PHYSIOLOGY OF ENTAMOEBA HISTOLYTICA

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INTRODUCTION

On morphological evidence the species of Entamoeba fall into four groups according to the number of nuclei present in the mature cyst: 1 nucleus in E. polecki and E. bovis; 4 nuclei in E. histolytica, E. hartmanni, E. invadens, and E. moshkowskii; 8 nuclei in E. coli, and E. muris; no cyst formation in E. gingivalis. There are sufficient physiological differences, such as degree of pathogenicity, critical temperature for survival and growth, host-restriction, metacystic development, development of the newly hatched amoeba, etc., which could be used to characterize these species (Neal, 1966). Furthermore, each species may consist of a series of races adapted to a particular host. In Entamoeba histolytica, a small race and a large race have been observed. The small race is not pathogenic while the large race may or may not be. The small race was usually called E. hartmanni, (Levine, 1961). In it's virulent phase the large race invades the tissues; the trophozoites of this phase are large; in it's commensal phase it remains in the lumen of the intestine, feeding on bacteria, saprozoically; the trophozoites of this phase are small. Brumpt (1925) further divided the

large race into pathogenic E. dysenteriae and non-pathogenic E. dispar. However, the question whether there actually are completely non-pathogenic strains of E. histolytica which cannot be induced to become pathogenic has not yet been answered satisfactorily (Levine, 1961). Concomitant bacteria, nutritional deficiency and other factors effect the pathogenicity of the amoebae.

A lot of different strains of E. histolytica were discovered and named. "Laredo" strain has been maintained since 1958 at both 25°C and 37°C. Entner and Most (1965) isolated two more strains, "JA" and "AG," which are capable of growing at 25°C and 37°C. Huff strain has been maintained in culture (35-37°C) in several laboratories since about 1956 (Richards, et al., 1965). They differ from E. histolytica K9 and 200 by their low virulence and ability to grow at room temperature. There are many other strains of E. histolytica. Two strains used in this experiment, K9 and 200, were kept by National Institutes of Health lab since 1951 and 1948. These amoebae grow well at 37°C (Richards, et al., 1965).

Since E. histolytica has been cultivated in vitro, many investigators studied its nutritional requirement, physical factors, and physiological characteristics. The relation between the associated bacteria and E. histolytica, their virulence and invasive ability, were established at

the same time (Nakamura, 1953; 1954; Blumenthal et al., 1954; Benham and Isabella, 1958; Karlson, 1952; Cabrera, 1958; Phillips and Gorstein, 1966).

The present studies were made on some physiological characteristics of two strains of E. histolytica. Special emphasis was placed on their surface requirement, because amoebae always move by attaching to a surface. It would be very interesting to find out whether, and how, growth and multiplication of E. histolytica would be effected if their chance of contacting a surface is disturbed. Other experiments, like change of pH value in the media used, temperature, horse serum requirement, and pinocytosis induction were also tested.

CULTURE MEDIUM

As early as 1916, Penfold et al. (Nakamura, 1952) claimed to have cultivated E. histolytica in a medium containing nutrient broth and a pancreatic digest preparation. The first successful cultivation of the amoebae without great difficulty, upon special media was demonstrated by Boech and Drbohlav (1925). Their medium became known as the Locke-egg-serum medium (L. E. S.).

After the publication of Boech and Drbohlav appeared, several modifications of their medium became known. In England, Dobell and Laidlow (1926) modified the Boech-Drbohlav medium by adding starch to the culture without dextrose, stating that the starch particles could be ingested by the amoebae, thus supplying an abundant carbohydrate source.

In view of the finding that the amoebae grew in the liver of an amebic patient, Cleveland and Collier (1930) used a liver infusion agar slant covered with serum and saline.

Balamuth and Sandza (1944) prepared a standardized fluid culture medium from an infusion of coagulated yolk made up in a buffered salt solution. This medium was

almost transparent and wholly liquid; when Wilson's liver concentrate powder was added, the growth of the amoebae was accelerated. These were advantages of this liquid medium over a diphasic medium.

Since the primary site of invasion for E. histolytica in man is the mucous membrane of the intestinal tract, mucous might contain some elements which, in whole or in part, would support the growth of E. histolytica. Dalkart and Halpert (1958) developed a monophasic medium using powdered gastric mucin. Excellent growth of two strains of E. histolytica with bacterial associates was obtained in this medium.

All the media described above contained bacteria together with the amoebae. It seemed to become an acceptable fact that the amoebae required the bacteria and that if the bacteria were eliminated the amoebae would not survive.

Phillips and Rees (1950) first eliminated the bacteria from amoeba cultures and replaced them with Trypanosoma cruzi and found that this haemoflagellate could support growth of E. histolytica in this monophasic culture medium. Trypanosoma lewisi, Leishmania donovani, L. enriettii, L. brasiliensis, and Endotrypanum schaudinni were also found to support the growth of E. histolytica (Pan, 1960).

The best condition to study the physiological characters of E. histolytica is to eliminate all the other living organisms in the culture. Many attempts were tried. The first advancement was made on development of monoxenic cultures by Jacobs (1947). He grew the amoebae in a medium in which bacteria were not obviously evident. The medium, the usual Locke's egg slant solution, had been conditioned by the cultivation of E. coli for 24 hours, after which bacteria were killed by heat. After heating, heat killed bacteria and rice powder were added. A relatively bacteria-free culture of E. histolytica was maintained in this medium for a period of several months. Some workers worked on antibiotics. Shaffer et al. (1948) cultivated E. histolytica with a 24 hour culture of Streptobacillus sp. and an undefined bacteria, "t organism," in a fluid thioglycolate, glucose, and rice flour medium plus normal horse serum as a support. By adding penicillin, the growth of bacteria was inhibited. After his work, many authors claimed successful cultivation of amoebae under the action of penicillin (Reeves et al., 1960; Karlson, 1952; Faust et al., 1950; and Gleason et al., 1960).

It was anticipated that certain desirable objectives might be more readily attained if it were possible to employ in the amoeba cultures bacterial cells which were incapable of continued vegetative growth and multiplication. An

immediate advantage would be that all antibiotics could then be eliminated from the culture fluid; a further gain would be that the metabolic process in such inactivated bacterial cells must surely be less complicated, than in cells capable of employing the whole cycle of normal growth and multiplication. Numerous procedures for killing the vegetative growth capability of bacterial cells were investigated, and at the same time the effects of the treatment upon the ability of the cells to support amoeba growth were studied. The most successful results were obtained with radiation to inactivate the cells (Reeves et al., 1960). This method produced bacterial cells which appeared not to be able to continue vegetative growth or multiplication, but did not lose their capability to support the growth and multiplication of E. histolytica.

