

ELECTROKINETIC PROPERTIES AND  
BACTERIAL INTERRELATIONSHIPS OF  
ENTAMOEB INVADENS RODHAIN, 1934

by

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## INTRODUCTION

It has become increasingly apparent during the past fifty years that amebiasis is not a problem limited strictly to the tropics. The epidemics of amebic dysentery in Chicago in 1933 and 1934 which resulted in some 1,409 cases are but an indication of the possible severity of amebiasis in the northern United States (Burrows, 1959). Between 1946 and 1953 there were 150 deaths annually from amebic dysentery in the United States. In the tropics, incidences of 30 to 60% are not uncommon, and in one period incidences of amebiasis approaching 100% were reported (Chandler and Read, 1961, p 62). It must be pointed out, however, that in many cases no distinction has been made by the observers between Endamoeba hartmanni and Entamoeba histolytica (Burrows, 1957). In 1961, Burrows came to the conclusion that previous estimates of 10 to 20% prevalence of amebiasis in the United States were much too high, and that 5% prevalence or less would be more nearly correct. Using the figure of 5% prevalence in the United States, it is readily apparent that the disease occupies a position of high significance in the United States and certainly in the world.

Among the very complex factors affecting the severity of amebiasis in man and experimental animals are the complex inter-relationships between the parasitic amebae and the organisms associated with them in the lumen of the intestine. Several recent studies have shown that bacteria influence the course and intensity of infections of man with E. histolytica, and more recently the same relationship has become evident in studies of

infections of snakes with the closely related ameba Entamoeba invadens. Many studies have been made of the monobacterial cultural properties of E. histolytica, but very little has been reported on the monobacterial cultural properties of E. invadens (Porter, 1953). It would seem desirable to obtain greater information on E. invadens, since it provides an excellent means for the study of the parasitic amebae without the necessity of working with an organism pathogenic for man. It fits most requirements for research excellently, since it can be grown in large numbers in axenic cultures (Diamond, 1960).

This study has been undertaken in an effort to provide some additional information about the relationship between E. invadens and bacteria. Four phases of study were undertaken: (a) studying the growth of E. invadens when associated with single species of living bacteria; (b) studying the growth of E. invadens by optical absorbance measurements when associated with single species of killed bacteria in various fluid media; (c) determining the electrophoretic mobility and charge of E. invadens and its relationship, if any, to the phagocytosis of bacteria by the ameba; and (d) the use of the phase contrast microscope and photomicrography to illustrate the structure of the ameba and the phagocytosis of bacteria.

#### REVIEW OF LITERATURE

E. invadens was first described by Rodhain in (1934) as a parasite of snakes. Geiman and Ratcliffe (1936) confirmed Rodhain's work and described the ameba and its life cycle completely.

The ameba trophozoite was described as  $9.2-36.6 \mu \times 9-30 \mu$  with an average diameter of  $15.9 \mu$ . It exhibits rapid motility in one direction in a slug-like manner with explosive protrusion of clear ectoplasmic pseudopodia upon change of direction. The trophozoite feeds on leucocytes, liver cells, cell debris, bacteria and starch grains and produces lesions in the alimentary tract (stomach, duodenum, ileum, and colon) and the liver. The nucleus is  $3.5-7.3 \mu$  in diameter, averaging  $4.78 \mu$ . Peripheral chromatin granules are evenly distributed under the thin nuclear membrane. The central karyosome is surrounded by a small halo and 'achromatic' cloud. The ameba's nucleo-cytoplasmic ratio in different strains is  $0.26-0.34$ , averaging  $0.295$ . The cyst is  $11-20.2 \mu$  in diameter, averaging  $13.88 \mu$ . It contains 1-4 nuclei, a glycogen vacuole and acicular, rod-like or cylindrical chromatoids. Encystation and formation of quadrinucleate cysts takes place in the intestine or post-mortem in the liver. Excystation requires 7-24 hr in the snake's intestine; the majority require 12-24 hr. The temperature range for maximum growth in cultures is  $20-30 \text{ C}$ . All of the above description can be attributed to Geiman and Ratcliffe (1936).

E. invadens has been found to infect the following snakes: Varanus salvator, Varanus varius, Tiliqua scincoides, Pseudoboa clelia, Lampropeltis getubus, Ancistrodon mokasen, Natrix rhombifer, Natrix sipedon, Natrix sipedon sipedon and Natrix cyclopion (Geiman and Ratcliffe, 1936). All of the above species were reported as having shown spontaneous amebiasis. Meerovitch (1961) used Tiliqua sirtalis as a vehicle for the study of the effects

of temperature on infections of snakes with E. invadens. Meerovitch is of the opinion that E. invadens does not infect snakes in nature, and that 'naturally infected' snakes become so through water and utensil contamination in captivity. Among the reasons given for this opinion are the fact that the normal temperature of snakes is not such as to support infections of the ameba and that Meerovitch was unable to find a single case of infection in wild snakes.

The excellent study of E. invadens by Geiman and Ratcliffe brought out the very great similarity between E. invadens and E. histolytica, both in their morphology and in their pathogenicity. The only readily apparent difference between the two species is in their optimum temperatures for growth. E. invadens has a temperature range of 20-30 C with maximum growth above 25 C whereas E. histolytica has an optimum temperature of 37 to 38 C. E. invadens will not grow at 37 C as several attempts to induce it to do so have shown (McConnachie, 1955). Meerovitch (1960) obtained results in experimental infections of snakes with E. invadens which established the fact that the ameba is unable to establish infections in snakes at a temperature of 35 or 37 C. McConnachie has suggested that E. invadens, E. histolytica, E. moshkovskii and E. aulostorni may constitute a series of subspecies in view of their morphological identity and the fact that the amebae of these groups form a series growing at increasingly higher temperatures.

The description of E. invadens resulted in a large number of studies of this ameba as a tool for the study of E. histolytica

which is both more difficult to culture and is also dangerous to work with routinely. Many attempts have been made to produce media capable of supporting the axenic growth of E. invadens in order that the physiology of the ameba might be studied without the effects of the bacteria or other associated organisms. Axenic cultivation is used here as meaning the indefinite subcultivation of amebae in an environment free of metabolizing bacteria, fungi, protozoa or metazoan cells. Stoll, in 1957, was the first to report the axenic cultivation of E. invadens and Diamond, in 1960, described an axenic medium which is clear and free of gross particulate matter. The technique used by Diamond in obtaining an axenic culture was as follows: the amebae were first isolated in axenic culture and then monoxenized with the Culbertson strain of Trypanosoma cruzi by the technique of Phillips (1950). A 1 ml sample of a seven-day-old ameba-trypanosome culture was introduced into the bottom of a tube of medium containing 0.05% agar. One ml subcultures were made every two weeks for the first 12 weeks and weekly thereafter. It was found that the trypanosomes originally introduced with the amebae disappeared between the second and third transfer. With about the seventh transfer the agar was successfully eliminated from the medium.

Since many studies had shown that the associated bacteria affect the course and intensity of infections of experimental animals with E. histolytica, similar studies were carried out using E. invadens (Luttermoser, 1952; Phillips and Wolfe, 1959). Miller (1953) observed that bacteria influenced the course and

intensity of E. invadens infections, but unfortunately, he did not identify the bacterial associates. Meerovitch reported in 1961 that his studies had shown that monoxenic amebae are less pathogenic than polyxenic amebae.

McConnachie, (1955) studied the cultural properties of E. invadens in a diphasic horse-serum-Ringer's eggwhite medium. He found that optimum growth of the ameba occurred in the medium in the presence of Bacterium coli (Escherichia coli), at 24-30 C. The amebae could not be induced to grow above 35 C. The amebae ingested normal rat erythrocytes, but not those of chick or frog and were adversely affected by aerobic conditions. In 1956, McConnachie reported obtaining a bacterial free culture from a monobacterial strain of E. invadens and Staphylococcus pyogenes var. albus, (Staphylococcus epidermidis). This was accomplished by maintaining the monobacterial strain of E. invadens plus S. epidermidis in saline-liver medium containing antibiotics (penicillin and streptomycin). Since his media contained fresh chick liver, these cultures could not be considered to be axenic. He reported achieving a monobacterial strain of E. invadens with E. coli. The two monobacterial strains were prepared from cultures containing Organism X and either E. coli or Bacillus megaterium. In 1958, McConnachie reported, in addition to those reported previously, the growth of E. invadens when associated with Bacillus subtilis, Aerobacter aerogenes and Proteus vulgaris. Of these species, E. coli and Organism X were the most favorable for amebic growth and mass encystation in his saline liver medium. Balamuth (1962) has reported that E. invadens



grows and encysts well in his medium in monobacterial culture with Clostridium perfringens.

In summary, growth of E. invadens in monobacterial cultures has been reported as occurring with the following bacteria: E. coli, B. megaterium, B. subtilis, S. epidermidis, A. aerogenes, P. vulgaris, C. perfringens and Organism X.

Very little information is available on the physiology of E. invadens despite the fact that it is now available in axenic culture. Perhaps the only extensive work to date is that of Balamuth (1962) in which he studied the effects of some environmental factors upon the growth and encystation of the ameba. Balamuth could not demonstrate a differential shift from continued growth to encystation associated with changes in temperature, viscosity, oxygen tension, or crowding. His experiments did confirm the observations of Geiman that E. invadens is a fastidious anaerobe growing in association with C. perfringens at an  $E_h$  of approximately -400 millivolts.

It has already been pointed out that available evidence indicates that bacteria influence the course and severity of infections with E. invadens, and that polyxenic amebae are more pathogenic than monoxenic amebae. The role of the bacteria in contributing to the pathogenicity of E. invadens has not yet been established nearly so exactly as it has for E. histolytica. It has been shown that it is probable that E. histolytica may in the absence of bacteria be a harmless microbe incapable of independent survival in the intestine (Phillips, et al., 1955). Phillips (1959) found that intestinal amebiasis was not produced

in the germfree guinea pig, but when E. histolytica was associated with any one of three known species of bacteria, it was able to produce ulcerative lesions. The three species of bacteria used in order of the severity of lesions were B. subtilis, A. aerogenes and E. coli.

Until the work of Stoll, E. invadens had not been cultivated free either of an associated organism or of fresh liver. The role of the bacteria in such a dependent association as the above, where, if they are removed or killed leave the amebae unable to survive, is an interesting problem. Very little work has been carried out on this problem with regard to E. invadens, although a great deal of work has been carried out with regard to the same problem with E. histolytica.

Several concepts have developed from studies of E. histolytica as to what the bacteria may furnish the amebae in culture. One such concept is that the bacterial flora may provide suitable anaerobic conditions or reduced O-R potentials (Balamuth and Howard, 1946). However, if reduction of the O-R potential were the sole requirement which bacteria fulfilled, certainly a number of reducing agents are available which could be added to the medium, or the atmospheric oxygen could be excluded in order to achieve the same effect. It is noted that in the most successful axenic medium for E. invadens to date, a reducing agent is added to the medium, in this case the amino acid cysteine (Diamond, 1960). Also, McConnachie achieved good growth in his media using sterile fresh chick liver and a pyrogallol plug to absorb oxygen. However, several authors had used

pyrogallol plugs previously and had been unable to culture the ameba without either microbial associates or fresh tissues. A second concept is that the products of bacterial metabolism are used by the amebae (Balamuth and Howard, 1946). This concept is widely accepted. Rees, et al. (1941) postulated that bacteria attack substrates in the test tube and furnish metabolic products similar to those found in the intestinal tract. This concept gains support from the successful axenic cultivation of E. invadens by Stoll (1957) and Diamond (1960). Stoll's medium utilizes fresh liver which contains factors which apparently can replace the metabolic products of bacteria. Diamond's axenic medium utilizes both concepts presented so far in that a reducing agent, cysteine, is added to the medium, and the enzymatic product trypticase (Baltimore Biological Laboratories) is added.

Another possibility is that the bacteria themselves serve as food for the amebae. Although some limited success has been achieved in culturing E. histolytica on killed bacteria, this technique has often been attempted without success (Jacobs, 1947; Reeves, et al., 1960). On the other hand, it is clear that the parasitic amebae do phagocytize the bacteria (Geiman & Ratcliffe, 1936; Hoare, 1952). They are also capable of showing selection in their phagocytosis (Shaffer and Iralu, 1961). Growth factors for E. histolytica have been found in dead bacterial cells (Jacobs, 1950). A fourth concept is that the bacterial flora may provide enzymic systems which the amebae require to utilize food material in the medium (Nakamura, 1953). It is difficult in the case of E. invadens at least, to differ-

entiate between this concept and the concept of the utilization of products of bacterial metabolism. This is especially so, since both E. invadens and E. histolytica have now been cultured in axenic media. It is clear from the above that much work remains to be done in characterizing the role of the bacteria in the complex interrelationships between the bacteria and the parasitic amebae.

A particularly useful method of gaining information about the electrokinetic activities of microorganisms is the microscopic method (Uber, 1950). Electrophoresis was discovered by Reuss in 1808 but did not come into wide spread quantitative use until about 30 years ago (Abramson, 1934).

Several different microscopic electrophoresis instruments have been described. The first practical instrument used a flattened glass capillary for the electrophoresis cell (Northrop & Kuntz, 1925). This instrument was modified and improved greatly by fusing glass microscope slides to form the electrophoresis cell and by using all glass construction (Abramson, 1929). The all glass construction of this instrument had two great advantages over previous instruments in that it permitted the instrument to be easily cleaned, and it prevented vibration due to rubber tubing connections between parts. A standard method for microscopic electrophoresis was suggested in 1936 by Moyer and has been used widely since that time. Since 1936, various modifications have been made in the design of the Abramson cell, although it has continued to be the one most frequently used. The greatest improvement in design to date has been the

design and construction of microscopic electrophoresis cell which allows it to be used in a water bath, thus providing accurate temperature control (Gittens & James, 1960).

Measurement of the electrophoretic mobility of particles involves measurement of the time required to move a set distance as indicated by a calibrated ocular micrometer. The mobility of the particle must be determined at the 0.21 or 0.79 level of the electrophoresis cell in order that electroosmosis will not either increase or decrease the velocity of the particle (Abramson, 1934). The field strength must be determined carefully, and the mobility of particles is recorded as either  $\text{cm}^2/\text{v} \cdot \text{sec}$  or as  $\mu/\text{sec}/\text{v}/\text{cm}$ . Although much emphasis has been placed upon calculation of the zeta potential and the net charge per unit area, the mobility measurements can reveal a considerable amount of information about the particles. Particles migrating with equal mobilities are considered to possess equal zeta potentials and charge densities (Uber, 1950). It cannot necessarily be assumed, however, that particles having the same mobility necessarily possess the same shape, size and conductivity as is sometimes mistakenly assumed (Abramson & Michaelis, 1929).

Although a great number of studies have been made of the electrophoretic mobilities of bacteria, only a few studies have been made of the electrokinetic properties of protozoans. These were summarized by Kudo in 1954 as follows:

Amoeba show negative reaction to the anode and move toward the cathode either by reversing the cytoplasmic streaming (Verworn) or by turning around the body, (Jennings). The free-swimming ciliates move mostly toward the cathode, but a few may take a transverse position

(Spirostomum) or swim to the anode (Paramecium, Stentor, etc.). Of flagellates Verworn noticed that Trachelomonas and Peridinium moved to the cathode while Chilomonas, Cryptomonas, and Polytomella, swam to the anode. When Paramecium caudatum was exposed to a high frequency electrostatic or electromagnetic field, Kuler, Chalkley and Voegtlin (1929) found the effect was primarily caused by a temperature increase in the organism. By subjecting Pelomyxa carolinensis to a direct current electric field, Daniel and May, (1950), noted that the time required for the rupture of the body in a given current density is directly correlated with the size of the organism and that calcium increases the time required for rupture at a fixed body size and current density, but does not alter the size effect.

From this summary, it can be seen that no studies have been made of the parasitic amebae, and further that no studies to date have determined the electrophoretic mobilities or the electrostatic surface charge of any of the protozoans. Paramecia have been described as moving towards the cathode (Jahn and Jahn, 1949). The movement of protozoans toward a particular electrode is called galvanotropism by Jahn and Jahn. There is obviously a lack of information about the properties of the protozoans, and it would therefore seem desirable to acquire more information about these electrokinetic properties.

## MATERIALS AND METHODS

### Cultural Methods

Axenic Culture. A strain of E. invadens (strain P Z, clone I V) was obtained from the laboratory of parasitic disease of the National Institutes of Health, Bethesda, Maryland. This strain had been monoxenized with the Culbertson strain of Trypanosoms cruzi by the method of Phillips, 1950. The ameba

was then established in axenic culture (Diamond, 1960). Diamond's medium contains the following ingredients: trypticase (BBL), 2.0 g; yeast extract (BBL), 1.0 g; maltose, 0.5 g; L-cysteine hydrochloride, 0.1 g; L-ascorbic acid, 0.02 g; potassium phosphate, monobasic, 0.08 g; potassium phosphate, dibasic, anhydrous, 0.08 g; distilled water to make 90 ml; horse or sheep serum (inactivated 56 C, 30 min), 10 ml. The pH is adjusted to 7.0 to 7.2. The ameba was subsequently maintained in axenic culture in his medium. Transfers were made weekly of 1 ml (approximately 20,000 amebae) of a 7-day-old axenic ameba culture to fresh axenic medium. Inactivated sheep, horse or bovine serum was used in the medium. Incubation was carried out at room temperature of 27 to 30 C.

Choice of Medium for Xenic Cultures. The medium desired for these studies was one incapable of supporting the continued growth and multiplication of E. invadens in axenic culture; further it should be a standard medium for the xenic culture of E. histolytica, and it should lend itself readily to the enumeration of the amebae. Locke's egg medium for amebae was selected on the basis of the above considerations (Boeck and Drobohlav, 1925; Reardon and Rees, 1939). This medium has the advantage that it does not require serum, and it is recommended as a standard medium for laboratory use by the Communicable Disease Center of the United States Public Health Service, (CDC Bulletin, Oct.-Dec. 1948). This medium, usually referred to as LER medium (Locke's-egg-rice) consists of a solid egg base and an overlay of Locke's solution. It is suitable for the cultivation of many

of the intestinal protozoa, and it will support the growth of a very large number of types of either anaerobic or aerobic bacteria.

