "mild extraction" technique (*4*). Embryos are quick-frozen in a Dry Ice–alcohol bath and placed in the refrigerator, and 1 hr later 3 ml of autoclaved, chilled, buffered saline (NaCl, 5.0 g; KH_2PO_4, 1.6 g; Na_2HPO_4, anhydrous, 1.6 g; distilled water, 1000 ml or as much as needed, *p*H 6.8) are added per gram of tissue. Extraction is carried out for 96 hr at 3°C. The extract is then strained through sterile cheesecloth and centrifuged for 30 min (1000*g*, 0°C). The supernatant fluid is collected aseptically, distributed in 10-ml portions to screw-capped tubes, quick-frozen in Dry Ice and alcohol, and stored at −22°C. This material, 25-percent, mild, chick embryo extract, is referred to hereafter as $CEEM_{25}$.

In the other process, boiling buffered saline is poured over the embryos (3 ml/g). The embryos are then boiled for 2 min, cooled to room temperature, and refrigerated (3°C) for 24 hr. Further processing is similar to that described for the preparation of $CEEM_{25}$. The second extract, 25-percent, heated, chick embryo extract, is hereafter referred to as $CEEH_{25}$. Each batch of extract is checked for microbial contaminants by the procedure which is outlined below.

For detection of bacterial and fungal contaminants of extracts and cultures, the following media are used: fluid thioglycollate, brain-heart infusion with 0.1 percent agar, Sabouraud's broth, trypticase soy agar plates with 5 percent sheep blood, and PPLO (*5*) agar plates (Difco) with 20 percent human blood. The fluid media, in 50-ml quantities, are inoculated with 0.25-ml samples, the plates with 0.05-ml samples. Fluid thioglycollate and brain-heart infusion are incubated at 25°, 35°, and 55°C; Sabouraud's broth and agar plates are incubated at 25° and 35°C. All are kept under observation for 7 days. In testing cultures containing antibiotics, 1-ml samples are transferred from the first set of liquid test media to a second set and also incubated for 1 wk. To detect trypanosomatids, tubes of STB medium (*6*) are inoculated with 0.25-ml samples, incubated at room temperature, and observed for 14 days.

Amoebas (NIH strain 200) grown in association with a trypanosomatid of the genus *Crithidia* (*7*) were used to initiate axenic cultures in medium containing $CEEM_{25}$. The monoxenic source cultures were started with amoebic cysts isolated with a micromanipulator after a wash in dilute HCl. Two sets of axenic cultures (I and II) were established, the first with inocula of 500,000 amoebas, the second with 150,000. Incubation was carried out at 35°C. First transfers were made after 24 or 48 hr, second and third at intervals of 48 or 72 hr. At this point multiplication of the amoebas slowed, and it was necessary to extend the time between transfers to 5 and even 7 days. After the seventh transfer, cultures showed renewed vigor, and thereafter transfers were made alternately at 72 and 96 hr. In well-established cultures, inocula of 50,000 amoebas yielded 150,000 to 200,000 organisms in 72 hr. Subcultures were made by removing the relatively amoeba-free upper half of overlay and transferring 0.5 to 1.5 ml of the remainder to fresh medium.

Set I cultures were initiated in the presence of penicillin G and dihydrostreptomycin sulfate (250 units and 0.25 mg, respectively, per milliliter of over-

lay). This set has undergone 50 transfers during a 6-mo period. After the 16th transfer a subline was started in antibiotic-free medium. It has undergone 34 transfers. No antibiotics were employed in set II cultures; this was to rule out possible effects of these agents in preventing detection of certain bacterial contaminants. Set II cultures were discontinued after the 15th transfer, when tests showed clearly that axenic cultivation had again been achieved.

Repeated sterility checks failed to uncover any evidence of cryptic microbial contamination of the amoebic cultures. Gross contamination occurred in approximately 2 percent of some 800 tubes used and was traceable in each instance to airborne contamination which occurred during subculturing. Tests with STB medium demonstrated that *Crithidia* introduced with the original inoculum of amoebas died out prior to the third transfer.

The effect of heat on growth-promoting factors present in embryo extract was investigated. A subline from set I cultures was started in medium containing $CEEH_{25}$. Yields from this subline (still in existence after 29 transfers) were equal to those obtained with unheated extract. These results were unexpected in light of earlier studies (*2*, *8*) which showed that even milder heat treatment destroyed growth-promoting activity. The highly supplemented nature of the diphasic medium may explain, in part, the results obtained. Baernstein *et al.* (*8*), in working with amoebas grown in microcultures, discovered that enriching their medium with vitamins, amino acids, and ribonucleic acid partially restored factors that had been lost through heating the extracts (*9*).