Shaffer and his colleagues (Shaffer and Siekiewicz, 1952; Shaffer et al., 1953) had reported that E. histolytica could be cultivated without other living organisms, in medium containing minced chick embryo, and this method had been used successfully by Rees et al. (1953). Considerable effort has been made during recent years toward the goal of axenic cultivation. While there are many reports on the axenic culture of Entamoeba, the first consistent and confirmed results were Stoll's (1957). The species was E. invadens from snake, and the medium used at room temperature

was a monophasic liquid incorporating substances from natural sources. Subsequently, Diamond (1960) reported a clear monophasic medium which supported this amoeba as well as the related species E. terrapinae, which is also a parasite of reptiles. Diamond used a diphasic medium at 37°C for the cultivation of the human parasite E. histolytica, in 1961. Recently Diamond and Bargis (1965) reported a new monophasic transparent medium which was used in the present experiments. In this medium he eliminated tryptose and replaced yeast extract with liver extract, which were in the liquid portion of the previous diphasic medium. Other components were also varied in quantity. Jackson and Stoll (1964) tried to grow E. histolytica from diphasic cultures into the original monophasic medium for E. invadens with the addition of heat in-activated serum agar and NCTC 107, only one or two subcultures could be obtained. Serum was found to be an intrinsic requirement of this human parasite.

GROWTH BEHAVIOR AND MULTIPLICATION RATE

The growth of E. histolytica follows the classical pattern of microorganisms which is divided into several phases: lag phase, log phase, stationary phase, and death phase. The duration of lag phase depends on various physical conditions, size of inoculum, different types of medium, and different strains of amoebae, etc. Harinasuta and Harinasuta (1955) found that the lag phase in the early stages of amoebic growth in vitro was considerably shortened if the medium was preconditioned by allowing growth of the associated bacteria to take place before the amoebae were inoculated. Growth of amoeba populations was found to be more prolific in such preconditioned medium, than in fresh medium.

Boech and Drbohlav (1925) reported that the amoebae grew mainly at the bottom of the culture tubes in the bacterial sediment. Faust et al. (1950) grew E. histolytica on Shaffer-Frye medium and found that the population in small Erlenmeyer flasks had almost ten times that in tubes which contained the same amount of medium and received the same size of inoculum. This observation suggested the possibility of investigating various physical factors

which might influence the population growth of cultures in Shaffer-Frye medium or other kind of media. They also set-up 24, 16 x 150 mm test tubes containing 5 ml of freshly prepared medium and 0.5 ml of medium with strain "22" E. histolytica, and put glass tubes of 10 x 30 mm in 12 cultures to increase the vertical surface area. All tubes were sealed with a layer of melted petroleum to maintain the anaerobic condition. These tubes were divided into four groups: 1) tubes with glass and agitated, 2) tubes without glass and agitated, 3) tubes with glass but not agitated, 4) tubes without glass and not agitated. It was found that there was no significant difference in growth between them. They thus postulated that the failure to produce better growth, when the area was increased vertically, may be due to the action of the growth of the E. histolytica on the products in the media or of themselves, particularly since the amoebae were not observed to migrate through the entire length of the inner vertical wall.

Balamuth and Isabella (1958) also reported that in the use of Shaffer-Frye medium, in Koch flasks, the motile amoebae adhered to the glass.

Reeves (1962) tested some surface active agents such as quaternary ammonium salts, cetyltrimethyl ammonium bromide, dimethyl ammonium bromide, cetylpyridium chloride,

cetyl benxyl dimethyl ammonium chloride and Tween 80 on E. histolytica growth. By increasing the concentration of these surfactants, both multiplication and surface tension diminished gradually until, the multiplication of amoebae ceased finally. The results of his work indicates that amoeba growth might be strongly affected by the presence of surface active agents. However, the increased property of air-liquid surface tension of the cultures did not bear a simple relationship to amoeba growth. When a surfactant was added to the medium the relationship between the air-liquid surface tension and amoeba multiplication could be markedly altered and the result depended upon the type of surfactant. He concluded that it might be that the surface of amoebae contained sites which selectively bound cations, and when the cation was also a surfactant the amoebae were seriously impaired. Microscopic observation indicated that all of the surfactants were capable of rupturing the outer membrane of the amoebae and caused them to disintegrate. A suitable concentration was required. Low concentrations did not produce this effect.

PHYSICOCHEMICAL FACTORS

The various physicochemical factors that enter into the physiology of an organism appear to be especially critical for E. histolytica.

1) pH factor: The variation in pH has marked effect on the ability of E. histolytica to propagate. Shaffer and Sienkiewicz (1952) obtained 6.0 as the optimum pH. A value of 7.9 was observed by Baernstein et al. (1957) as the optimum value. Rees et al. (1960) got maximum average yields at a pH of 6.4 by using another medium. He concluded that the results would seem to suggest the optimum pH for propagation of E. histolytica to be determined by the medium or other conditions rather than by some exact requirement of the organisms.

2) Temperature: Entamoeba coli has a survival time of less than a day at 3°C and 18°C and disappear within 8 to 10 hours at these temperatures. Entamoeba coli is very sensitive to cold while E. histolytica was less easily killed by lower temperatures. Entamoeba coli is more resistant to higher temperatures (40-45°C) than the histolytica group. At 33°C all the strains of E. histolytica and E. coli under investigation by Cabrera

and Porter (1958) gave a luxuriant growth, and at 32°C all the histolytica strains with the exception of the F-22 and SB strains under observation had a longer survival time than at 37°C. Several strains of E. histolytica were found to be able to adapt to lower temperatures by periodic lowering of the temperature, always less than 0.5°C each time. These strains could keep growing at 29°C for longer than 18 months. Adaptation of these organisms to high temperatures was also accomplished (Cabrera, 1958). All three strains of E. histolytica (UC, 201,202) grew at a little above 42°C, but when the temperature was raised to 43°C, all adapted strains failed to grow. Siddiqui (1963) studied the effect of temperature on E. histolytica (DSB and Laredo strains), E. moshkovskii (DSR strain), and E. invadens (IP strain) and found the DSB strain was very sensitive to temperatures and DSR strain of E. moshkovskii and Laredo strain of E. histolytica were similar in their range of temperature tolerance. Individual trophozoites from the 30°C-adapted strain grown at 37°C. Amoebae of the IP strain of E. invandens adapted to 35°C were smaller than those of the original strain grown at 30°C.