Procedures Used in Xenic Growth Studies. Two tubes of LER medium for each species of bacteria to be tested were conditioned by inoculation with the bacteria and incubation at 25-30 C for 24 hr before the addition of the amebae. Approximately 30 mg of sterile rice starch were added to each of the tubes either before or after inoculation with bacteria. In earlier experiments rice starch was added to the tubes with a small sterile wire loop at the time they were inoculated with bacteria. In later experiments a rice powder dispenser was used, and the rice starch was added just before the tubes were inoculated with bacteria.

One ml of the stock axenic cultures of E. invadens was added to each of the cultures after 24 hr incubation. At the time of inoculation and at approximately 48 hr intervals thereafter, the number of amebae trophozoites per ml of culture was determined. The tubes were chilled for five minutes in cold water in order to free the amebae from the sides of the tubes. The cultures were then shaken to suspend the amebae at the base of the egg slants; the suspension of the rice starch was used as an indication of successful suspension of the amebae. A sterile wire loop was used to transfer a drop of the suspension to each side of a hemacytometer. The number of amebae in the entire field of each of the nine mm<sup>2</sup> rulings was determined (Paulson, 1932). Except where otherwise noted, only active trophozoites were counted.



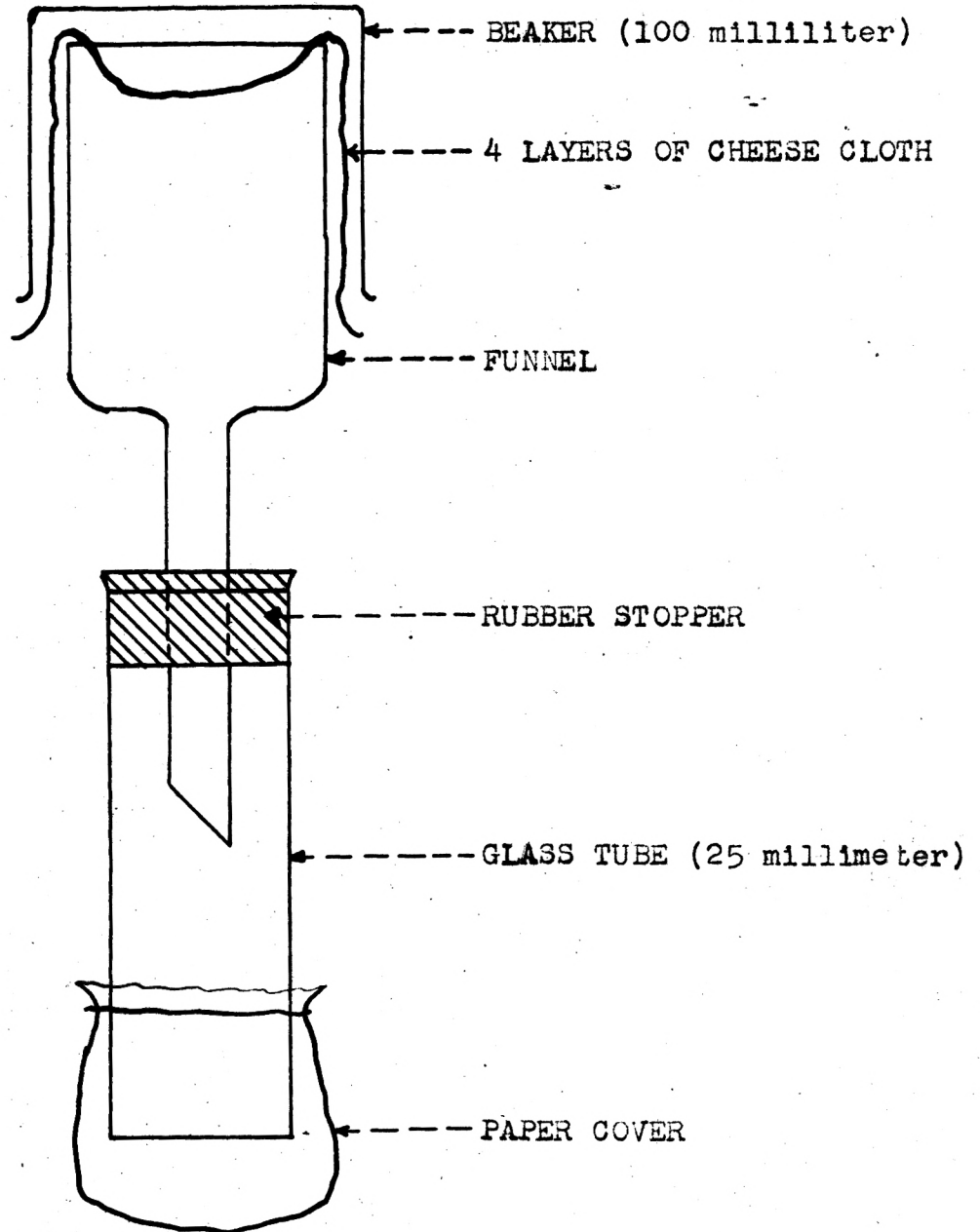
The rice powder dispenser is illustrated in Plate I and is based on a design in the Communicable Disease Center manual Laboratory Diagnosis of Parasitic Diseases (1955). The rice powder dispenser consists essentially of a straight wall 35 mm funnel with four layers of cheese cloth across the top of the funnel and a 100 ml beaker covering the funnel. The stem of the funnel protrudes through a rubber stopper into a 25 ml glass tube which extends approximately 4 cm below the end of the funnel stem. In order to use the device, the funnel is wrapped in heavy paper and sterilized in the autoclave. The dispenser is charged by removing the paper cover and the beaker and adding rice powder (previously sterilized with dry heat for two and one-half hours at 150 C). The beaker and paper cover are then replaced. The dispenser is used by removing the paper cover from the mouth, placing the culture tube under the opening and then tapping the dispenser once or twice to deliver the desired amount of rice starch. The paper cover is then replaced.

Optical Density Procedure Used for the Study of Bacterial Phagocytosis by Entamoeba invadens. Several different media were used for the study of the phagocytosis of bacteria by E. invadens. These ranged from initial studies with Locke's solution to complex media such as Diamond's axenic medium for E. invadens. In each case, a clear medium was selected and its optical absorbance curve determined. Since Locke's solution is simply a salt solution, its optical absorbance was negligible, and the optical absorbance of the cultures were measured at area of peak absorbance for the bacteria studied. These cultures were studied at

#### EXPLANATION OF PLATE I

Rice starch dispenser used for dispensing rice starch to tubes of Locke's egg medium. The rice starch is dry sterilized and added to the sterile cheese cloth in the dispenser. The rice starch is released by tapping the side of the dispenser. The paper cover is used to keep the dispenser sterile between periods of use.

## PLATE I



400  $m\mu$ . The remaining cultures used complex media as a base and were studied at 600  $m\mu$  in order to minimize the optical absorbance of the media.

Three species of bacteria were used in these studies: E. coli, B. megaterium and B. brevis. The bacteria were cultured for 24 hr on nutrient agar washed in Locke's solution and then resuspended in the Locke's solution and autoclaved for 15 min at 121 C. The optical absorbance curve for each of the three species of bacteria was determined with the bacteria suspended in the Locke's solution. All optical absorbance measurements were made with the Bausch & Lomb Spectronic 20 colorimeter.

Photographic Studies. Photomicrographic studies were made of E. invadens in various media using an American Optical phase-contrast microscope (dark-M phase) and a 35 mm camera.

#### Electrophoretic Methods

Determination of the Electrophoretic Mobility. Electrophoretic mobility was determined in the Abramson electrophoresis cell using the procedure described by Moyer (1936). The electrophoresis cell used in these investigations used plaster of paris plugs in connection with Cu-CuSO<sub>4</sub> electrodes.

The electrode system was modified by the addition of electrolyte reservoirs connected to the electrophoresis cell by means of rubber tubing. This system is illustrated for one electrode in Plate II. When in use, the reservoirs were nearly filled with the saturated copper-sulfate electrolyte and the rubber tubing and electrode tubes filled with the electrophoresis cell inverted. With both reservoirs filled, the electrophoresis

instrument was taped securely in place on the microscope stage and the reservoirs leveled with a leveling syphon. The level of the copper sulfate electrolyte was adjusted so that no positive pressure would be applied to the plaster of paris plugs. The copper electrodes were placed in the reservoirs. The use of electrolyte reservoirs offers four advantages: (a) the hydrostatic pressure on the plaster of paris plugs is exactly the same for both electrodes; (b) bubbles are more easily excluded from the electrode tubes; (c) a larger volume of electrolyte is used resulting in greater stability during operation; and (d) the rubber tubes permit the electrophoresis instrument to be inverted with less danger of disturbing the electrodes.

In making electrophoretic measurements the cells to be studied were first concentrated by centrifugation and then washed four times in buffer. The specific resistance was determined using an impedance bridge and a conductivity cell with vertical electrodes.<sup>1</sup> A portion of the cells suspended in buffer was placed in the electrophoresis cell which had been previously filled with buffer. It was found expedient to allow the displaced buffer to fill the rubber tube attached to the drain tube of the electrophoresis instrument. When sufficient sample had been added, the tubing was clamped at the end and squeezed several times in order to suspend the sample evenly in the instrument. The stopcocks were then turned to the electrode position and the current turned on. Either of two rectifiers delivering

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<sup>1</sup>The conductivity cell is described in the appendix.

105 and 300 volts, respectively, were used as power supply units in series connection with either a one-milliammeter or a ten-milliammeter. The current was adjusted by a resistance unit to a suitable value, the lower stationary level located, and the cells which came into focus were individually timed over a set distance. An attempt was made to measure the mobility in each direction an equal number of times. This was not always possible because of the difficulties inherent in working with such large cells.

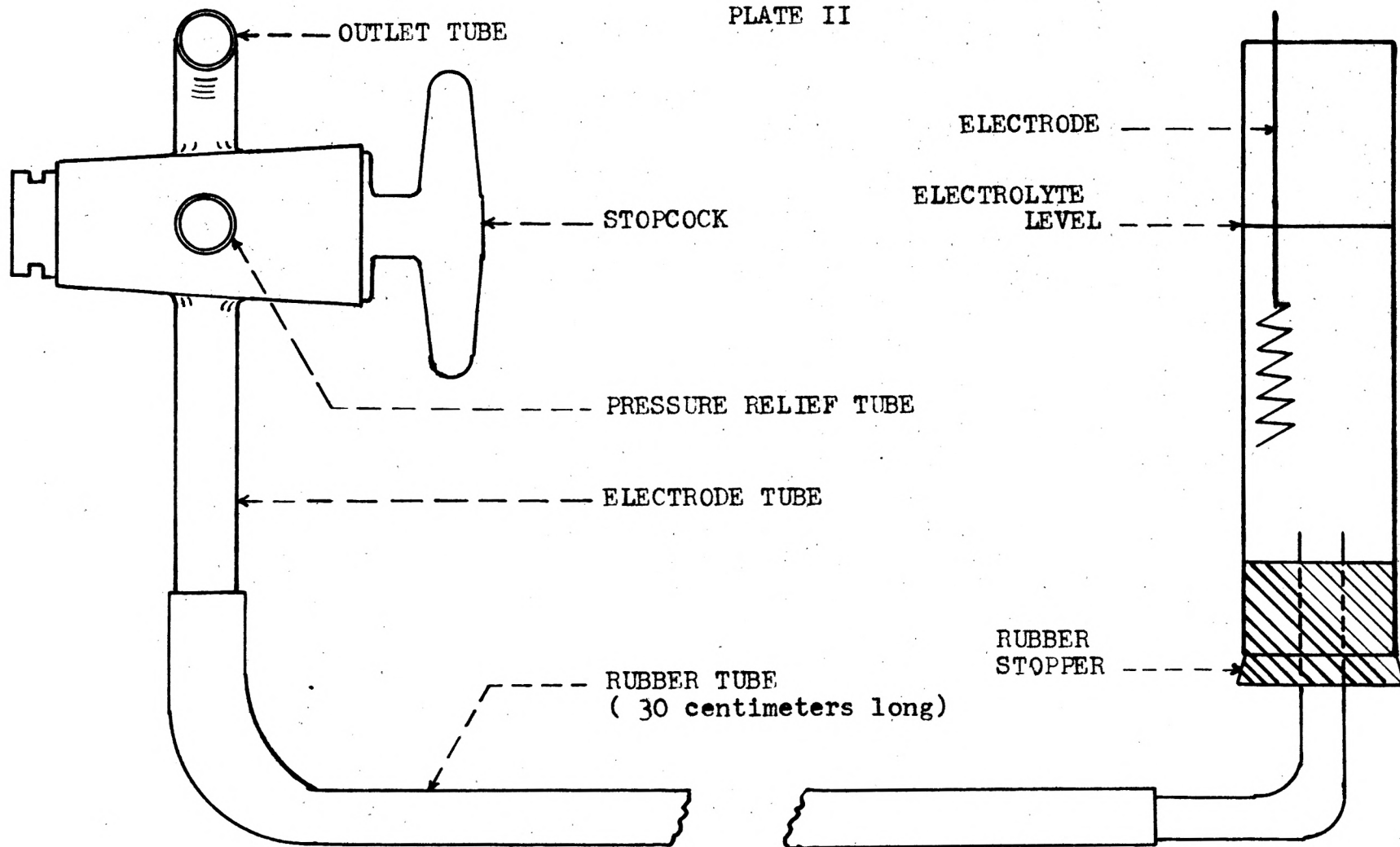
The large size of the amebae and their tendency to settle very rapidly made it mandatory that as many counts as possible be made. To this end, the samples were resuspended several times in the manner described above. The electrophoresis cell was washed out with buffer after about 20 counts and refilled with a fresh sample. The bacteria and erythrocytes did not present the settling problem that the amebae presented and could be timed for greater distances. The technique used for the erythrocytes was to measure the time required for them to traverse two scale divisions ( $62.4\mu$ ) and return to their starting point with the current reversed. The bacteria were generally timed as they traversed five scale divisions.

Since the cross sectional area of the electrophoresis cell is difficult to determine by measurements, the area was determined electrically. The mobility of human erythrocytes has been found to be extremely constant and was used for the calibration of the electrophoresis cell. The mobility of erythrocytes has been reported as  $1.31\mu/\text{sec}/\text{v}/\text{cm}$  at pH 7.35 in M/15 phosphate

#### EXPLANATION OF PLATE II

The diagrammatic representation of one of the two electrolyte reservoirs used with the micro-electrophoresis instrument. The diagram shows the reservoir at its actual size. The reservoir is shown as it is connected to the micro-electrophoresis apparatus by means of the rubber tubing.

PLATE II





buffer (Abramson, 1929<sub>b</sub>). Thus it is relatively simple to measure the apparent mobility of human erythrocytes and from this determine the cross sectional area of the electrophoresis cell. For the work presented in this paper, 170 measurements were made of human erythrocytes.

The effect of carboxy-methy-cellulose in the buffer was also measured. For these experiments 0.5% by weight of the carboxy-methyl-cellulose was added to several buffers. The buffers were standardized by measurement of their pH and viscosity after the addition of the carboxy-methyl-cellulose. These studies were carried out in order to discover if materials such as carboxy-methyl-cellulose could be used to retard the settling of large particles thus allowing more accurate measurements to be made of the mobility of large particles.

## EXPERIMENTAL RESULTS AND DISCUSSION

### Axenic and Monoxenic Cultures

Monobacterial cultures of E. invadens in Locke's egg medium for amebae were prepared with each of the following bacteria: B. brevis, Bacillus cereus, B. megaterium, B. subtilis, Bacillus subtilis var. niger, Bacillus pumilus, E. coli, Escherichia coli "B", A. aerogenes, Aerobacter cloacae, Serratia marcescens, S. epidermidis, Micrococcus lysodeikticus, Salmonella typhosa, Proteus rettgeri, Proteus mirabilis, C. perfringens, Pseudomonas aeruginosa and Mycobacterium smegmatis. The effect of each bacteria species on the growth of E. invadens is summarized in

Table I. The trophozoite population is presented as growth curves in Plates III-XXIV with the original population in each case being arbitrarily established as one.

The following bacteria increased the multiplication and survival of E. invadens: B. brevis, B. subtilis, B. subtilis var. niger, E. coli, A. aerogenes, S. epidermidis, M. lysodeikticus, P. rettgeri, C. perfringens and P. aeurginosa. Of the above species, B. brevis, E. coli, in Locke's egg medium with added serum, A. aerogenes, M. lysodeikticus, P. rettgeri and P. aeurginosa had the greatest effect on the survival of the ameba. Two species, A. cloacae and S. marcescens had notably deleterious effects on the amebae causing a reduction of approximately 90% of the initial populations in two days.

Several axenic culture studies were made of the growth of E. invadens. As a basis for comparison to the monoxenic cultures, the ameba was cultured axenically in the Locke's egg medium with rice starch and in Diamond's axenic medium for E. invadens. The results of these experiments are presented in Table 2.

In Locke's egg medium the number of trophozoites in the two cultures without bacteria dropped to approximately 60% of their original number in two days. On the third day there was an apparent increase in the number of trophozoites, indicating that the amebae had become better adjusted to their new environment after two days. However, the trophozoite population decreased rapidly after the third day and by the twelfth day the trophozoite populations in the two tubes were about 1% of their original number. Thus it is evident that, although some multiplication did

Table 1. The growth of monoxenic cultures of Entamoeba invadens.