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Intracellular Study of Recurrent Facilitation

Abstract. Recurrent facilitation in the cat's spinal cord has been studied in deep peroneal and quadriceps motoneurons with the use of intracellular recording. The presence of facilitation was indicated by several criteria, among them increased firing index of the cells or decreased latency of firing. In many, but not all, facilitated cells the conditioning volley caused a small visible depolarization. Subthreshold synaptic potentials were frequently increased in magnitude by the conditioning volley, which also increased the effectiveness of a stimulus applied through the microelectrode. Facilitation was found in a large percentage of the motoneurons investigated and was clearly able to bring about pronounced changes in the excitability and firing behavior of these cells.

Previous experiments in this laboratory have shown that recurrent inhibition and recurrent facilitation are distributed in an organized manner in the cat spinal cord (*1*), and subsequent work has suggested that the facilitation may in fact be a disinhibition which enhances reflex discharge by decreasing background inhibitory activity (*2*). The purpose of the present investigation has been to record, in individual motoneurons, the intracellular changes which accompany recurrent facilitation of reflex discharge, in order to study the distribution of this phenomenon among motoneurons and to obtain further evidence on the synaptic mechanism of recurrent facilitation.

All experiments have been performed on cats whose spinal cords had been severed in the upper cervical region, and the preparation was the same as previously described (*1*, *3*). So far, studies have been made of motoneurons of the deep peroneal group and of quadriceps whose action is usually facilitated by antidromic stimulation of the nerve to gastrocnemius-soleus and biceps-semitendinosus respectively (*1*). Intracellular recording was by means of glass micropipettes with tip diameters less than 1 μ, filled with 2.7*M* KCl.

The cells that have been studied had action potentials ranging from 50 to 100 mv. Synaptic activity was frequently visible in the absence of electric stimulation and occasional firing was observed in several cells as a result of such activity. Many deep peroneal cells fired repetitively to single dorsal root shocks, in a manner similar to that described for cells whose activity was recorded in ventral root filaments (*4*); it was often possible to obtain as many as four to five responses, the intervals between spikes being as short as 2.5 msec. Such repetitive discharges were superimposed on long and complex synaptic potentials. Many of the penetrated cells had a monosynaptic firing index (*5*) of 100. Such cells were studied either by weakening the strength of the dorsal root volley or by restricting stimulation to a small dorsal rootlet. In this manner it was possible to make a cell respond either with an intermediate monosynaptic firing index or polysynaptically.

Facilitation of single motoneurons was studied only in preparations in which visible recurrent facilitation of the monosynaptic reflex was present. Upon penetration it was first essential to determine whether the cell was facilitated. This was done employing a number of criteria: in many cases a conditioning shock increased the firing index; at times the latency of a polysynaptic discharge was reduced; conditioning has also been seen to increase the number of spikes in the discharge.

Facilitation has been studied in 103 cells, and changes in membrane potential were looked for in a number of these. In many cases the conditioning shock caused small depolarizations, the largest being less than 3 mv. In other facilitated cells depolarizations were much smaller and in some cases they were either absent or vanishingly small. The depolarization shown in Fig. 1 is typical. While it is hard to determine its exact onset, it first becomes clearly evident about 6 msec after the conditioning stimulus and has a time course of approximately 50 msec. These values are compatible with the latency and duration of recurrent facilitation as exemplified in typical facilitation curves (*3*).

As a result of a conditioning volley, subthreshold synaptic potentials in facilitated cells were frequently measurably increased over a considerable part of

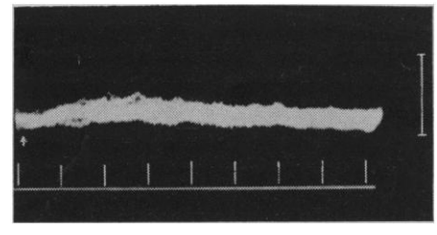

Fig. 1. Depolarization evoked in a deep peroneal motoneuron by an antidromic volley in the nerve to gastrocnemius-soleus. Conditioning stimulus delivered at the arrow. This record was obtained by superimposing approximately 15 faint traces. Time, 10 msec, voltage calibration, 10 mv