3) Anaerobiosis: Many workers had concluded that the pressure of free oxygen was definitely harmful to E. histolytica (Balamuth and Howard, 1946; Shaffer et al., 1948). Shaffer et al. (1948) were not sure whether the

oxygen was directly harmful to the amoebae or whether oxidation destroyed some essential oxygen-labile factor needed by the amoebae. A petroleum seal had been used to eliminate atmospheric oxygen. Jacobs (1950) thought that there was not enough evidence to conclude that the complete removal of oxygen was necessary for the growth of E. histolytica. Information currently available indicated that an anaerobic condition was necessary for optimum growth and metabolism, but small amounts of oxygen did not hurt the growth (Jackson and Stoll, 1964).

4) Other factors: E. histolytica can tolerate considerable changes of tonicity (Dobell and Laidlow, 1926), a medium with a salt concentration of 0.94% NaCl was shown to be optimal. Nelson and Jones (1964) showed prompt and vigorous growth of E. histolytica by adding sodium bicarbonate to the culture medium.

Sadun et al. (1950) found that a single irradiation exposure of the E. histolytica to 30,000 roentgen units did not effect their growth. Exposure to 6,000 and 120,000 r inhibited the amoebae, although viable amoebae were observed for 16 transfers after irradiation with 120,000 r. Reeves et al. (1960) showed that dosages of X-ray irradiation up to 120,000 r's did not greatly harm amoebae, nor did they cause the E. histolytica to lose its infectivity for guinea pigs. The factors that influence amoebic growth seem to be varied with various conditions as described above.

MATERIALS AND METHODS

The medium (Diamond and Bartis, 1965) consists of a nutrient broth supplemented with horse serum and vitamins. The nutrient broth (TP) has the following composition:

Trypticase	1	gm
Panmede, liver digest	2	gm
Glucose	0.5	gm
L-cysteine hydrochloride	0.1	gm
Ascorbic acid	0.02	gm
Sodium chloride	0.5	gm
Potassium phosphate, monobasic	0.06	gm
Potassium phosphate, dibasic	0.1	gm
Distilled Water	90	ml

To prepare TP broth, the ingredients were dissolved one by one in water in the order presented, the pH adjusted to 7.0 with 1 N sodium hydroxide, and the final mixture passed through #1 Whatman filter paper in a funnel. It was then autoclaved for 30 minutes, 15 lb., 121°C.

The complete medium (TPS-1) was prepared by combining under aseptic conditions 90 ml of TP borth, 10 ml of inactivated horse serum and 2.5 ml of vitamin mixture,

NCTC 107. The vitamin mixture was prepared from five primary stock solutions which were made as follows:

1. Water-soluble B. vitamins:

a. 62.5 mg niacin and 125 mg p-aminobenzoic acid were dissolved in boiling, glass distilled water and brought to a total volume of 150 ml.

b. 62.5 mg niacinamide, 62.5 mg pyridoxine hydrochloride, 25 mg thiamine hydrochloride, 25 mg calcium pantothenate, 125 mg i-inositol, and 1250 mg choline chloride were dissolved in glass distilled water and brought to a total volume of 150 ml.

c. 25 mg riboflavin was added to 75 ml of glass distilled water and dissolved with the aid of 0.1 N sodium hydroxide added drop by drop. The total volume was then brought to 100 ml.

Solutions a, b, and c, were then combined and the total volume brought to 500 ml with glass distilled water.

2. Biotin solution:

30 mg D-biotin was dissolved in 200 ml glass distilled water with aid of 0.1 N sodium hydroxide and the total volume brought to 300 ml.

3. Folic acid solution:

30 mg folic acid was dissolved in 200 ml glass distilled water with 0.1 N sodium hydroxide and the total volume brought to 300 ml.

4. Lipid-soluble vitamins A, D, and K:

a. 300 mg vitamin D_2, calciferol, was dissolved in 63 ml of 95 percent (v/v) ethyl alcohol. To this, 300 mg vitamin A, crystalline alcohol was added and dissolved.

b. 60 mg vitamin K, menadione sodium bisulfite, was dissolved in 300 ml of 5 percent (v/v) aqueous solution of Tween 80.

Solution b was then combined with solution "a" and the total volume brought to 3000 ml with glass distilled water.

5. Vitamin E solution:

25 mg vitamin E, alpha tocopherol acetate, was dissolved in 250 ml glass distilled water.

The working mixture of vitamins was prepared by combining the five primary stock solutions in the following proportions: 500 ml water-soluble B vitamins, 250 ml biotin solution, 250 ml folic acid solution, 2500 ml lipid-soluble vitamins A, D, and K, and 250 ml vitamin E solution. Sterilization was accomplished by passing the mixture through a millipore filter (HA, pore size 0.45 μ).

The medium was distributed in 7.5 ml portions to 9 ml screw-capped tubes as stock cultures.

The amoebae used in these experiments were obtained from Park Davis and Company. Subcultures were made at

intervals of 72 or 96 hours alternatively. Cultures were chilled in the refrigerator for five minutes till cool, inverted several times to loosen the amoebae from the walls of the tube and centrifuged for 10 minutes at 230 X g. After this, the supernatant fluid was aspired from each tube and the remaining sediment of amoebae was transferred to fresh medium.

During the experiments, some tubes were silicone-coated immediately before the fresh medium was distributed into them. In the agar test, 0.02% (w/v) of Ionagar #2 (Oxoid) were added before autoclaving.

The number of amoebae was determined from an average of 3 culture tubes every 24 hours. After thorough mixing with a sterile pipette a small amount of the chilled fluid medium was withdrawn and placed in the counting chamber of a haemocytometer (AO spencer). The amoebae within the 9 large ruled squares of the chamber were counted.

Most of the experiments were run more than twice. The results obtained were more or less consistent.

RESULTS

The growth and multiplication of E. histolytica was similar to that of other microorganisms except that no lag phase was obtained, in the medium used. The length of log phase differed with the size of inoculum. A small inoculum took more time to reach the peak than the larger one did (Table 1). The inoculation size did not determine the maximum yields which were almost consistent with different initial inoculations used, except with a very small inoculum. For instance, with less than 1,000 amoebae per millimeter, it was hard to get the normal log phase of growth. This was true for both strains.