Age in days	0	2	3	4	5	6	7	8	10	12
populations in thousands per ml										
Monoxenized with <u>Bacillus brevis</u> (tube a)										
Tro <sup>1</sup>	28.3	54.4		31.6		27.8		39.4	23.8	15.6
						(tube b)				
Tro	33.3	53.8		32.8		35.5		31.1	40.5	16.7
						(tube c)				
Tro	3.33	6.12		3.89		1.85		2.22	0.37	0.37
Cys <sup>2</sup>	0	1.67		1.39		0		0	0	0
Tot	3.33	7.79		5.28		1.85		2.22	0.37	0.37
						(tube d)				
Tro	1.94	3.33		3.60		1.48		1.66	2.22	1.11
Cys	0	0		0.83		0		0	0	0
Tot <sup>3</sup>	1.94	3.33		4.43		1.48		1.66	2.22	1.11
Monoxenized with <u>Bacillus megaterium</u> (tube a)										
Tro	28.9	27.8	36.6		21.7			10.6	2.22	0.56
					(tube b)					
Tro	27.8	24.4	26.6		16.1			1.11	0	
					(tube c)					
Tro	8.05	8.61		6.94				4.72	3.33	2.22
Cys	0	0		0.56				1.39	0	0
Tot	8.05	8.61		7.50				6.11	3.33	2.22
						(tube d)				
Tro	1.39	1.39		3.61				3.33	4.17	3.33
Cys	0	0		0				0	0	0
Tot	1.39	1.39		3.61				3.33	4.17	3.33
Monoxenized with <u>Bacillus cereus</u> (tube a)										
Tro	32.2	15.0	16.1		5.00					
					(tube b)					
Tro	28.9	10.0	12.8		3.89			0.56	0.56	0
Monoxenized with <u>Bacillus subtilis</u> (tube a)										
Tro	26.6	40.0		15.5		11.7		1.67	0.56	0
						(tube b)				
Tro	23.9	15.6		34.4		4.44		2.78	0	0.56
Monoxenized with <u>Bacillus subtilis var. niger</u> (tube a)										
Tro	25.6	61.1		32.8		16.1		0.56	0.56	1.67
						(tube b)				
Tro	24.4	43.3		16.1		7.78		0	0	

Table 1. (cont.)

Age in days	0	2	3	4	5	6	7	8	10	12
populations in thousands per ml										
Monoxenized with <u>Bacillus pumilus</u> (tube a)										
Tro	28.4	23.9		10.5		10.0		4.44	8.33	8.33
				(tube b)						
Tro	29.4	15.0		11.1		2.22		3.33	0.56	0.56
Monoxenized with <u>Escherichia coli</u> "B" (tube a)										
Tro	74.4	50.0	40.0		10.6			0		
				(tube b)						
Tro	81.1	43.3	30.0		8.89			1.66	0.56	0.56
Monoxenized with <u>Escherichia coli</u> (tube a)										
Tro	22.5	42.5		2.78			0.56			0
Cys	0	6.67		2.78			0			0
Tot	22.5	49.2		5.56			0.56			0
				(tube b)						
Tro	20.2	30.0		8.33			1.39			4.44
Cys	0	3.33		2.78			2.92			1.67
Tot	20.2	33.3		11.1			4.31			6.11
				(tube c) Medium : L.E.S.						
Tro	3.33	6.12		7.23				2.50	2.59	1.11
Cys	0	0		0.56				1.39	0	0
Tot	3.33	6.12		7.79				3.89	2.59	1.11
				(tube d) Medium : L.E.S.						
Tro	10.0	7.50		6.67				2.78	0.37	1.85
Cys	0	0		0				0	0	0
Tot	10.0	7.50		6.67				2.78	0.37	1.85
Monoxenized with <u>Aerobacter aerogenes</u> (tube a)										
Tro	1.11	2.78		3.61		1.11		0.56	0	0.37
Cys	0	0.83		0.28		0.37		0	0	0
Tot	1.11	3.61		3.89		1.48		0.56	0	0.37
				(tube b)						
Tro	2.78	1.94		2.50		3.70		3.06	1.48	1.85
Cys	0	0.28		0		0		0.28	1.11	3.33
Tot	2.78	2.22		2.50		3.70		3.34	2.59	5.18
Monoxenized with <u>Aerobacter cloacae</u> (tube a)										
Tro	17.8	0.55	0		1.10			0		
				(tube b)						
Tro	38.9	0	2.22		1.10			0		

Table 1. (cont.)

Age in days	0	2	3	4	5	6	7	8	10	12
populations in thousands per ml										
Monoxenized with <u>Serratia marcescens</u> (tube a)										
Tro	9.45	1.11		0						
										(tube b)
Tro	1.66	0		0						
Monoxenized with <u>Staphylococcus epidermidis</u> (tube a)										
Tro	12.5	21.1		15.8			1.94			2.22
Cys	0	0.56		1.67			1.67			1.11
Tot	12.5	21.7		17.5			3.61			3.33
										(tube b)
Tro	15.8	26.1		11.1			2.96			0.55
Cys	0	1.11		2.50			1.11			1.67
Tot	15.8	27.2		13.6			4.07			2.22
Monoxenized with <u>Micrococcus lysodeikticus</u> (tube a)										
Tro	5.56	6.67		13.3				5.83	2.22	1.11
Cys	0	0		0				3.89	5.18	6.30
Tot	5.56	6.67		13.3				9.72	7.40	7.41
										(tube b)
Tro	8.89	7.50		15.0				6.12	3.33	0.74
Cys	0	0		0.28				8.33	5.56	3.33
Tot	8.89	7.50		15.3				14.4	8.89	4.07
Monoxenized with <u>Salmonella typhosa</u> (tube a)										
Tro	15.8	25.0		9.45			2.50			0.55
Cys	0	0		1.67			0			0.56
Tot	15.8	25.0		11.1			2.50			1.11
										(tube b)
Tro	17.8	9.45		6.40			1.39			4.82
Cys	0	0		1.67			0.28			1.67
Tot	17.8	9.45		8.07			1.67			6.49
Monoxenized with <u>Proteus rettgeri</u> (tube a)										
Tro	2.22	4.44		8.62		7.50		3.89	2.22	0.37
Cys	0	0		0		0.28		3.06	3.33	0
Tot	2.22	4.44		8.62		7.78		6.95	5.55	0.37
										(tube b)
Tro	2.78	3.05		5.00		3.89		6.67	5.56	1.48
Cys	0	0.28		0		0.56		2.78	5.18	4.07
Tot	2.78	3.33		5.00		4.45		9.45	10.7	5.55

Table 1. (concl.)

Age in days	0	2	3	4	5	6	7	8	10	12
populations in thousands per ml										
Monoxenized with <u>Proteus mirabilis</u> (tube a)										
Tro	1.66	1.67		1.39		0		0	0	
Cys	0.56	1.39		1.85		0		0	0	
Tot	2.22	3.06		3.24		0		0	0	
(tube b)										
Tro	2.78	2.78		0		0.37		0	0	
Cys	0	2.50		1.39		0.37		0	0.74	
Tot	2.78	5.28		1.39		0.74		0	0.74	
Monoxenized with <u>Clostridium perfringens</u> (tube a)										
Tro	11.6	20.5		2.50			0.83			0
Cys	0	0		0.83			0			0
Tot	11.6	20.5		3.33			0.83			0
(tube b)										
Tro	13.6	17.2		3.05			2.50			0
Cys	0	1.11		1.39			0.28			0
Tot	13.6	18.3		4.44			2.78			0
Monoxenized with <u>Pseudomonas aeruginosa</u> (tube a)										
Tro	3.89	2.78		3.89				6.12	7.03	1.48
Cys	0	0		0.83				2.22	6.67	3.33
Tot	3.89	2.78		4.72				8.34	13.7	4.81
(tube b)										
Tro	6.39	4.44		5.28				5.83	6.30	3.33
Cys	0	0		0				8.61	11.1	6.67
Tot	6.39	4.44		5.28				14.4	17.4	10.0
Monoxenized with <u>Mycobacterium smegmatis</u> (tube a)										
Tro	2.50	1.94		0.83		0		0	0	
Cys	0	0.83		0		0.74		0	0	
Tot	2.50	2.77		0.83		0.74		0	0	
(tube b)										
Tro	3.89	1.94		1.39		0		0	0	
Cys	0	0		0.28		0		0	0	
Tot	3.89	1.94		1.67		0		0	0	

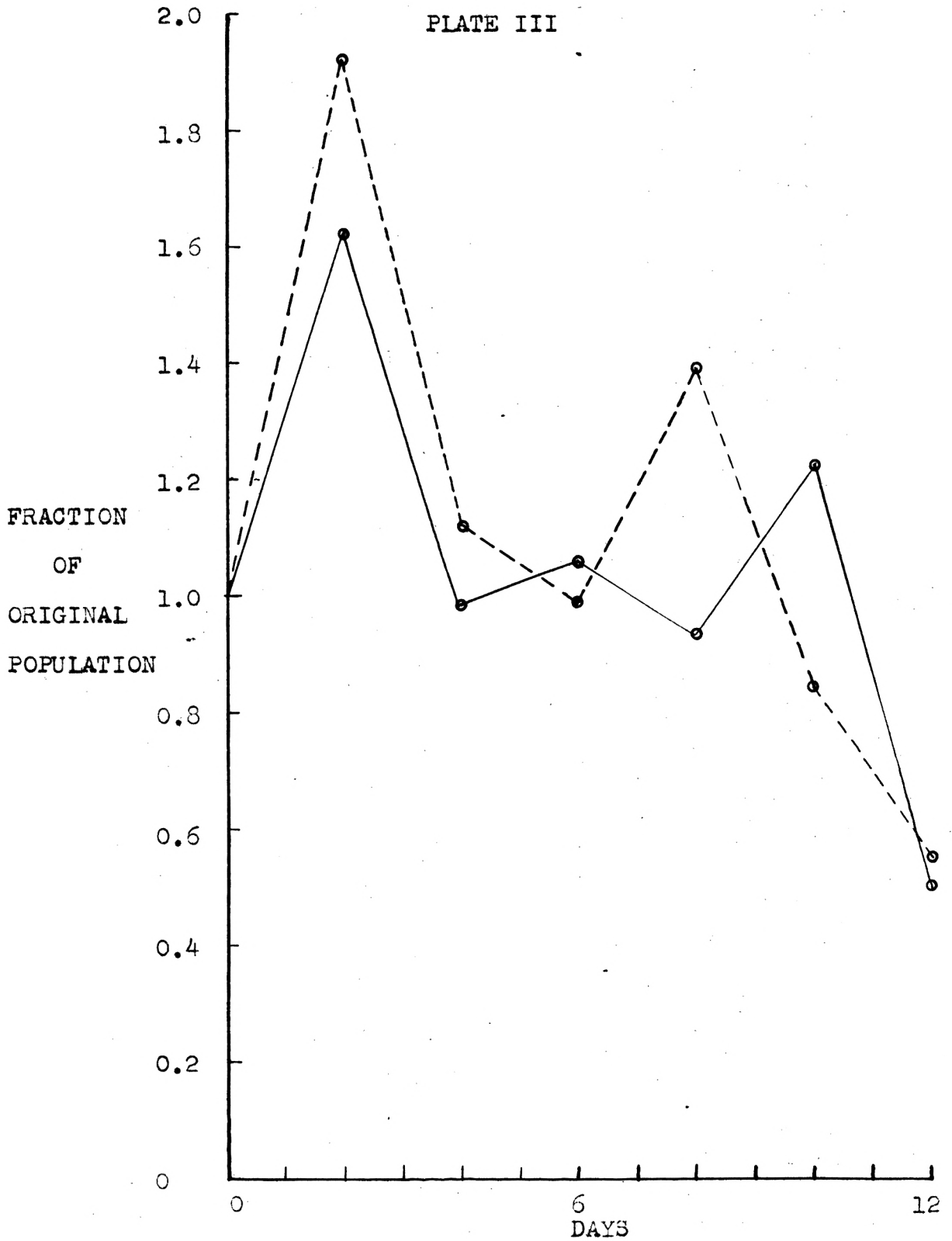
1 Tro stands for trophozoites.

2 Cys stands for cysts.

3 Tot stands for the total population.

#### EXPLANATION OF PLATE III

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus brevis. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.

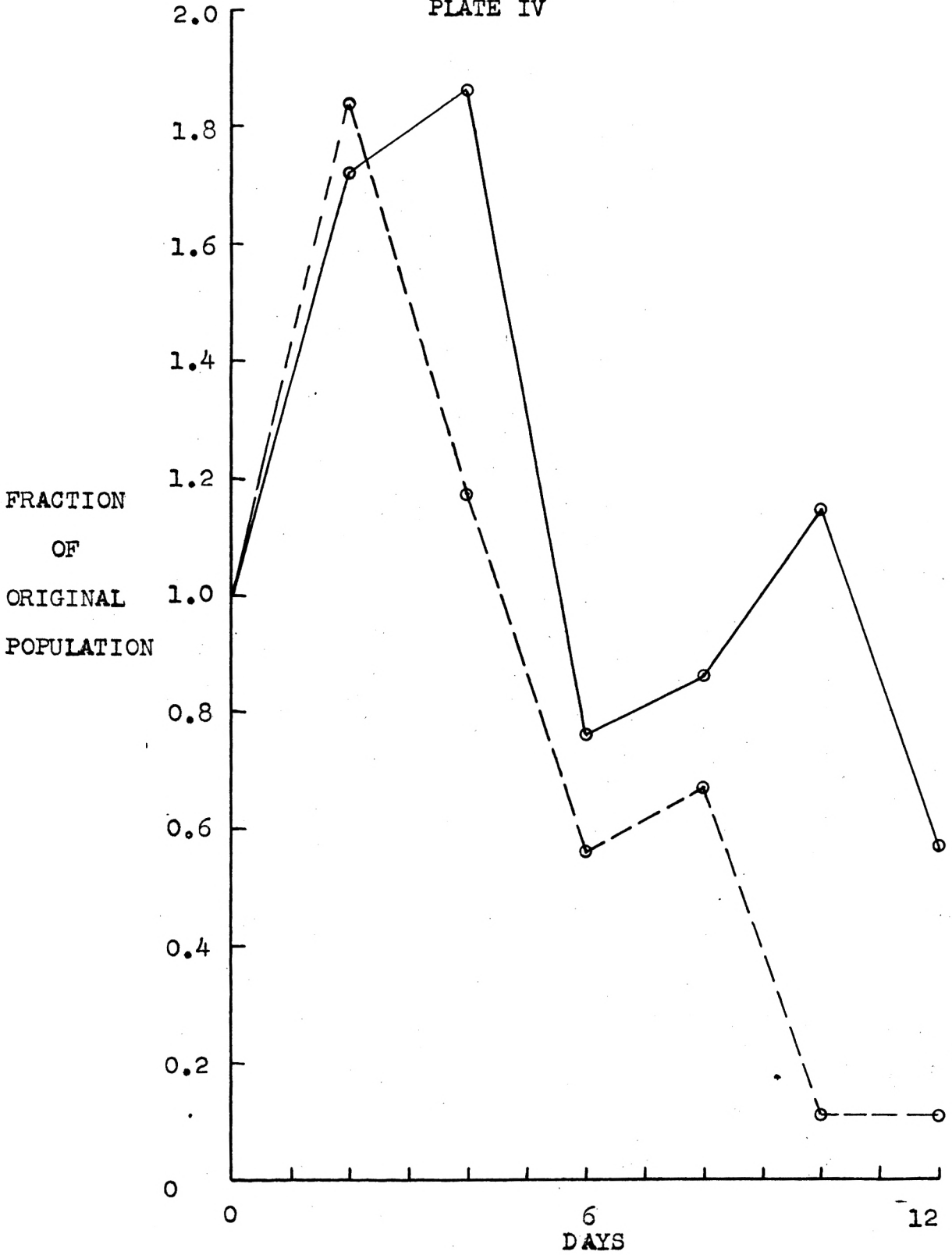




#### EXPLANATION OF PLATE IV

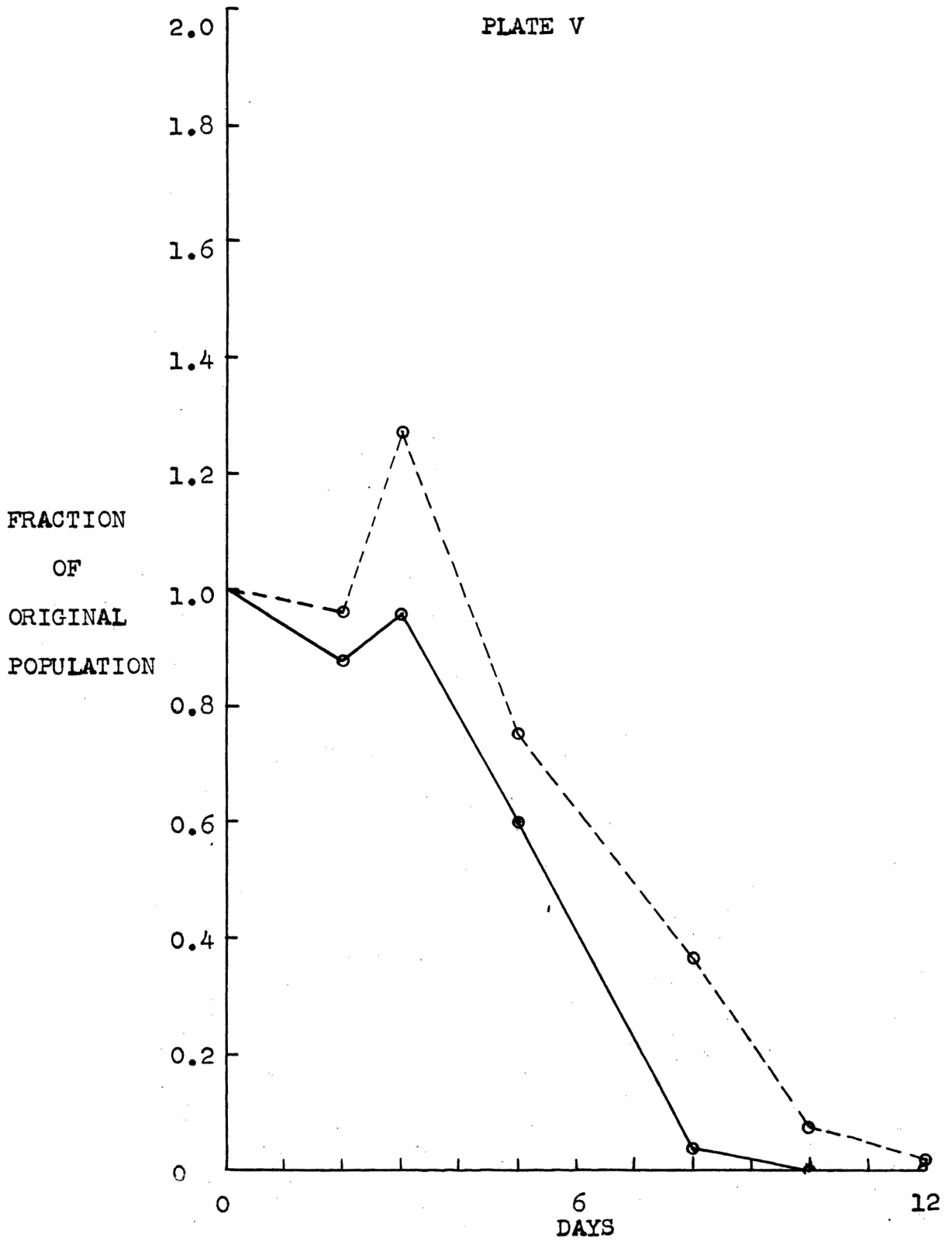
The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus brevis. The dashed line represents culture tube c, and the solid line represents culture tube d in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.

PLATE IV



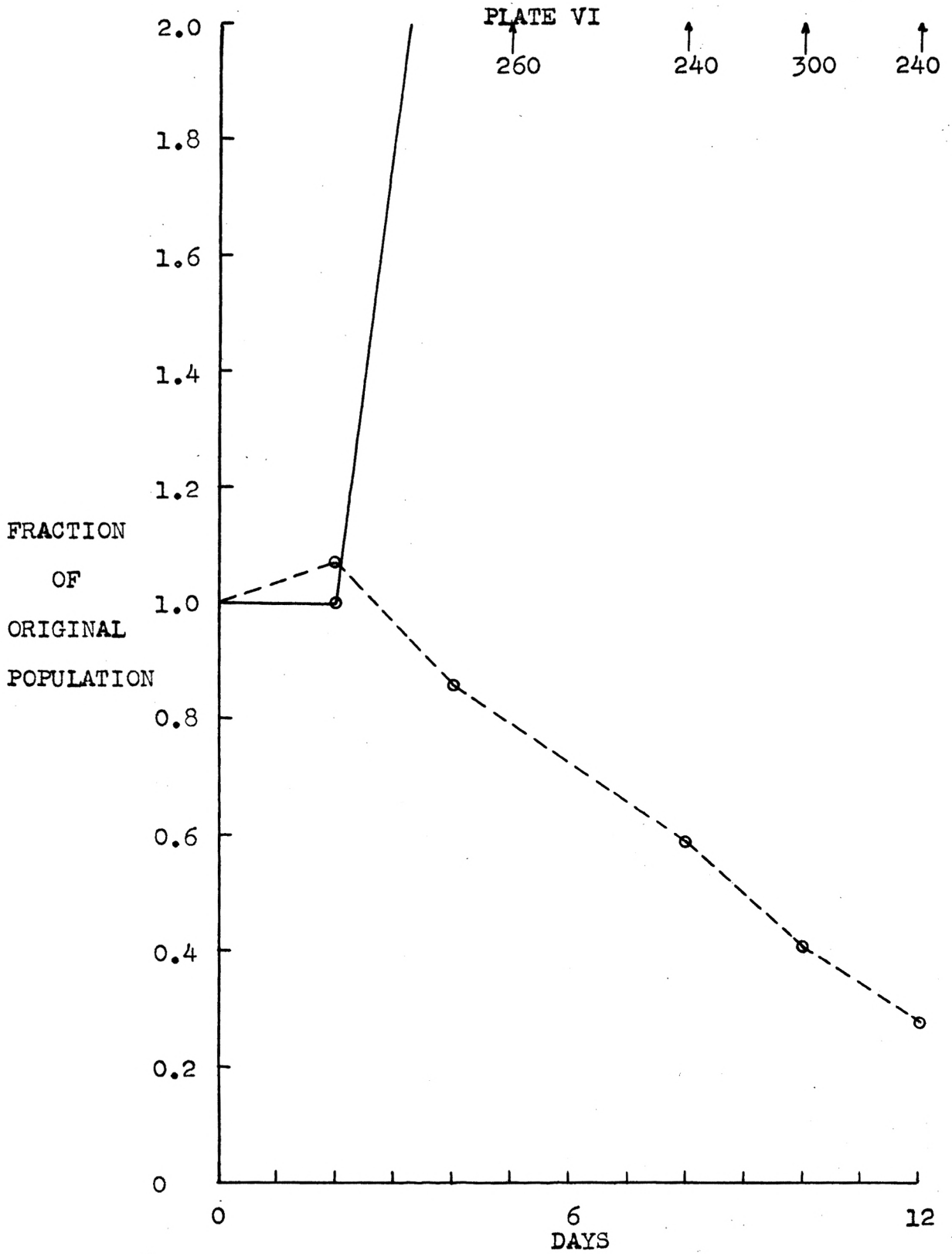
#### EXPLANATION OF PLATE V

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus megaterium. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.



#### EXPLANATION OF PLATE VI

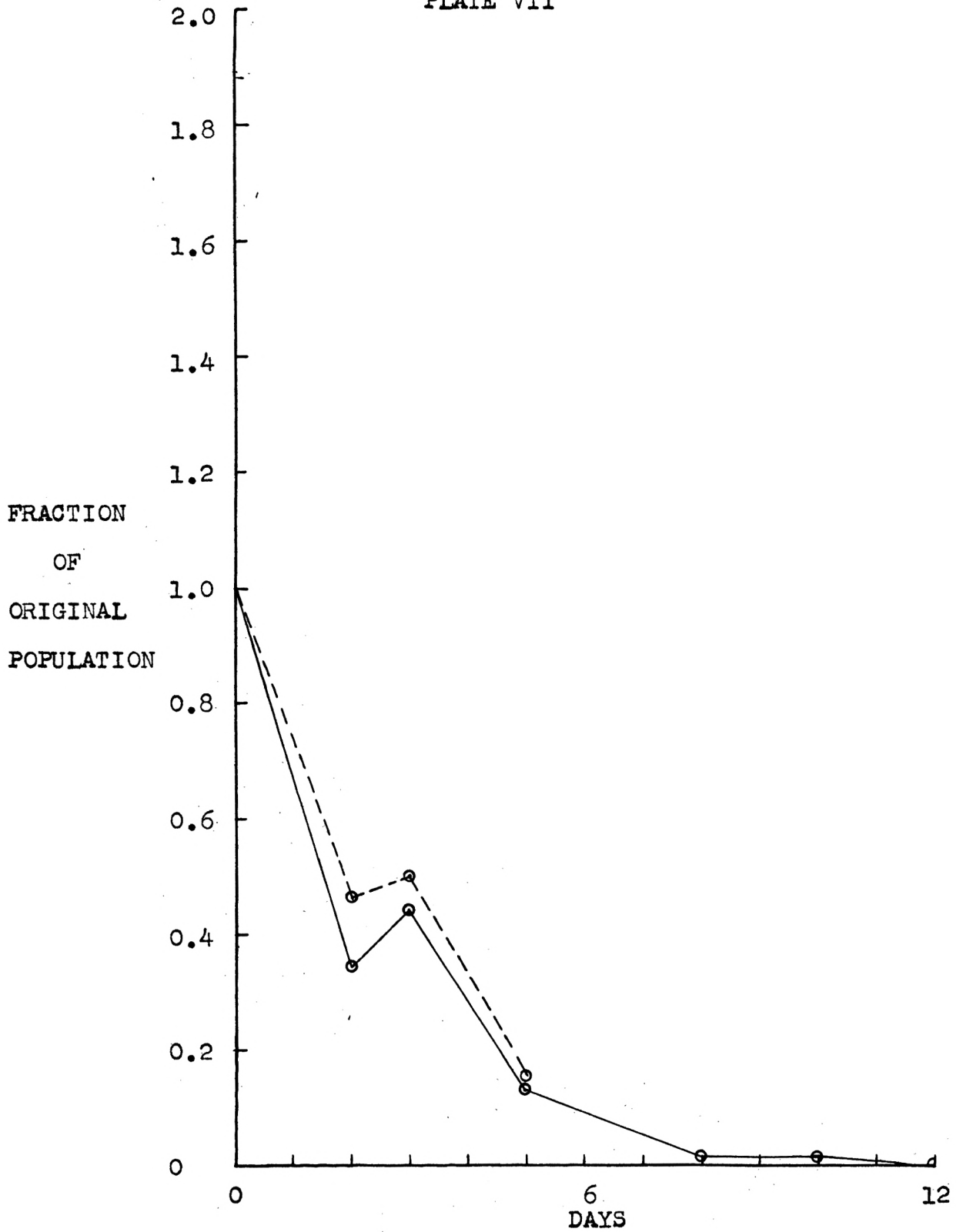
The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus megaterium in Locke's egg medium containing ten percent blood serum. The dashed line represents culture tube c, and the solid line represents culture tube d in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.



#### EXPLANATION OF PLATE VII

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus cereus. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.

PLATE VII

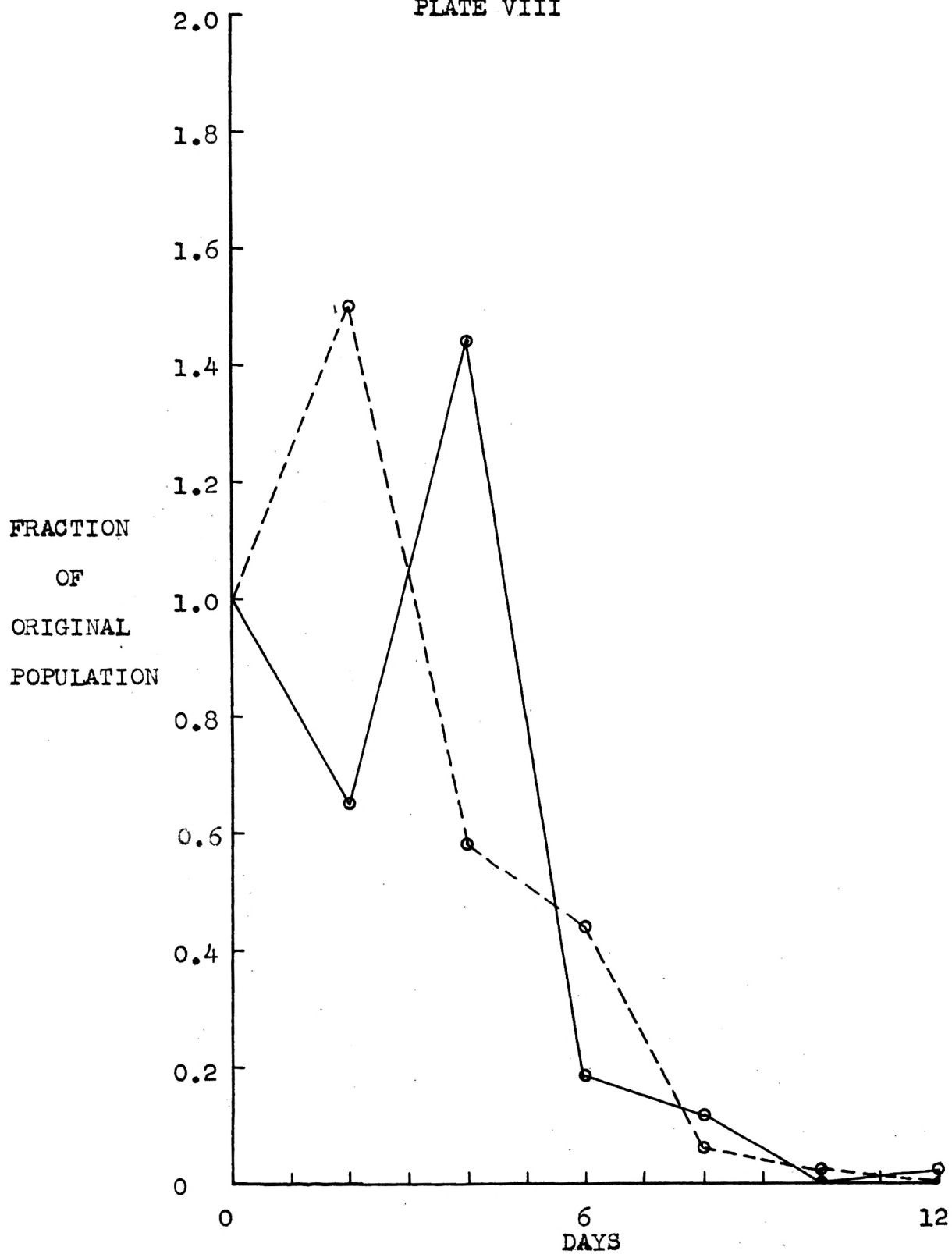




#### EXPLANATION OF PLATE VIII

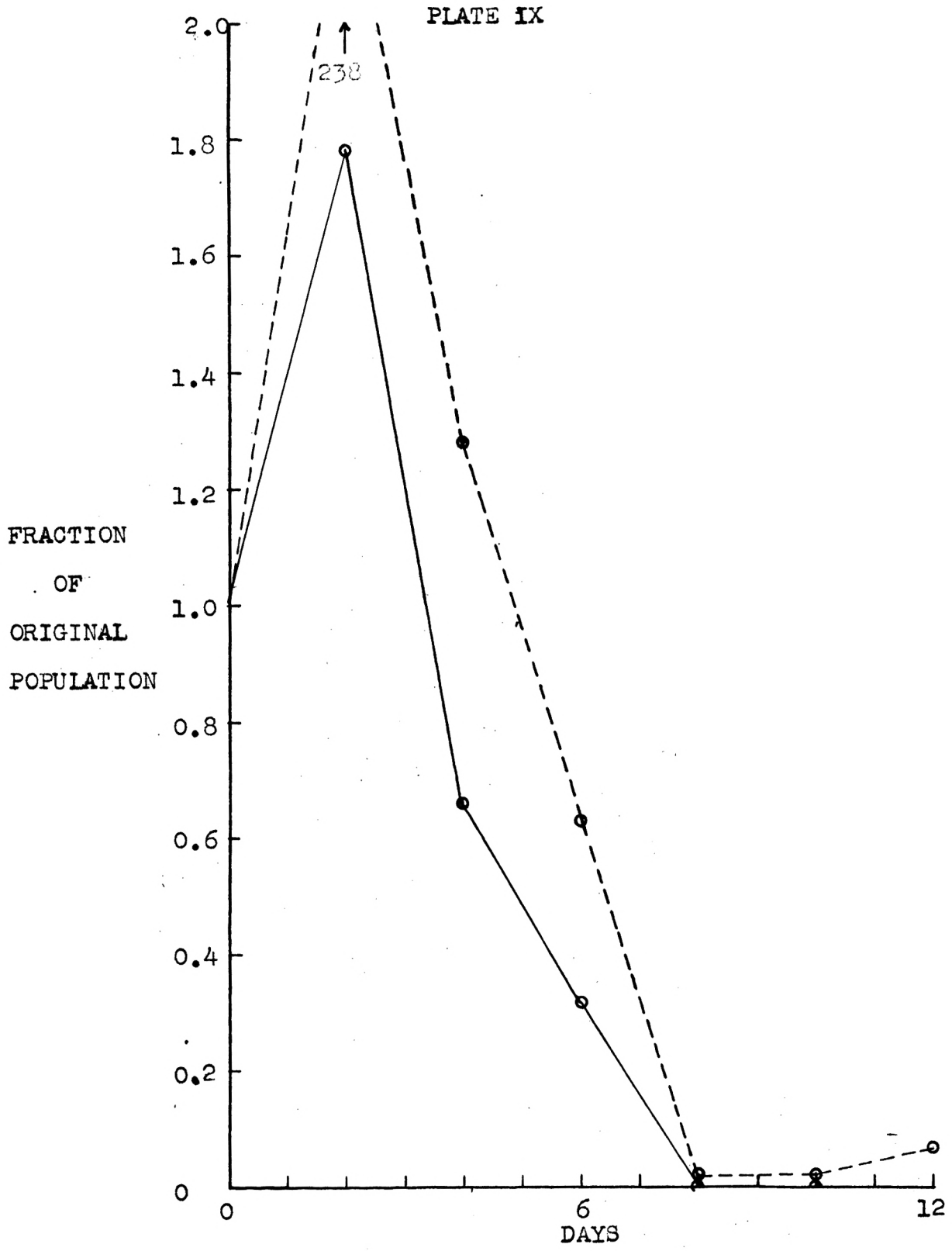
The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus subtilis. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.

PLATE VIII



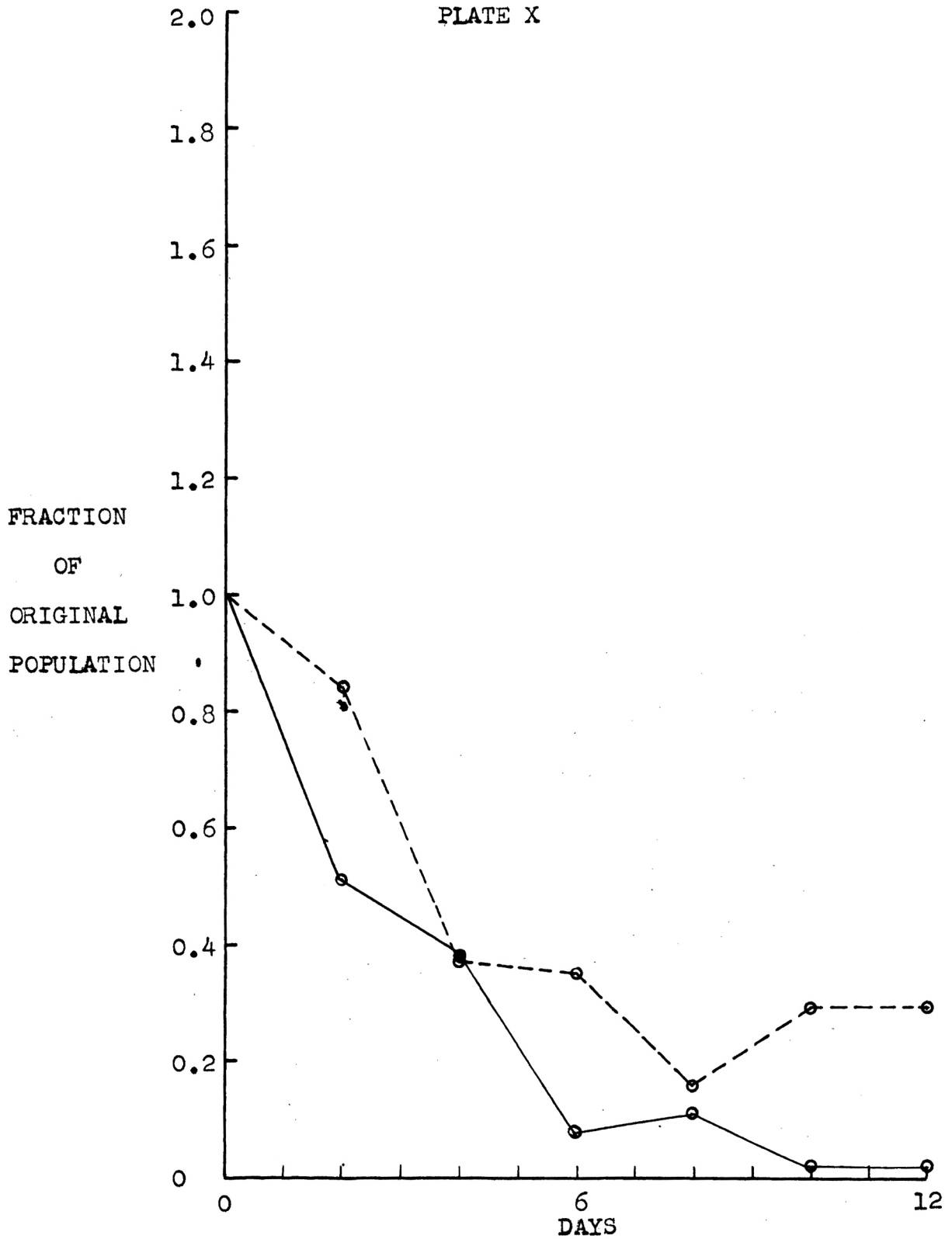
#### EXPLANATION OF PLATE IX

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus subtilis var. niger. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.