The growth rate of the two strains was different, K9 amoebae grew more rapidly than the 200 strain. With the same inoculum, for instance, 17,700 amoebae per ml, the K9 strain took 72 hours to get to the peak while 200 amoebae needed 96 hours in the same medium (Table 1).

During active growth, round forms were found. They were round, non-motile, and did not exclude dyes. Round amoebae were most commonly found during and after the stationary phase (Table 1, Fig. 1). The stain taking character indicated that they were dead amoebae. These

TABLE 1. Length of time to reach maximum yields by different inoculation size and the relation between the maximum yields and the number of round forms

Strains	Inoculation Size (x 10^4/ml)	Maximum number (x 10^4/ml)	Time taken to reach maximum (hour)	# of dead cells at half maximum (x 10^4/ml)	# of dead cells at peak (x 10^4/ml)
K9	0.33	17.4	120	0.1	2.66
	0.44	18.6	120	0.4	7.4
	0.55	18.1	96	0.3	2.2
	1.77	14.7	72	0.2	3.0
	2.99	25.7	48	0.4	2.9
	3.22	17.4	48	0.1	2.4
	3.33	29.3	48	0.2	2.2
	3.44	20.2	48	0.1	1.1
	3.66	25.2	48	0.2	3.0
	4.44	19.8	48	0.1	2.0
	4.55	20.8	48	0.3	3.4
	4.88	20.7	48	0.1	2.0
	5.33	18.8	48	0.1	3.3
	9.77	26.2	24	0.7	4.0
200	0.77	15.1	120	0.1	1.8
	0.88	7.0	96	0.1	0.8
	0.99	7.0	96	0.1	2.2
	1.77	21.8	96	0.2	2.3
	1.99	26.2	96	0.1	1.1
	2.22	17.9	72	0.1	0.8
	2.66	26.7	72	0.2	2.7
	3.33	28.7	72	0.2	2.2
	4.22	27.2	72	0.2	2.2
	5.77	28.1	48	0.1	2.2
	5.88	26.7	48	0.1	1.6
	6.44	20.0	48	0.3	4.4
	7.66	16.6	48	0.2	3.0

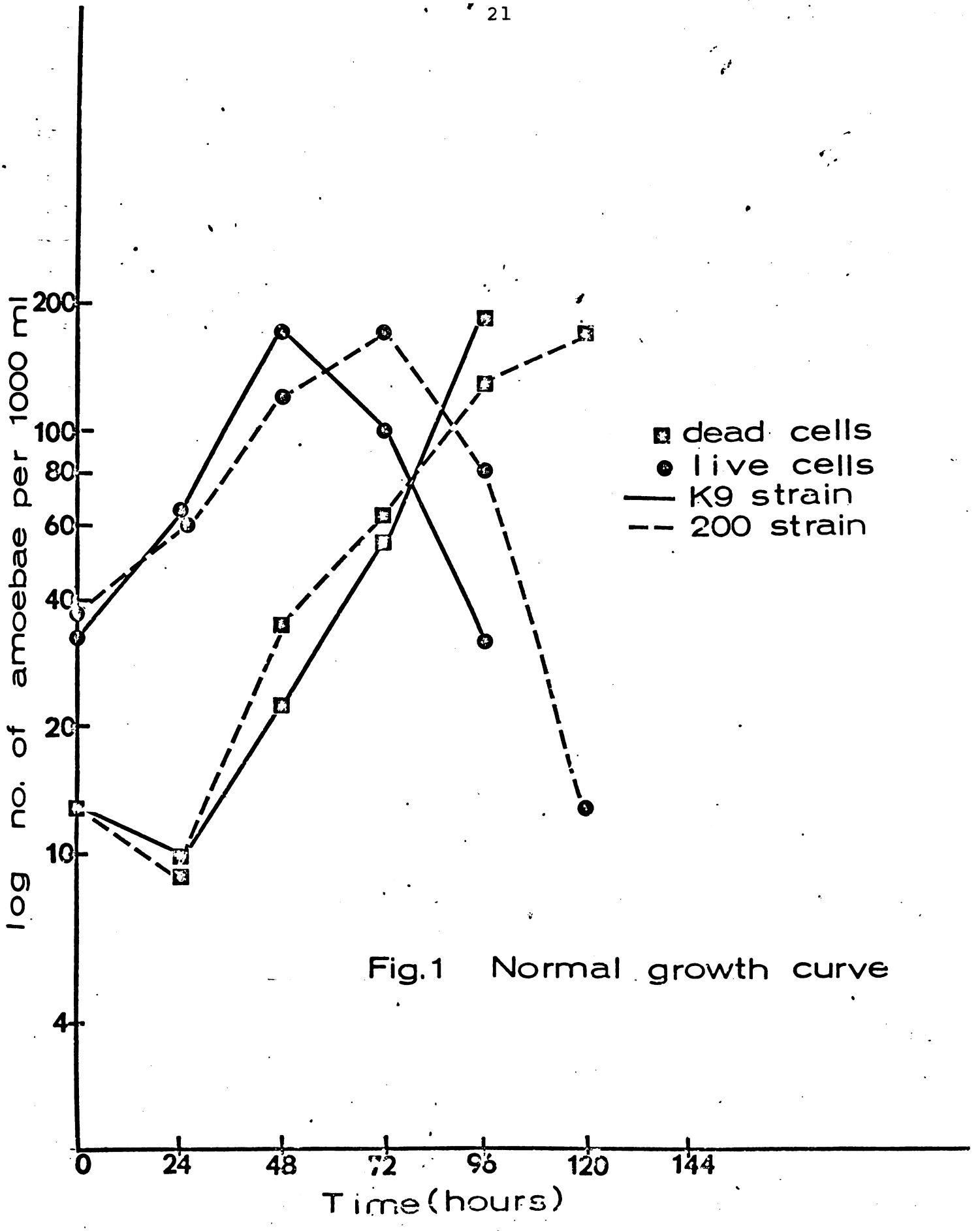

Fig.1 Normal growth curve

dead organisms decreased in size in the following days and finally disintegrated.

By direct observation, all the amoebae were seen to adhere to the bottom of the test tubes. Amoebae tended to pile up and form small clumps as the number increased near maximum and the available space for their attachment became limited.

In order to test whether multiplication of E. histolytica required a surface, 9 groups of tests were set up:

1) control tubes having the normal TP broth medium
2) medium with 0.02% Ionagar added
3) silicone-coated tubes with normal medium
4) silicone-coated tubes with 0.02% Ionagar in the medium

These tubes were put in slanted position. The other 4 groups (5-8) had the same constitution as groups 1-4 but were put in a rotator with a speed of 1/5 rpm.

9) Vertical tubes with normal medium

The results were shown in Table 2. Growth rate was calculated as:

$$\frac{\log N - \log N_o}{\text{Time}}$$

in which, N_o = initial No. N = No. of amoebae after a certain time later. G value was calculated every 24 hours.

TABLE 2. Growth rate of E. histolytica under different conditions*

Strains	Conditions	Generations/hr.	Maximum No. (x 10^4/ml)	# of dead cells at peak (x 10^4/ml)	# of dead cells at 1/2 max. (x 10^4/ml)
K9	(1)	0.029±0.009	18.6±1.3	3.6	0.1
	(2)	0.036±0.01@	15.8±2.5@	1.1	0.3
	(3)	0.019±0.01¢	12.5±0.8¢	2.3	0.2
	(4)	0.038±0.008@	15.3±2.2@	4.4	0.2
	(5)	0.019±0.003¢	11.2±0.9¢	2.9	0.4
	(6)	0.034±0.005@	18.3±3.3@	9.9	0.5
	(7)	0.021±0.011¢	7.3±2.2¢	2.0	0.3
	(8)	0.026±0.008@	17.7±2.6@	8.4	0.5
	(9)	0.014±0.002¢	10.3±1.8¢	8.0	0.2
	(10)	0.023±0.009@	7.6±1.1¢	3.3	1.1
	(11)	0.050±0.011¢	20.2±0.5@	2.8	0.2
	(12)	0.026±0.013@	16.5±2.7@	1.1	0.1
200	(1)	0.023±0.007	19.3±1.0	5.3	0.4
	(2)	0.0265±0.011@	20.7±4.2@	3.8	0.3
	(3)	0.020±0.007@	12.2±3.1¢	1.8	0.1
	(4)	0.033±0.010@	17.4±3.3@	4.4	0.4
	(5)	0.011±0.008¢	13.3±0.5¢	2.3	0.2
	(6)	0.024±0.012@	21.8±1.6@	3.4	0.2
	(7)	0.021±0.007@	13.3±0.6¢	1.7	0.1
	(8)	0.026±0.009@	20.0±1.0@	5.9	0.5
	(9)	0.014±0.015¢	14.0±1.5¢	1.4	0.3
	(10)	0.019±0.0075@	7.7±1.0¢	2.2	0.1
	(11)	0.017±0.004@	17.7±2.5@	1.4	0.1
	(12)	0.039±0.0078¢	21.0±3.4@	3.9	1.7

*Data were taken from the average of 6 tubes: (1)-(4) slanted position; (5)-(8) rotating tubes; (1) and (5) normal medium; (2) and (6) agar medium; (3) and (7) silicone-coated tubes with normal medium; (4) and (8) silicone-coated tubes with agar medium; (9) vertical tubes with normal medium; (10) 0.9 µg poly-D-lysine per 7.5 ml in the medium; (11) 0.3 µg poly-D-lysine per 7.5 ml medium; (12) 0.1 µg poly-D-lysine per 7.5 ml medium; @--statistical insignificant in comparison with the control (1) at 95% level; ¢--statistical significant in comparison with the control at 95% level.

Amoebae grew in the medium with 0.02% Ionagar, no matter whether silicone-coated or not, or whether in slanted position or in rotation, they had the same growth pattern and growth rate or slightly faster than the control culture.

Growth and multiplication was delayed in silicone-coated tubes which had no agar in the medium. All rotated tubes showed a slower growth rate of E. histolytica. Not only the growth rate but the maximum number they reached were less than those in the controlled tubes (Table 2).

By direct observation, very few amoebae adhered to the bottom of the silicone-coated glass wall. Entamoeba histolytica formed a lot of small and big clumps in the medium. The same tubes in the rotator also had few amoebae stuck to the glass; a very big mass of clumps that looked like sediment or precipitation was seen on the very bottom of the test tubes. Slanted tubes without silicone coat had more amoebae adhering to the wall, however, clumps also were found, at the later stages as the amoebae crowded together. These clumps were the same as those described in silicone-coated tubes.

All the tubes with agar appeared the same. With the semi-solid agar floating in the medium, no amoeba was found to attach to the glass wall, but instead, they were seen suspended in the agar medium. Of course, the agar

mass always concentrated near the lower part of the tube because of gravity. No clump of amoebae was ever found.

In vertical tubes the amoebae settled on the bottom, very few E. histolytica climbed on the tube wall. Because of the limited space for attachment, organisms settled on the very bottom, no floating clumps were seen. Their maximum yields never exceeded 12 x 10^4 and the initial inoculum was between 2 x 10^4 to 4 x 10^4 per ml. Growth rate also decreased.

When the "t" test was used to test statistical significance, the maximum yields of silicone-coating, vertical incubation, and rotation showed significantly at the 95% level less in number of amoeba than the controlled condition in both K9 and 200 strains of E. histolytica. The growth rate of K9 amoebae under these conditions also expressed statistical significance in comparison to the control, but the 200 strain did not show this kind of significance except in the case of rotation (Table 2).

Other experiments were set up later. The amoebae were inoculated into a spinner flask which had been silicone-coated immediately before it was filled with 30 ml of the normal culture medium. Two different speeds of agitation, 56 rpm and 88 rpm were tested. Amoebae multiplied at a very low rate when agitated. The maximum yields in this large volume of medium gave only 63,327

amoebae per ml with an initial inoculum of 20,000 at 55 rpm, and died out after 7 days. When 88 rpm speed was tested, E. histolytica showed only a slight increase in their population. With an inoculation size of 35,552 amoebae per ml used, the organisms could only reach a maximum yield of no more than 52,400 amoebae per ml of the medium. A lot of big clumps were seen suspended in the medium. Aggregation was stronger than in the case of silicone-coated and rotating conditions, so that sometimes agitation for sampling could not break them apart.

Entamoeba histolytica living under the agitated condition in the spinner flask looked healthy and acted normally. Very fine pseudopodia protruded from the posterior end. However, this did not frequently happen in the rotating tubes and vertical tubes and were not seen in the control cultures.