#### EXPLANATION OF PLATE X

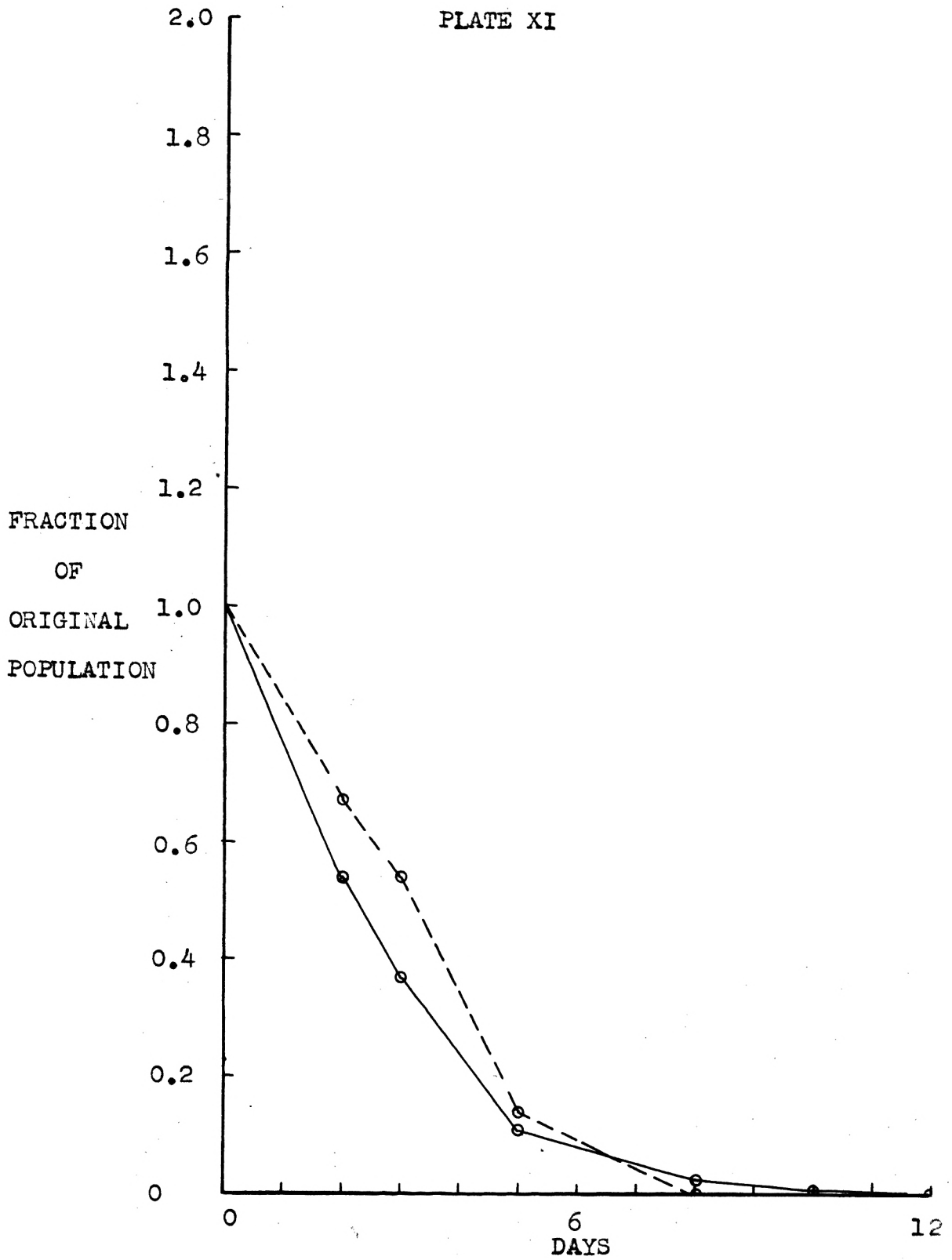
The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Bacillus pumilus. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.



#### EXPLANATION OF PLATE XI

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Escherichia coli "B". The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.

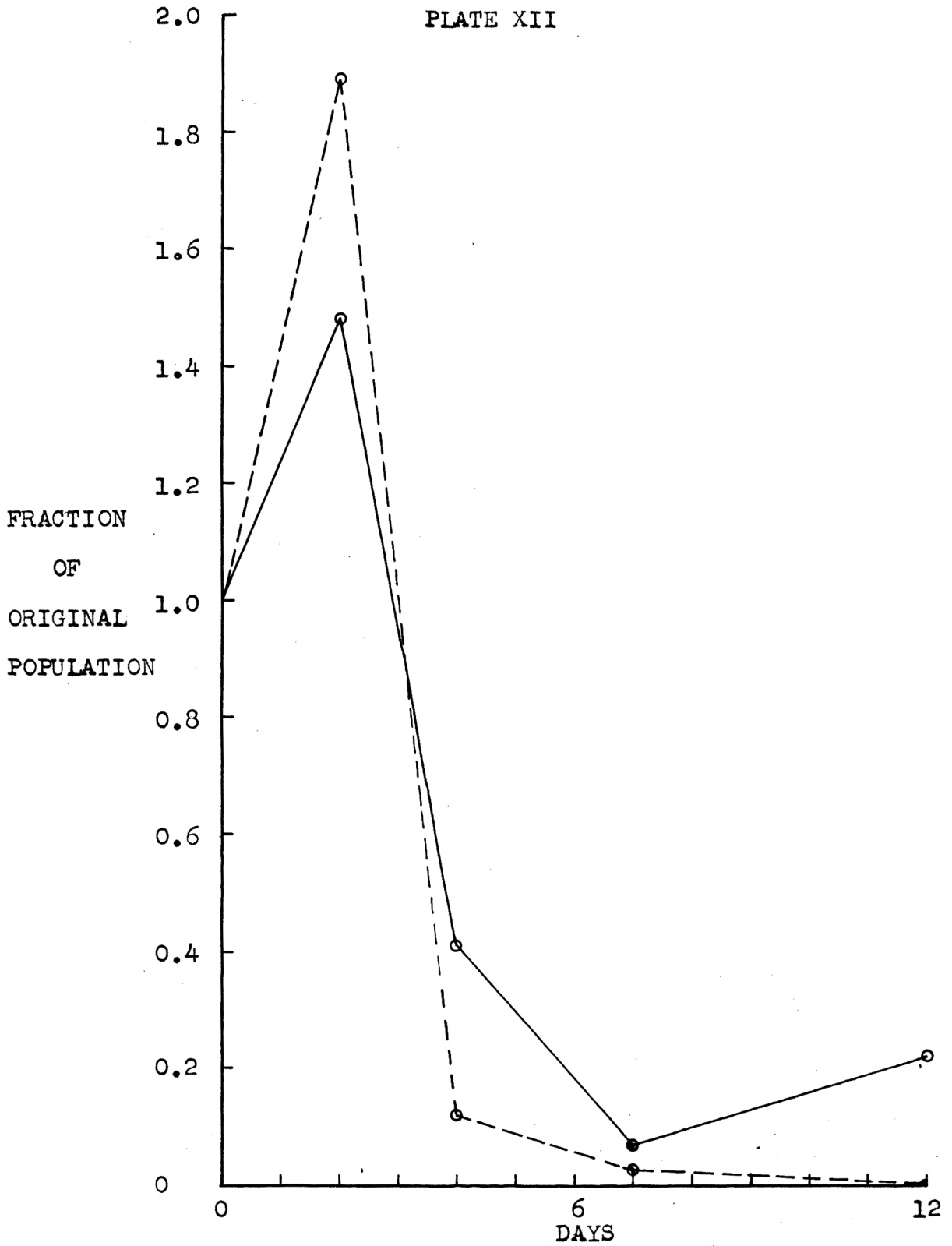
PLATE XI





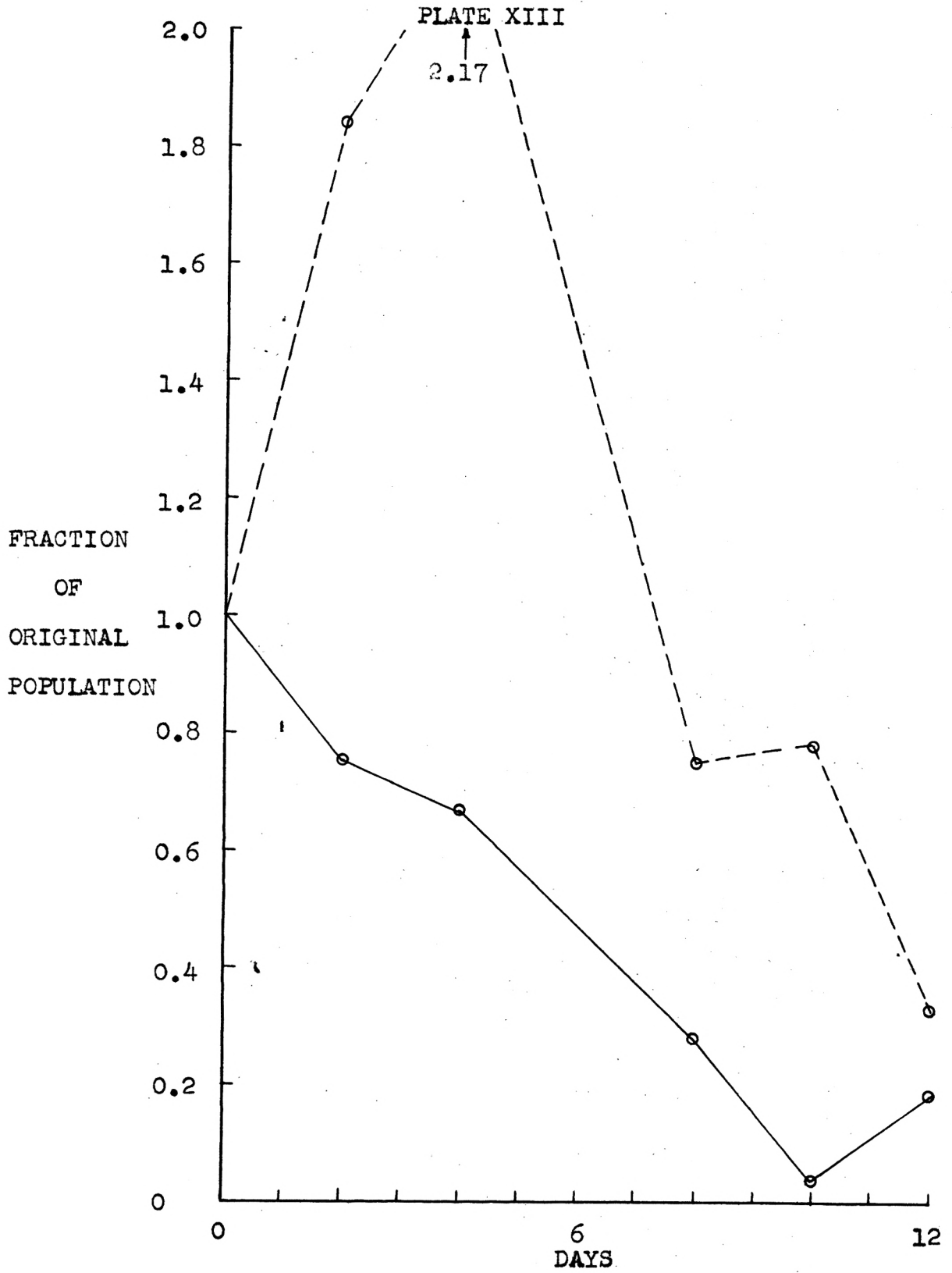
#### EXPLANATION OF PLATE XII

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Escherichia coli. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.



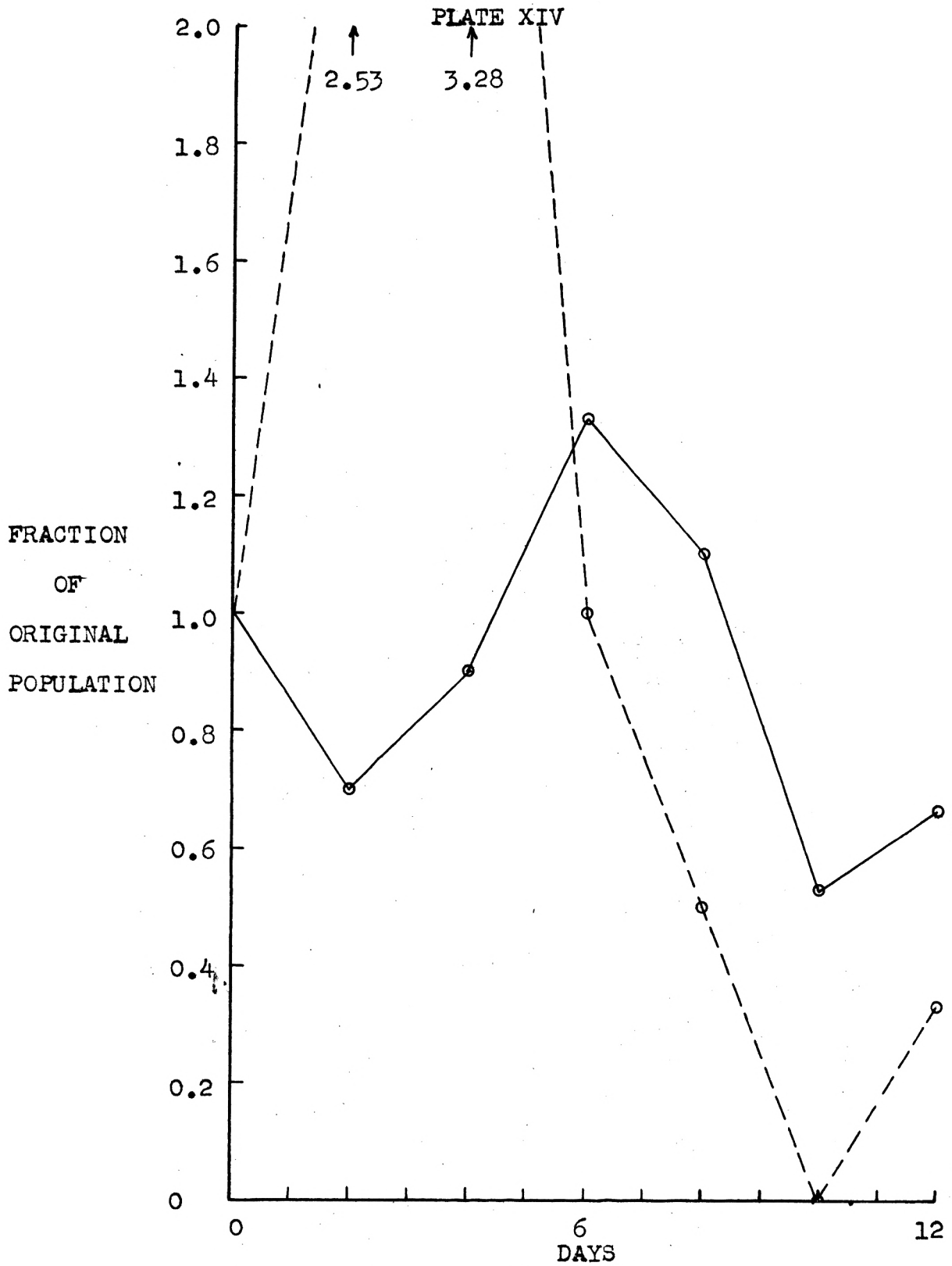
#### EXPLANATION OF PLATE XIII

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Escherichia coli in Locke's egg medium containing 10% blood serum. The dashed line represents culture tube c, and the solid line represents culture tube d in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.