By staining fixed amoebae with methyl green pyronine, 2, 3, 4, or even 5 nuclei could be found to occupy a single mass of cytoplasm in the case of spinning. This multinuclearity was uncommon in other conditions. In silicone-coated tubes, only 1/10th of the E. histolytica were seen to have two nuclei and only a few amoebae with three nuclei were found in rotating tubes and rare binucleate cells in the slanted tubes. Multinuclearity was not seen in the control cultures. All these examinations

were made after 3 days cultivation. All the results described were true for both K9 and 200 strains of E. histolytica.

Poly-D-lysine was added to the culture medium. A concentration of 0.9 µg/7.5 ml interrupted the growth of E. histolytica of both strains; 0.3 µg/7.5 ml concentration enhanced growth in K9 strain of E. histolytica but inhibited the growth in 200 strain amoebae. When 0.1 µg/7.5 ml concentration was used, strain 200 amoebae grew rapidly while the K9 strain showed approximately the same rate of growth as in the controlled condition.

Entamoeba histolytica treated with poly-D-lysine were more or less active than the control when a suitable concentration was added (0.3 µg/7.5 ml in K9 and 0.1 µg/7.5 ml in 200 amoebae). Higher concentrations slowed the activity (Table 2, Fig. 2, 3).

Horse serum-free medium was also tried. The serum was replaced with the same amount of mammalian saline. There was no increase in the number of amoebae, instead, all the 200 organisms died within 3 days although there were still a few K9 strain of E. histolytica alive in the 3rd day. Horse serum seems to possess a factor that supports the growth and propagation of E. histolytica.

Through the experiment, E. histolytica was kept at 36.5-37°C. These organisms were not very sensitive to

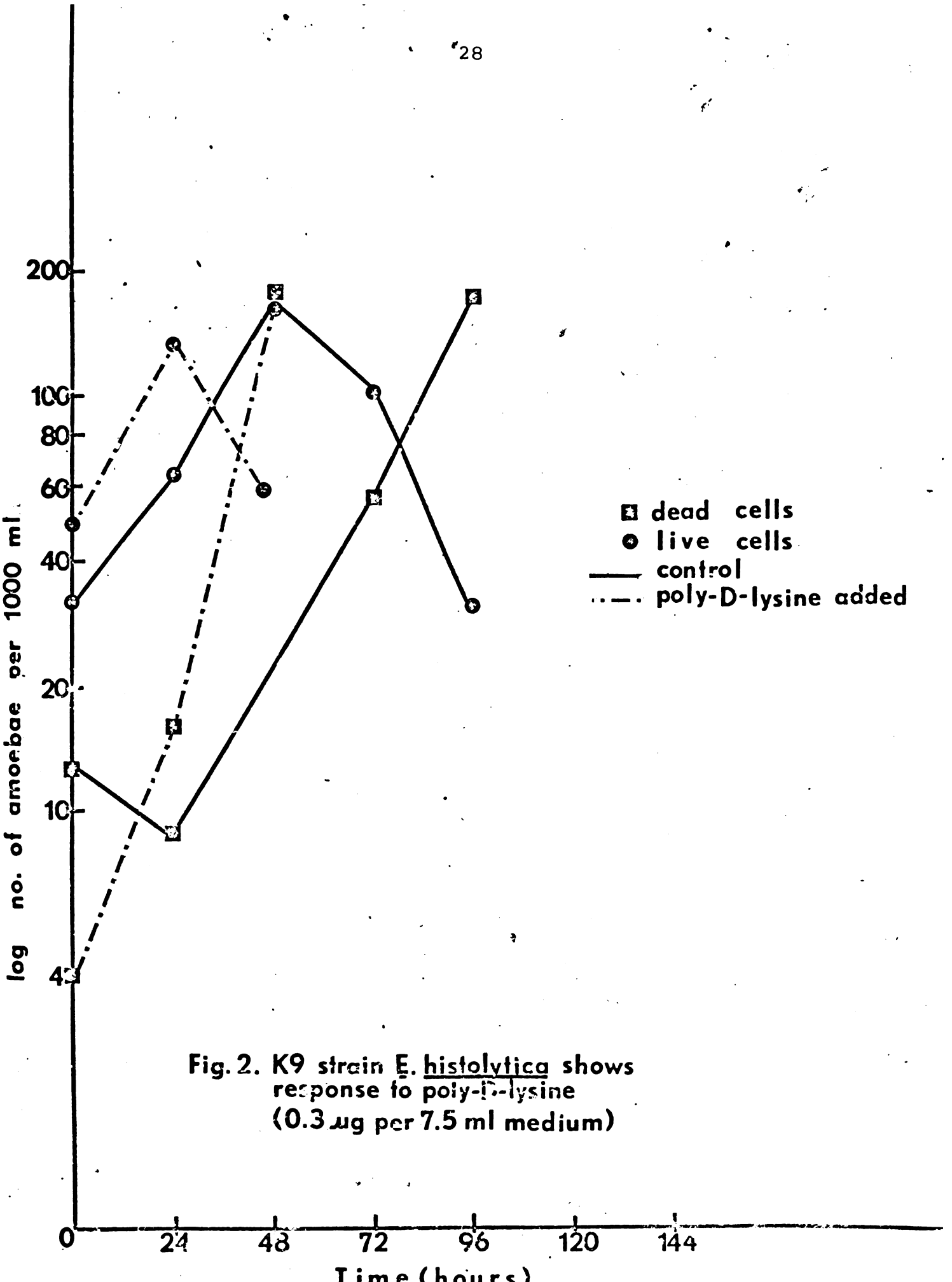

Fig. 2. K9 strain E. histolytica shows response to poly-D-lysine (0.3 µg per 7.5 ml medium)

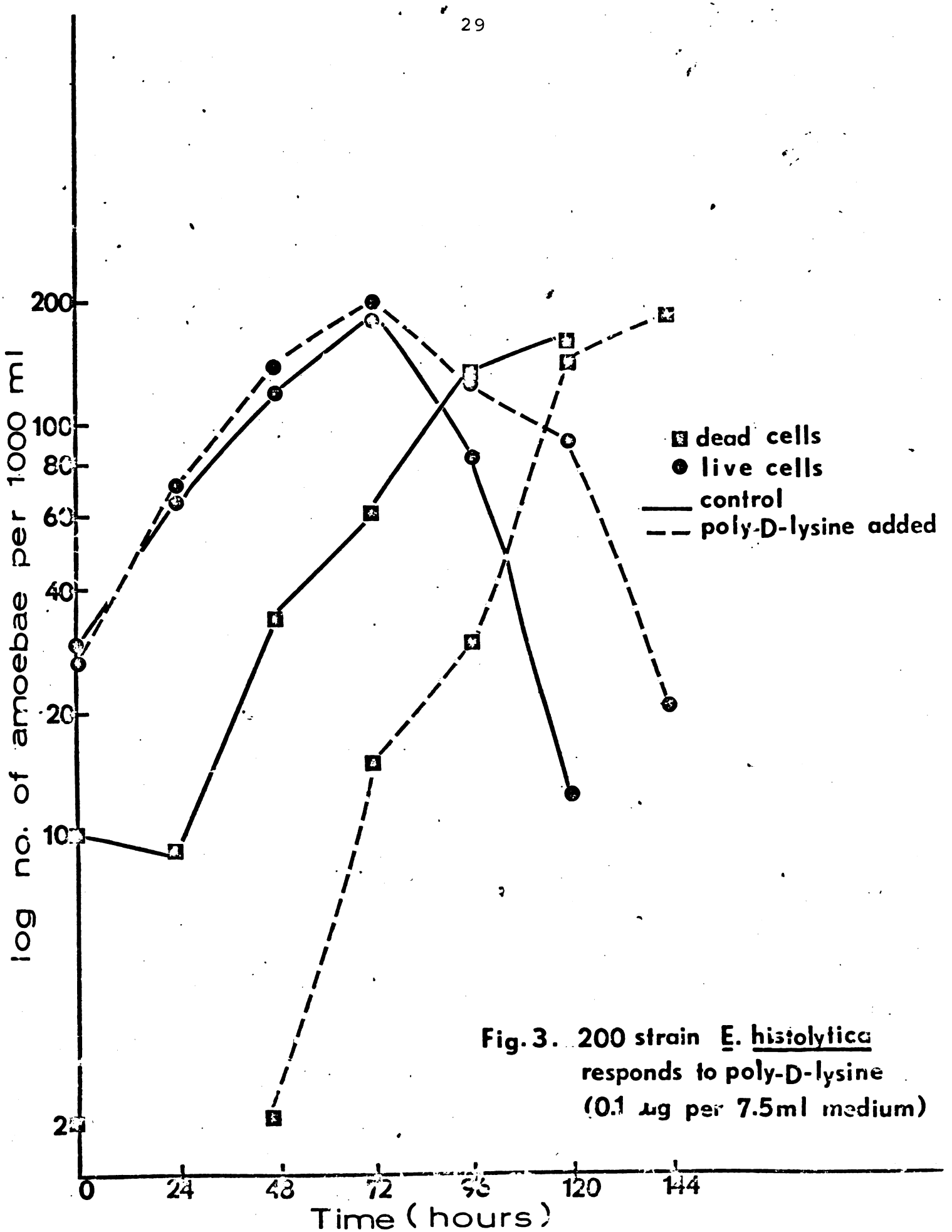

Fig. 3. 200 strain E. histolytica responds to poly-D-lysine (0.1 µg per 7.5ml medium)

lower temperatures. Amoebae can survive for several hours at room temperature. When kept at 30°C, 200 strain died out within 3 days, although a few K9 amoebae were still found to be alive under this lower temperature, growth was poor. A temperature of 38°C killed both strains in 2 days.

DISCUSSION

When Shaffer and Sienkiewicz (1952) grew E. histolytica (strain 200) on Shaffer-Frye medium, with small inocula of 30, 90, and 150 amoebae per ml., there tended to be a lag of several hours before active propagation occurred. With larger inocula the lag phase was shortened, there being a considerable increase in the number of amoebae during the 24 hours of incubation. With the exception of the smallest inoculum he found the maximum number of amoebae was reached after 72 hours. The inocula within the range of 150 to 300 amoebae per ml were most likely to yield consistent results. Sadun et al. (1950) used other strains of E. histolytica (22, 19, and NRS) and found that the greatest increase in population seemed to take place up to 96 hours but the maximal growth was very less in Shaffer-Frye medium. The NRS strain was found to gradually decrease in number in the same medium. With the same initial inoculum they found the growth in Locke-egg-serum medium was strikingly similar. Karlson et al. (1952) thought that the active principle was contained only in the bacterial cells. He found that the heat-killed bacterial cells contained a substance necessary

for the survival of E. histolytica and came to the conclusion that there was no stimulus in any bacteria-free filtrate. He explained that the amoebae obtained the stimulating principle only by ingestion of the bacteria. Harinasuta and Harinasuta (1955) claimed that the lag phase in the early stages of amebic growth in vitro was considerably shortened in the media by allowing growth of the associated bacteria to take place before the amoebae were inoculated. Growth of amoebic populations were more prolific in such preconditioned media than in fresh media. The optimal time required for preconditioning was 4-6 hours. They concluded that the stimulating effect of preconditioning might be closely connected with the adjustment of pH and oxidation-reduction potential, resulting from the growth of the associated bacteria. In the preconditioned media the optimum physical requirements of growth were at once available to the amoebae and growth was thereby enhanced. So, either the bacteria themselves or the environment favors the growth of E. histolytica.

In the present investigations, no lag phase in the growth of E. histolytica was observed in this axenic medium. On the contrary, according to the growth rate measured, amoebae proliferated more or less at the same rate in the first 24 hours as the later stages. The pH never dropped more than 0.2 through the period. This

indicated that both the physical and nutritional conditions in the medium used were good enough to support the immediate proliferation of E. histolytica. The large inoculation size was another reason why the lag phase could not be observed in the experiments. The use of an extraordinarily small inoculum (under 1,000 amoebae per ml) failed to follow the normal growth. There must be a density threshold for E. histolytica to multiply. They may require a certain amount of cells, at least, to counteract the damage during the transferring procedure.