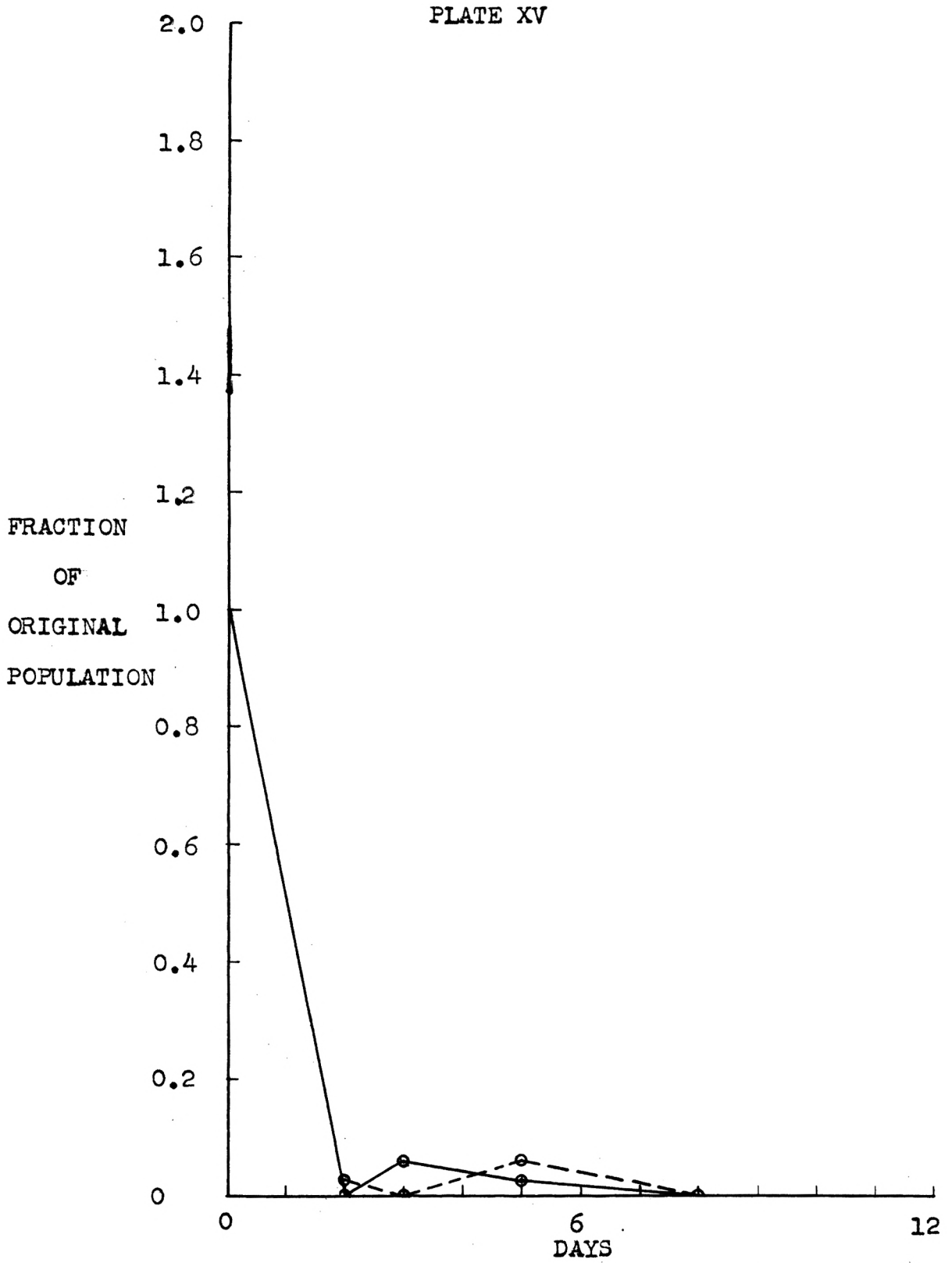
#### EXPLANATION OF PLATE XIV

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Aerobacter aerogenes. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.



#### EXPLANATION OF PLATE XV

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Aerobacter cloacae. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.

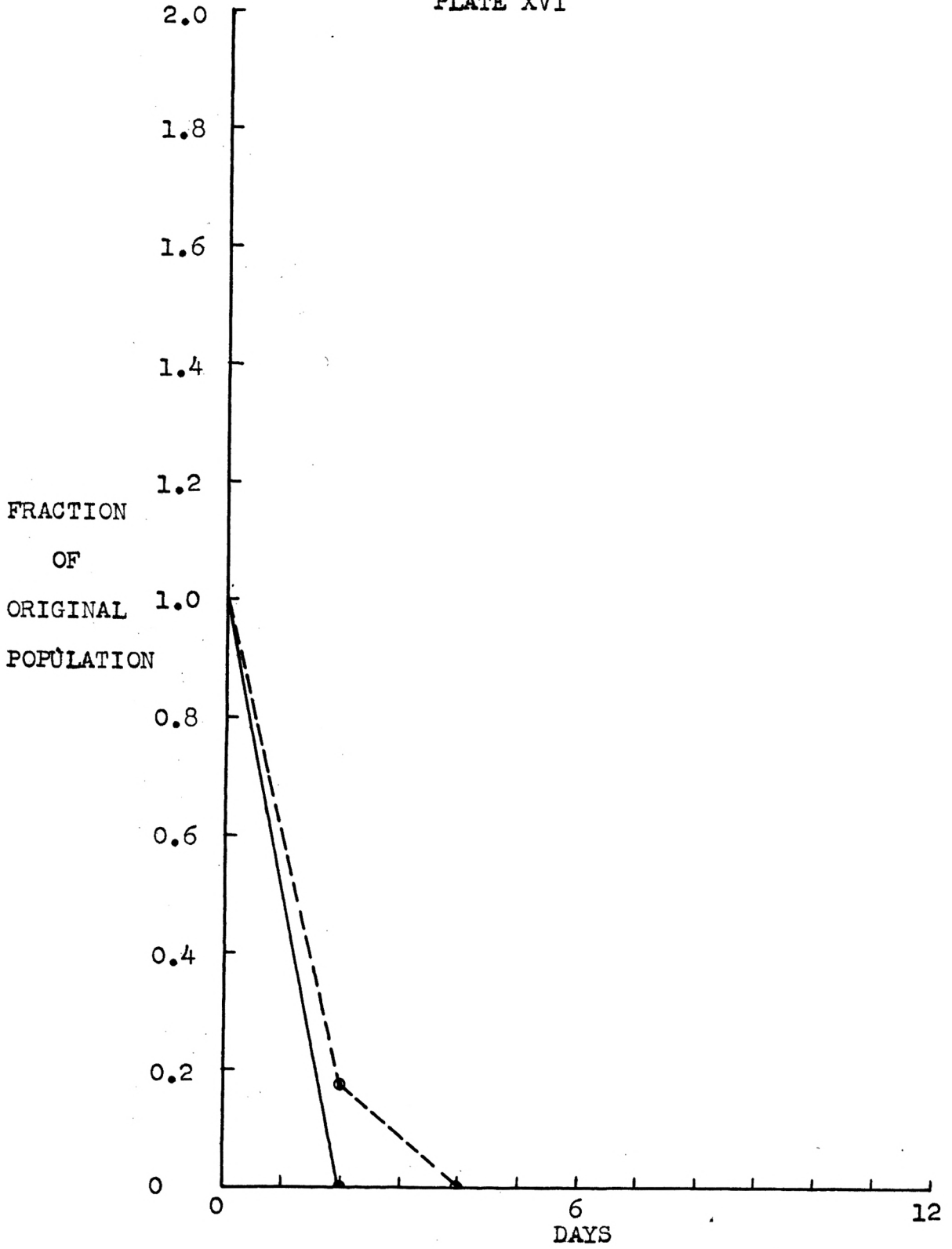




#### EXPLANATION OF PLATE XVI

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Serratia marcescens. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.

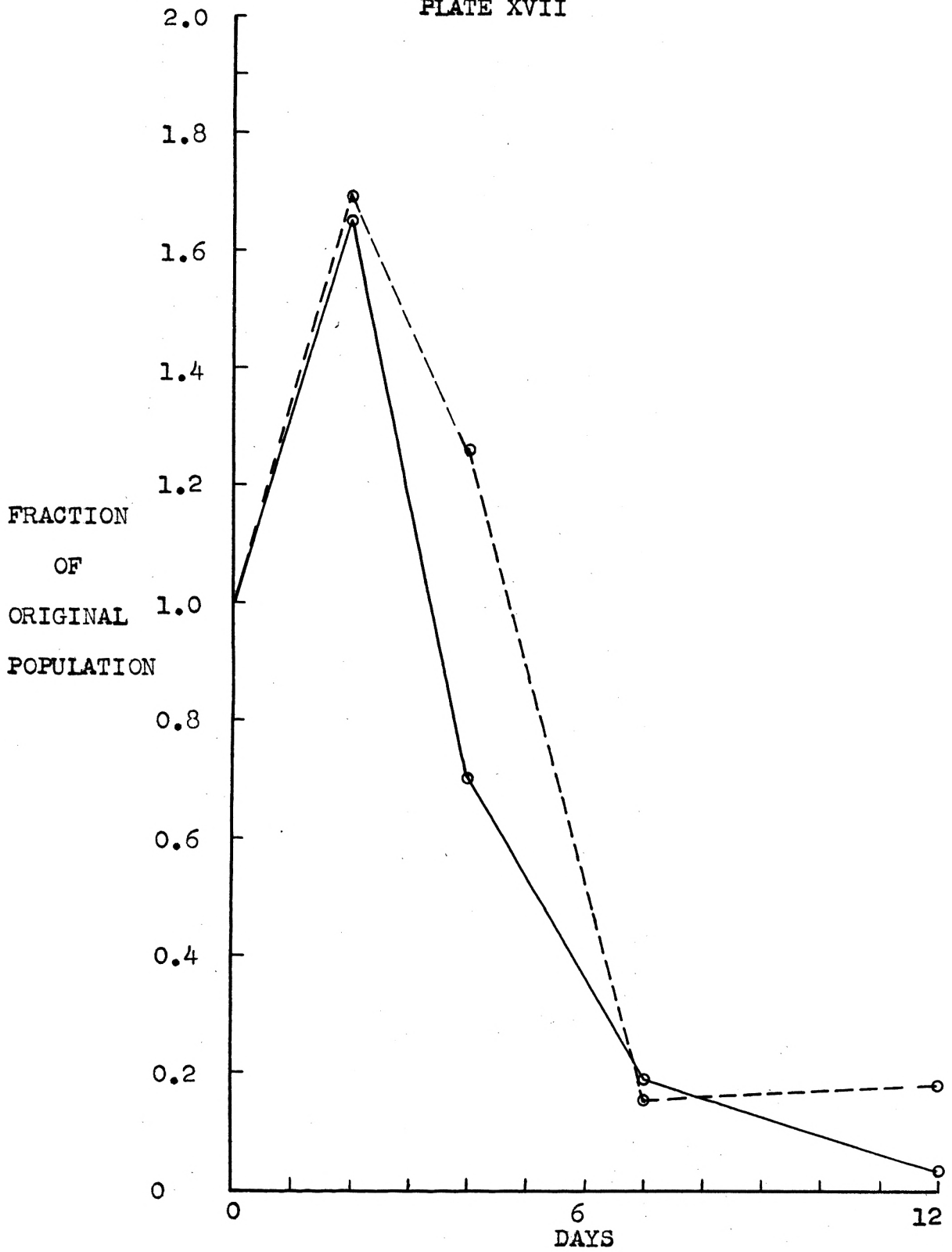
PLATE XVI



#### EXPLANATION OF PLATE XVII

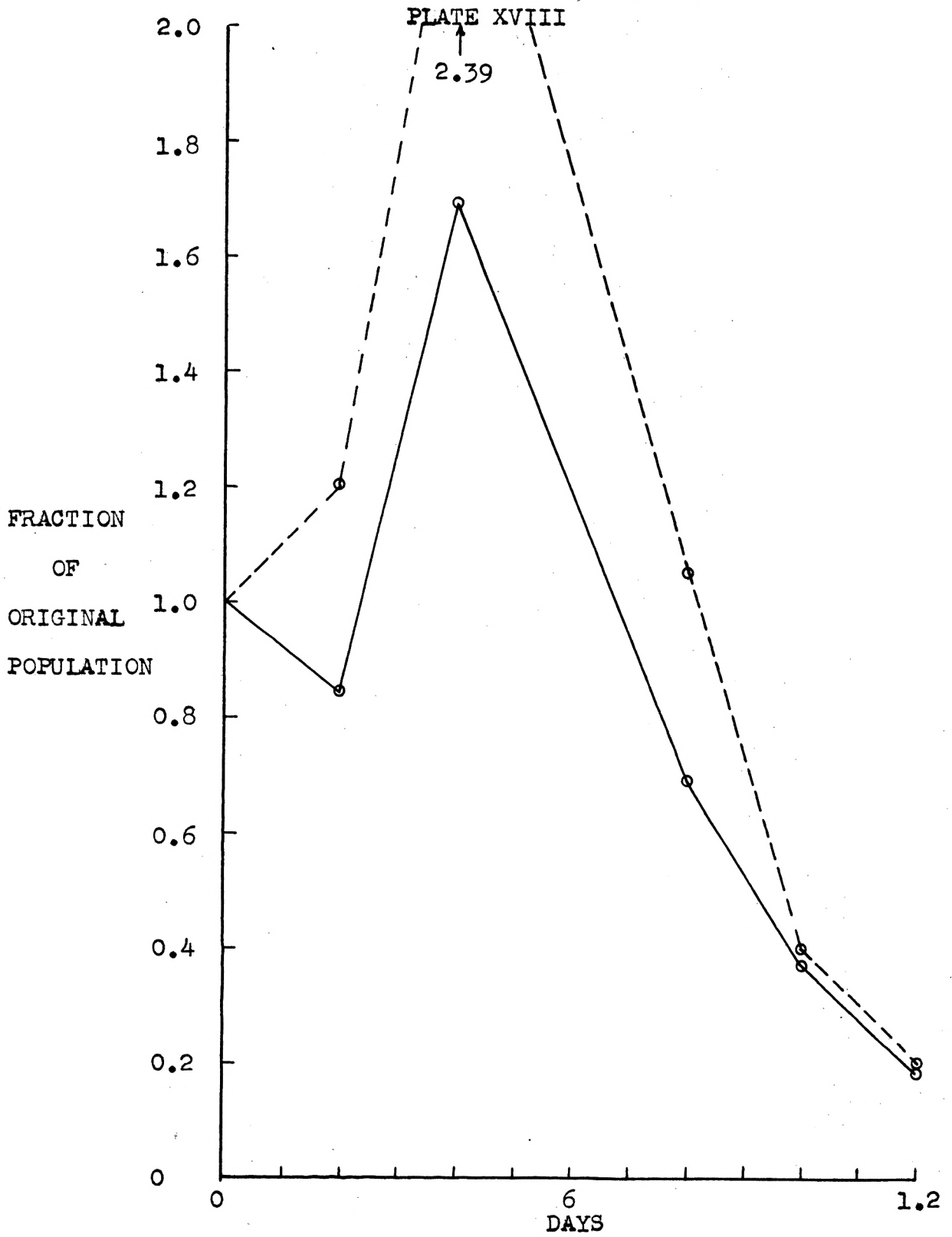
The trophozoite growth curve for Entamoeba  
invadens in monoxenic culture with Staphylococcus  
epidermidis. The dashed line represents culture  
tube a, and the solid line represents culture  
tube b in Table 1. The results are plotted in  
the curve as the fraction of the original number  
of trophozoites in the culture.

PLATE XVII



#### EXPLANATION OF PLATE XVIII

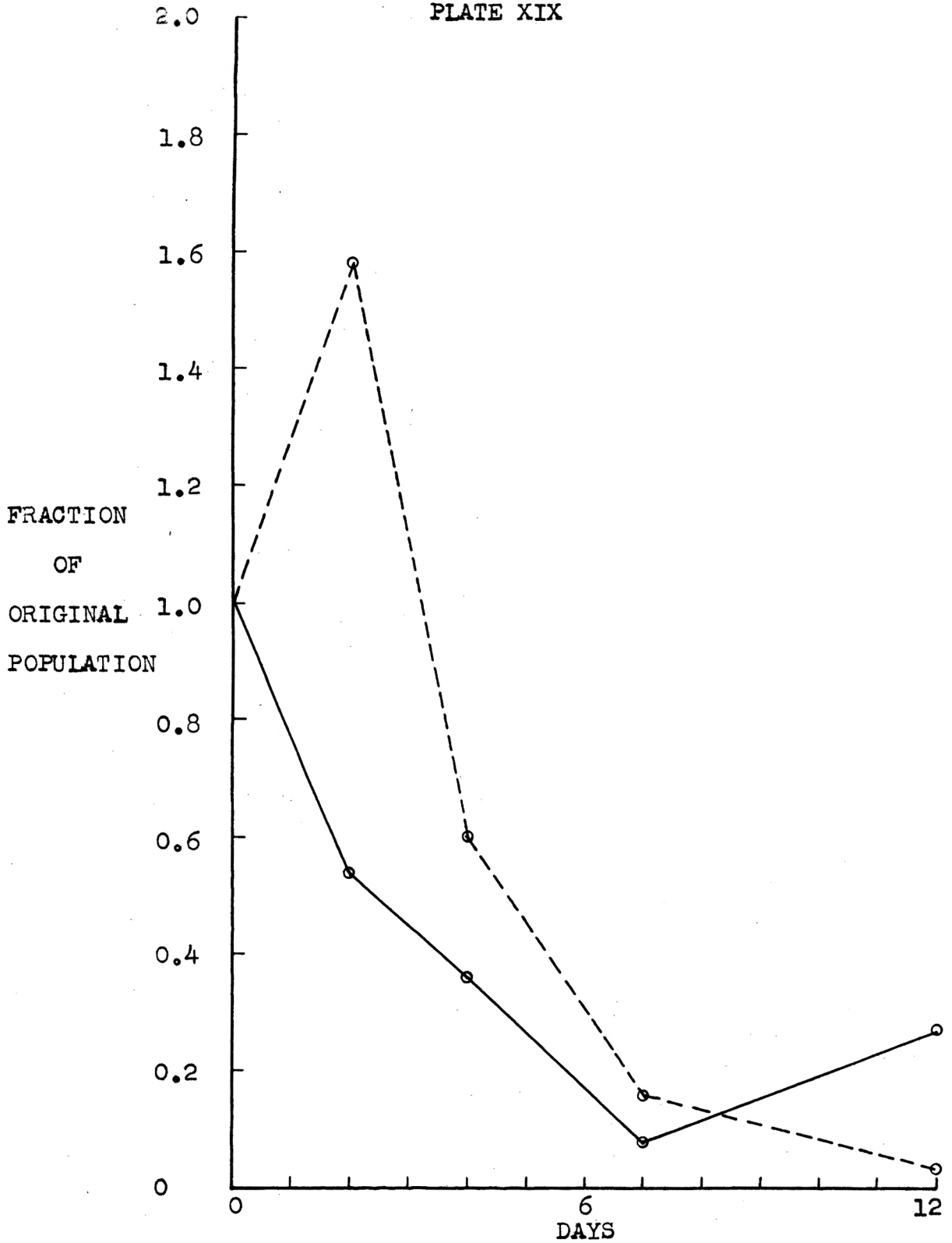
The trophozoite growth curve for Entamoeba  
invadens in monoxenic culture with Micrococcus  
lysodeikticus. The dashed line represents  
culture tube a, and the solid line represents  
culture tube b in Table 1. The results are  
plotted in the curve as the fraction of the  
original number of trophozoites in the culture.