Different strains showed different growth rates. In the same medium, K9 amoebae were found to have more rapid growth whereas in the 200 strain growth was slower. The former was also found to be more resistant during the 30°C incubation. In the series of experiments Shaffer did in 1953 (Shaffer et al., 1953), the 200 strain E. histolytica multiplied most rapidly and reached its maximum count routinely after 48-72 hours of incubation in Shaffer-Frye medium. The Luna strain multiplied more slowly in the same medium, reaching its maximum count between 72 and 96 hours at 37°C incubation. Harinasuta and Harinasuta (1955) grew E. histolytica in several media and found different growth rates in each medium during the cultivation under 37°C. The different growth rates of K9 and 200 strains of E. histolytica in the present test were

not surprising. The rate of growth of any strain of E. histolytica was thus seen to be determined by the medium used, the strain differences, and many other physical conditions. The adaptability of different amoebae varied. The K9 strain in this experiment could withstand longer periods of lower and higher temperatures, and the absence of horse serum as compared with the 200 strain. They were more resistant than the 200 strain. Cabrera (1958) treated several strains of E. histolytica with raised and lowered temperatures (3-45°C). The survival time for these different amoebae, at different temperatures, varied. The same thing was true for their critical temperatures. The differences were said to depend on the amoebae themselves, not on the environmental conditions. It might be true for any kind of physical stress that acted on E. histolytica.

All the surface robbing treatments (i.e. silicone-coating, vertical standing, rotating, spinning) showed inhibition of growth rate and maximum yields. These results were consistent with the experiments done by the other workers. Similar tests were set-up by Faust et al. (1950). They compared the results of increasing vertical and horizontal surfaces on which E. histolytica were grown and got the results that the former condition did not enhance the growth but the latter did. Benham and Havens (1958) got the same results by comparing the growth of

E. histolytica in test tubes and flasks. Reeves (1962) added surface active agents to the culture medium. These agents were found to diminish the multiplication of E. histolytica. It could be assumed that the surface was required by the organisms to settle down and multiply.

Because of the strong disturbing force, E. histolytica grown in spinning flasks showed only a slight increase, even during the active growing phase. Their multiplication was inhibited. Possibly in order to increase the surface for attachment, amoebae extended several filamentous pseudopodia at the posterior end. This character was rarely seen in the aggregating amoebae in clumps in the same flask. Most of the E. histolytica in the spinner flask that were multinucleate might have been the result of the failure of the cytoplasm of E. histolytica to divide, because of the unstable environment, although nuclear division was normal. Multinuclear E. histolytica were observed by many other workers during normal growth (Shaffer, 1965, Dubey and Das, 1966), they all agreed that nuclear and cytoplasmic division of E. histolytica did not have to occur at the same time. Band and Machemer (1963) described multinucleate Hartmennella rhysodes under agitated conditions. The explanation was "multinucleate organisms arose because the amoebae were unable to attach to the substrate in order to divide."

The same explanation could be applied to the phenomena described in the present investigation.

During exponential growth, in the presence of agar and the static conditions, E. histolytica appeared as uninucleate organisms. In the silicone-coated, either vertical or rotated tubes, multinucleate amoebae were not very commonly found. Any disturbing force was directly related to the growth rate and maximum yield. The number of E. histolytica decreased as the disturbing force was increased. The amoebae may have aggregated under surface contact interrupting conditions, in order to get certain attachment. Clumps formed in spinning flask had stronger adhesive ability in each amoeba since agitation did not break them apart. No chilling before sampling, in the case of spinning, was another reason that clumps were present occasionally in the sample to be counted. In the agar medium, heavier agar particles in the medium gave the organisms more chance in gathering around them. Large areas were found with amoebae clustered about the denser piles of agar particles. Sometimes this cluster complex appeared to move as a unit. These particles might serve as a surface that amoebae might require. The number of dead cells in the agar medium was more than in the control medium in the stationary phase. The agar particles prevented the dead cells from disintegrating and rupturing.

The greater number of dead E. histolytica might also indicate that the maximum number of E. histolytica was higher than the control. Because of the semi-solidity of the agar, particles were distributed unevenly into each tube, although difference in quantity was not obvious. This caused the variance in the maximum yields, although the differences were not statistically significant (Table 2). The agar tests showed that certain kinds of surfaces other than the glass wall could provide E. histolytica with their attachment requirement.

It was found that the agar medium had the same length of log phase as the normal growth. There are some other factors which cause the onset of the death phase. Whether this is because of the nutritional deficiency, genetic inheritance, physical factors or other factors is not known.

Poly-D-lysine is one of the agents (poly-amino acids) that induce pinocytosis of some cells (Ryser and Hancock, 1965). Higher concentrations of poly-D-lysine were found to cause abnormality of the cells which forces them to detach from the glass wall, increased clumping, and caused damage of the cell membrane. The basic macromolecules were taken up themselves and their own rate of uptake was found to be proportional to the transfer of other macromolecules (Ryser and Hancock, 1965).

Concentrations of poly-D-lysine below the threshold could not induce pinocytosis. Suitable concentrations enhanced the activity of cells. The threshold concentration of two strains of E. histolytica in this experiment was found to be different, K9 strain had a higher threshold than the 200 strain E. histolytica. This again indicated different adaptability between them.

According to the "t" test, K9 strain showed statistically significant differences between vertical standing, silicone-coating, and rotating experiments as compared to the controlled condition, but, the 200 strain E. histolytica had less response, as the environment changed, than the K9 amoebae.

Horse serum was found by Shaffer et al. (1952) to be beneficial to E. histolytica in a bacterial medium but it might not be an essential constituent. They stated that the multiplication of E. histolytica could occur without horse serum if a vitamin mixture was used to replace it. In the medium used for the present test, horse serum was found to be essential for the multiplication of E. histolytica. This inconsistancy might be in the use of different kinds of media and in the absence of associated bacteria.

SUMMARY

1. Entamoeba histolytica strains, K9 and 200, were grown in TPS-1 medium with and without agar particles. Test tubes, coated with silicone, rotating, vertical standing, and flask spinning consistently with a spinning rod, were used to grow them. Controlled tubes were set up accompanying each test.

2. Growth of E. histolytica was depressed by silicone-coating, rotating, vertical incubation and spinning.

3. Multinucleate E. histolytica were very commonly found in the spinning flask. This phenomenon indicated that cytoplasmic division of E. histolytica was inhibited but nuclear division remained normal.

4. Agar particles seemed to be able to replace the glass surface, required for the growth of E. histolytica.

5. The K9 strain was more resistant and had a higher adaptability than the 200 strain as the environment changed.

6. Poly-D-lysine, which may induce pinocytosis, enhanced the growth of E. histolytica; the threshold concentration for K9 and 200 strains were different.

7. Dead E. histolytica first appeared in the exponential growth phase although they were largely present in the stationary phase.

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