#### EXPLANATION OF PLATE XIX

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Salmonella typhosa. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curve as the fraction of the original number of trophozoites in the culture.

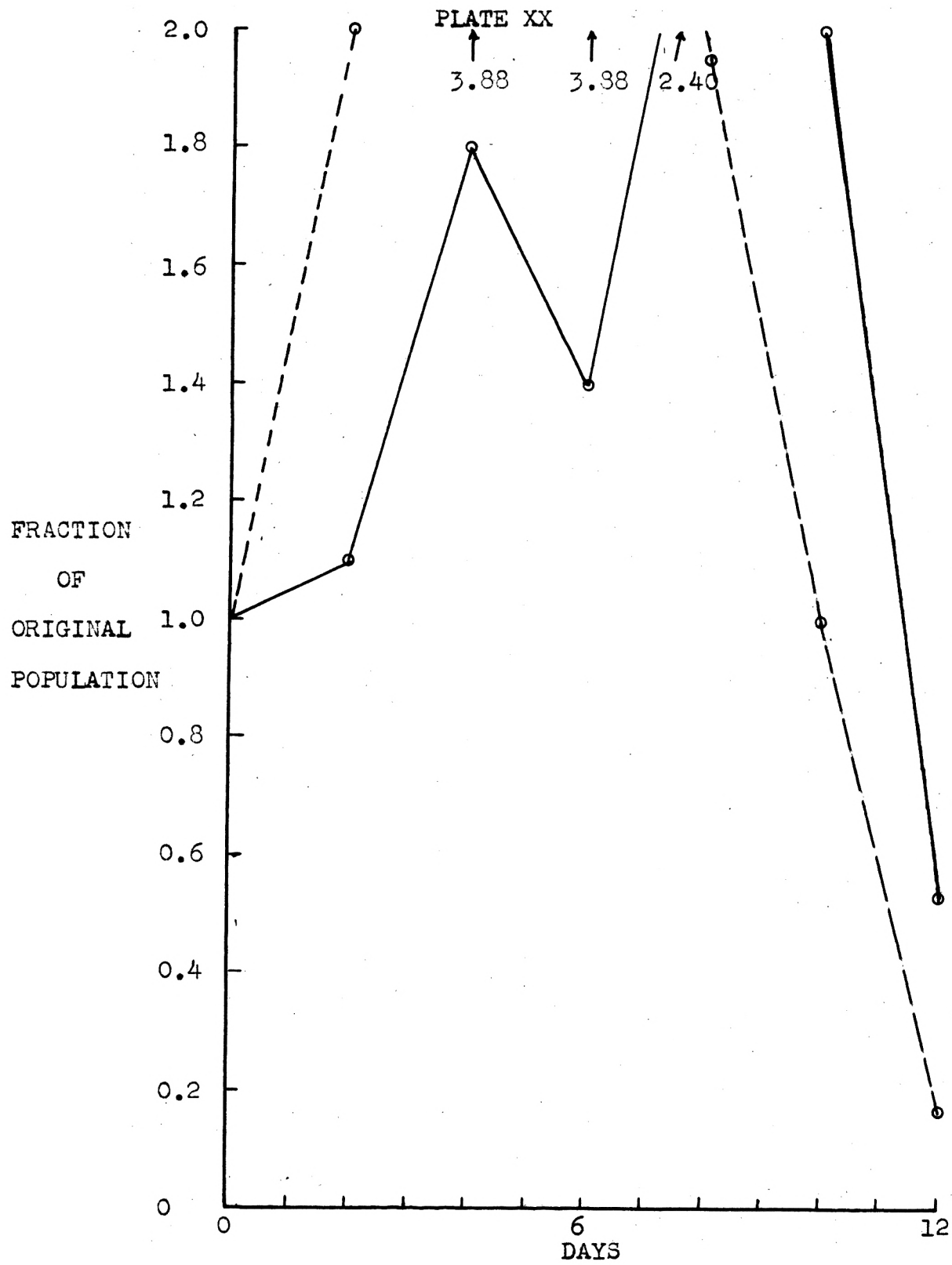
PLATE XIX





#### EXPLANATION OF PLATE XX

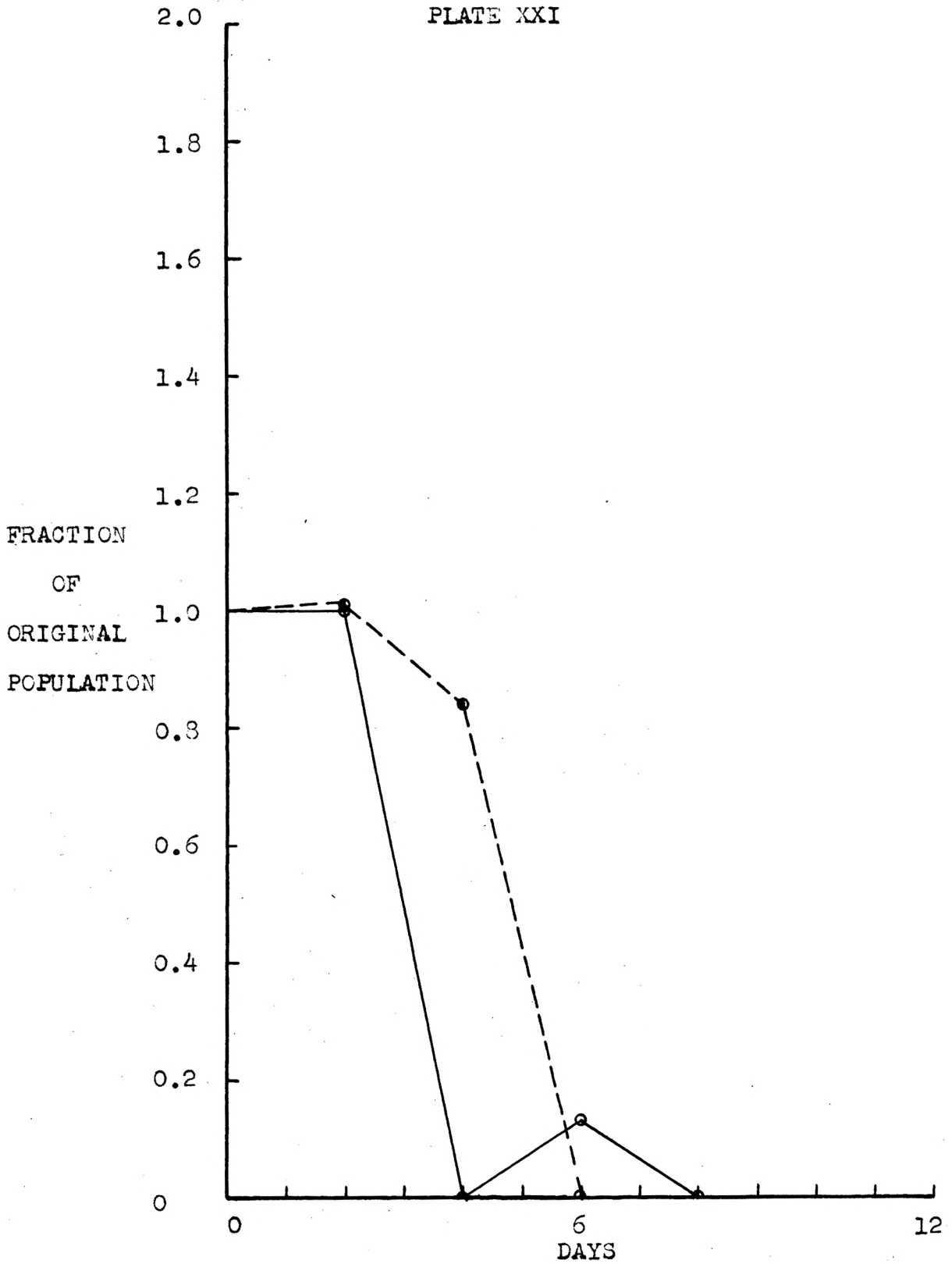
The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Proteus rettgeri. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.



#### EXPLANATION OF PLATE XXI

The trophozoite growth curve for Entamoeba  
invadens in monoxenic culture with Proteus  
mirabilis. The dashed line represents culture  
tube a, and the solid line represents culture  
tube b in Table 1. The results are plotted  
in the curves as the fraction of the original  
number of trophozoites in the culture.

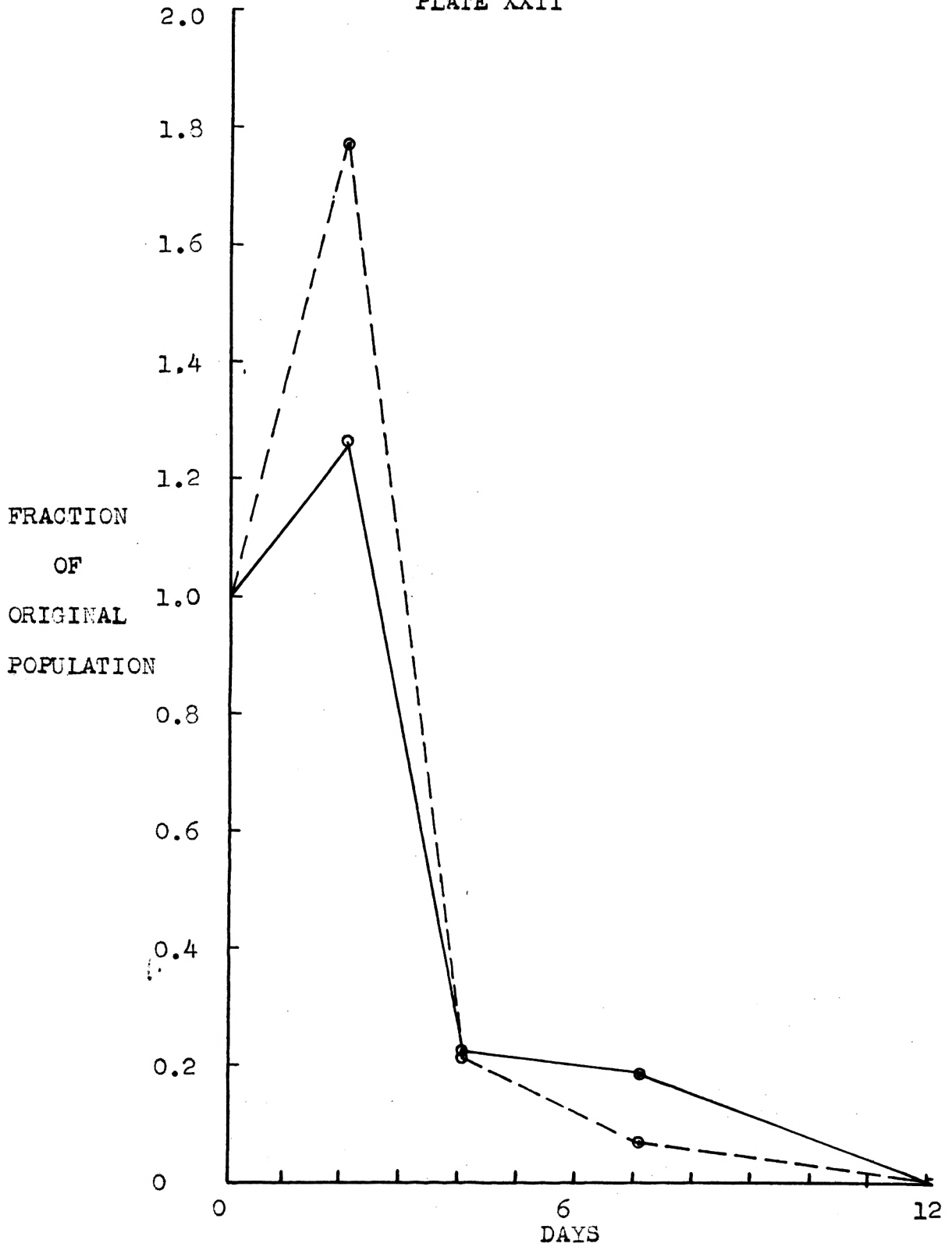
PLATE XXI



#### EXPLANATION OF PLATE XXII

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Clostridium perfringens. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.

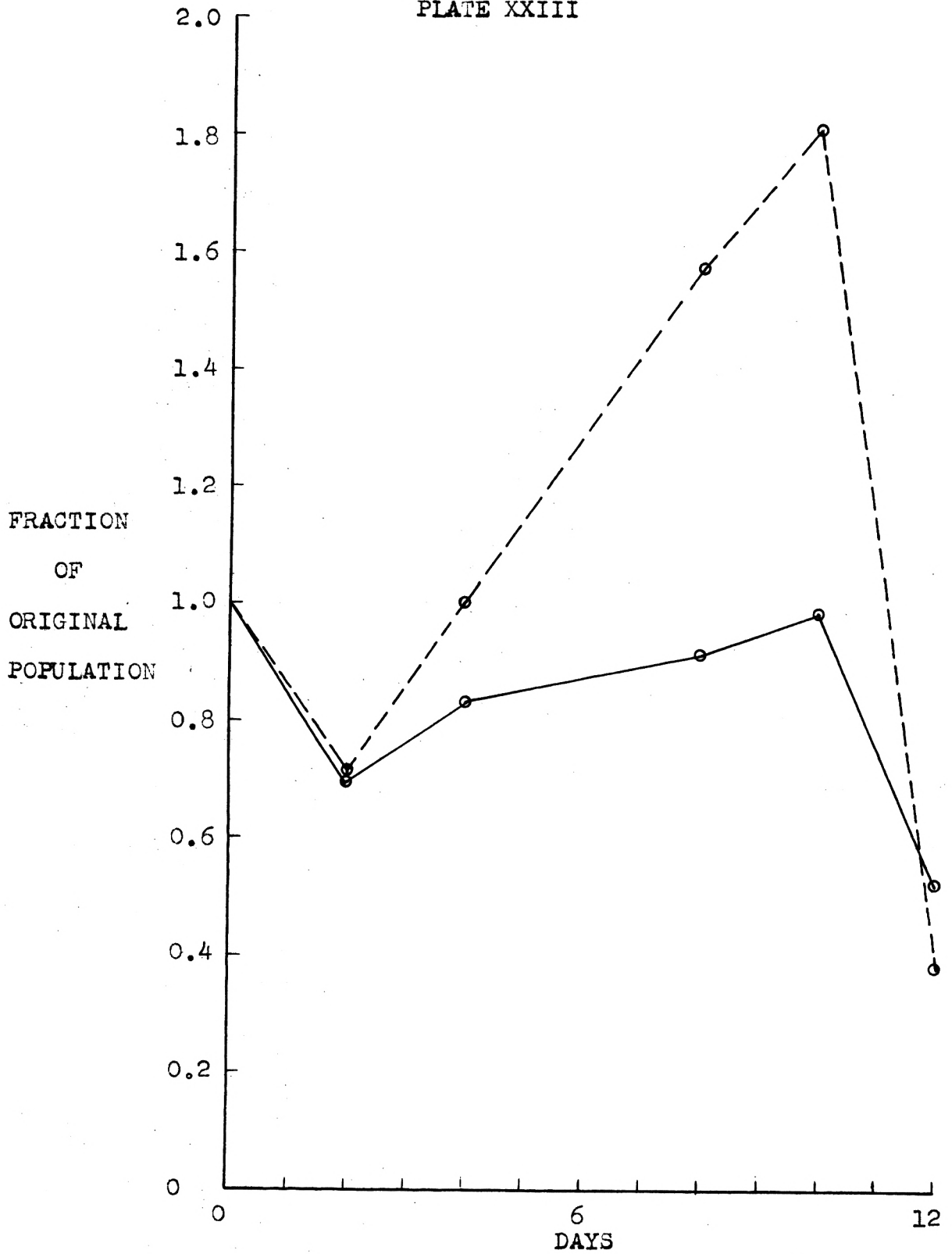
PLATE XXII



#### EXPLANATION OF PLATE XXIII

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Pseudomonas aeruginosa. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.

PLATE XXIII





#### EXPLANATION OF PLATE XXIV

The trophozoite growth curve for Entamoeba invadens in monoxenic culture with Mycobacterium smegmatis. The dashed line represents culture tube a, and the solid line represents culture tube b in Table 1. The results are plotted in the curves as the fraction of the original number of trophozoites in the culture.

PLATE XXIV

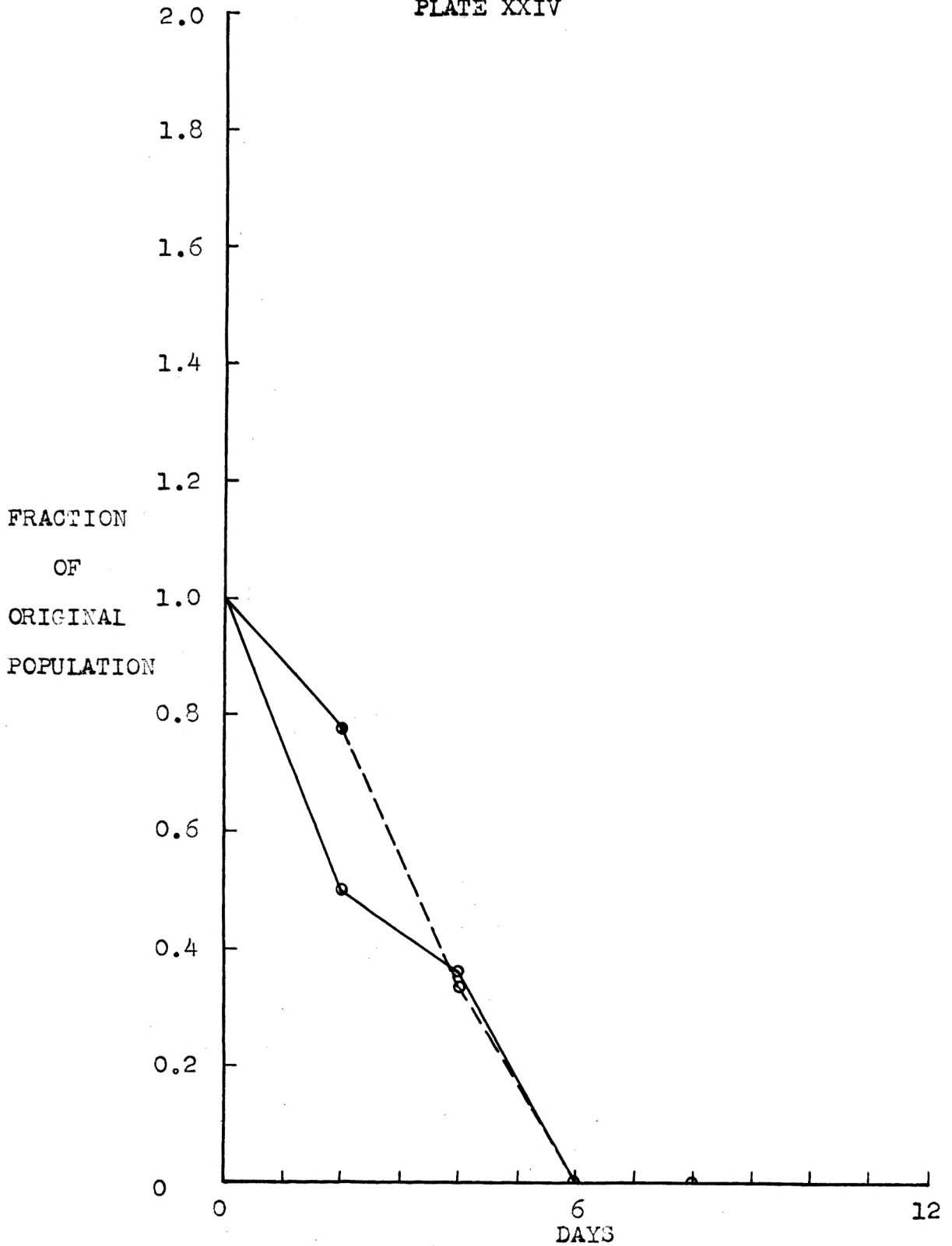


Table 2. The growth of axenic cultures of Entamoeba invadens.<sup>1</sup>

Medium: Diamond's Axenic for <u>Entamoeba invadens</u>							
Age (days)	0	1	2	4	5	6	
Tube a Tro <sup>2</sup>	19.2	30.6	53.1	76.4	75.8	88.7	
Tube b Tro	18.6	31.4	47.5	92.0	81.7	83.3	
Age (days)	7	8	9	10	11	12	
Tube a Tro	124	134	91.4	113	90.0	122	
Tube b Tro	85.0	123	111	119	87.8	145	
Medium: Locke's egg medium for amebae							
Age (days)	0	2	3	5	8	10	12
Tube a Tro	28.9	18.3	24.4	20.0	2.22	1.66	0.56
Tube b Tro	42.2	23.3	26.6	20.0	2.22	0.56	0

1 The populations are reported as thousands per milliliter.

2 Tro stands for trophozoites.

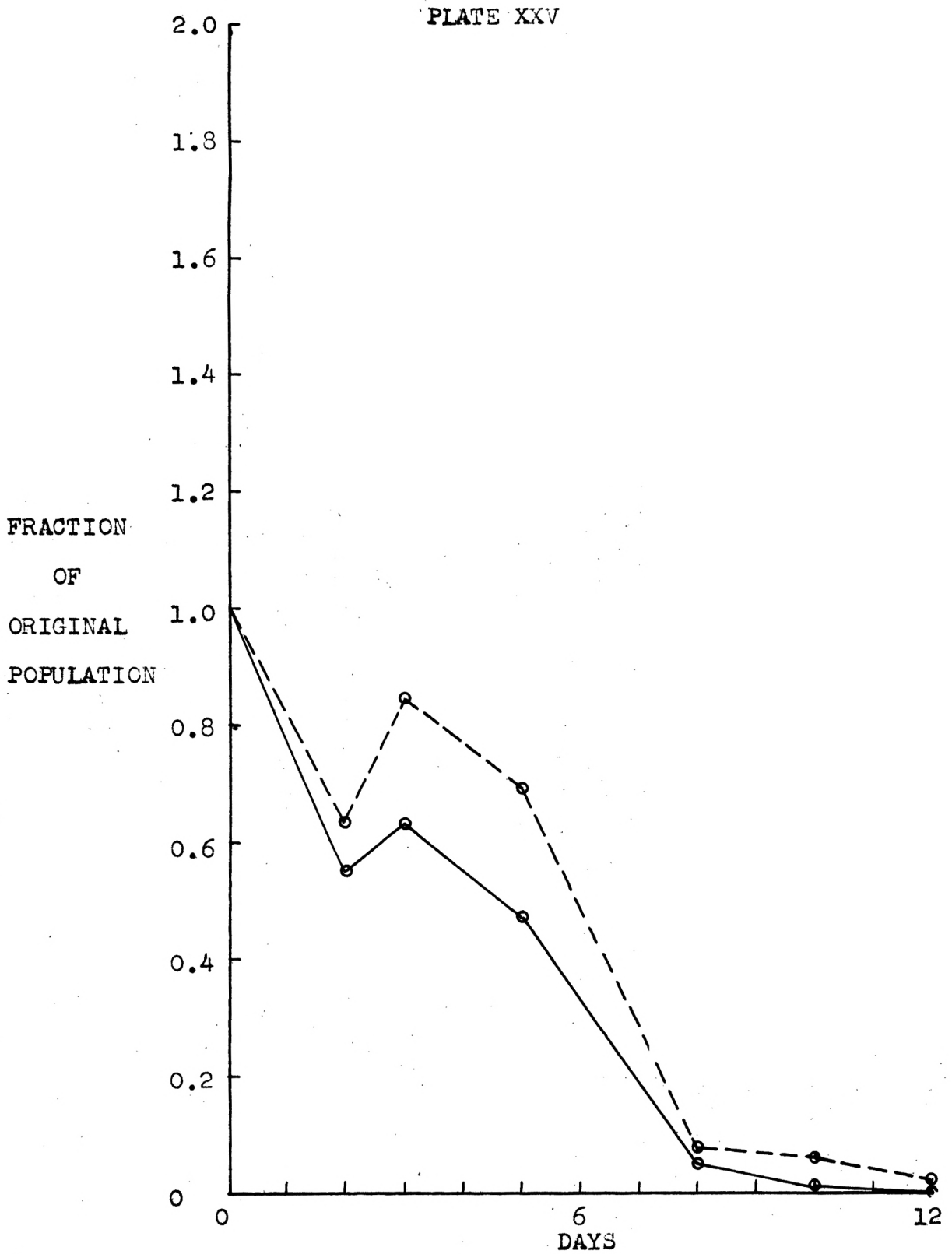
occur in the Locke's egg medium without bacteria, the medium is incapable of supporting the growth and multiplication of E. invadens for more than a few days.

In Diamond's axenic medium for E. invadens the number of trophozoites increased to approximately seven times the original population in a week. There was no evidence of encystation in the medium. It is evident that the ameba passes through a nearly logarithmic growth phase during the first four days, (See Plate XXVII). The population then levels off, and the ameba could be said to be in a roughly stationary phase of the growth cycle. The patterns shown in Plate XXVI and XXVII are apparently the normal growth pattern of the ameba when there is little or no encystation taking place.

Qualitative studies of axenic cultures of E. invadens

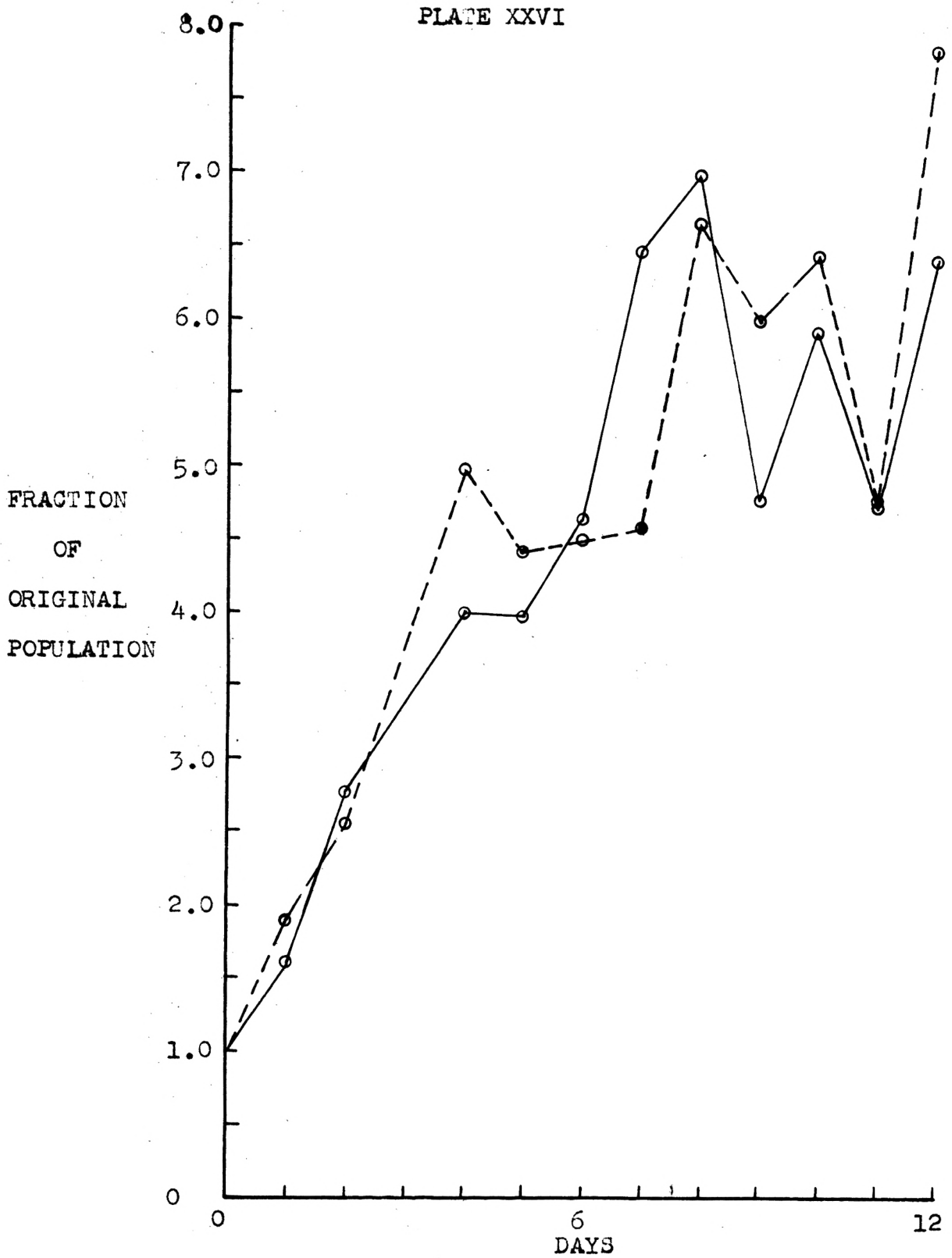
#### EXPLANATION OF PLATE XXV

The trophozoite growth curve for Entamoeba  
invadens in axenic culture in Locke's egg  
medium. The dashed line represents culture  
tube a, and the solid line represents culture  
tube b in Table 2. The results are plotted  
in the curves as the fraction of the original  
number of trophozoites in the culture.



#### EXPLANATION OF PLATE XXVI

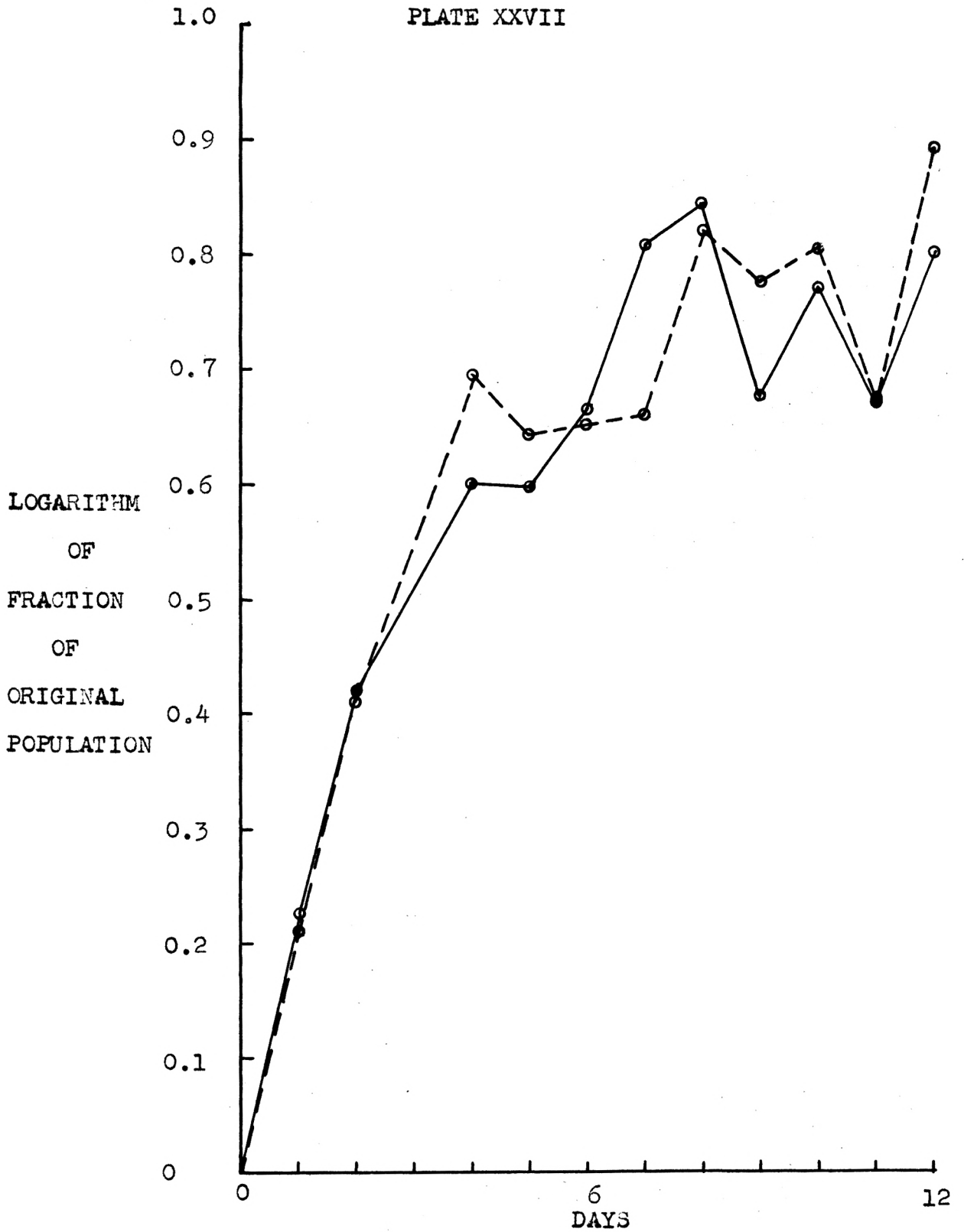
The trophozoite growth curve for Entamoeba  
invadens in axenic culture in Diamond's  
axenic medium. The dashed line represents  
culture tube a, and the solid line represents  
culture tube b in Table 2. The results are  
plotted in the curves as the fraction of the  
original number of trophozoites in the culture.



#### EXPLANATION OF PLATE XXVII

The trophozoite growth curve for Entamoeba  
invadens in axenic culture in Diamond's  
axenic medium. The curves are plotted as  
the logarithm of the fraction of the original  
population. The dashed line represents cul-  
ture tube a, and the solid line represents  
culture tube b in Table 2.





indicated the following: the amebae cannot survive for even a short time in nutrient broth or heart infusion broth, the ameba can survive in small numbers in thioglycollate broth, and that addition of serum to thioglycollate broth increases the survival of the amebae only slightly. The number of active trophozoites in both cultures of thioglycollate broth and serum (10%) decreased to 15% of the initial value in 24 hr.

From an examination of Table 2, it may be observed that relatively few of the bacteria tested appeared to support the growth of E. invadens. Two of the 18 species of bacteria had a definitely inhibitory effect on the multiplication and survival of the ameba. Six other species of bacteria apparently did not noticeably either increase or decrease the multiplication or survival of the ameba in the Locke's egg medium as compared to similar cultures without bacteria.

Examination of the properties of these bacteria as listed by Bergey's Manual of Determinative Bacteriology (Breed, et al., 1957) fails to reveal significant similarities between them. Several species are Gram-positive, and several others are Gram-negative. All but one, C. perfringens, are aerobic or facultatively anaerobic. Their optimal temperatures are in the range of 28-40 C with approximately one-half of these preferring a temperature close to 37 C. The bacteria are approximately evenly divided in motility and in the ability to ferment lactose and the ability to hydrolyze casein. All of the species are capable of utilizing glucose, and most are able to liquefy gelatin. A few of the bacteria are capable of hydrolyzing starch.

As a result of selection, all of the bacteria concerned are inhabitants of the soil, feces or sewage. With the exception then of E. coli and A. aerogenes, all of the ten species are proteolytic. Unfortunately the similarities of the bacteria supporting the growth and multiplication of E. invadens are also possessed by the bacteria which did not stimulate the growth of the ameba.

The results of this study certainly do not seem to support the concept that the primary purpose served by the bacteria is to provide suitable anaerobic conditions for the amebae. If this were the only function served by the bacteria, then every bacterial species with the possible exception of M. smegmatis should have supported the growth of the ameba. Another concept is that the bacteria attack the substrate in the test tube and provide metabolic products similar to those in the intestinal tract. This concept seems to be supported in part by the results obtained in this experiment, since most of the species studied were proteolytic. However, it must again be pointed out that many of those which did not support growth were also proteolytic. It is unlikely, however, that it is merely the ability to hydrolyze proteins that is involved, but rather it is the ability to form or release specific metabolic products needed by the ameba. It is most probably that both of the above concepts may be involved in the support of the growth of E. invadens.

Several of the bacteria which were found to support the growth and multiplication of E. invadens could be used to maintain the ameba in monobacterial cultures. B. brevis was used

for the cultural maintenance of the ameba for several weeks with weekly subcultures. Most of the monobacterial cultures of E. invadens reached a peak population between the second and fourth day, indicating that ideally for maximum population maintenance, culture transfers should be made on the third or fourth day after inoculation. The fact that the ameba was successfully maintained for several weeks with weekly subcultures, indicates that B. brevis is an especially good species to use for this purpose.

The initial experiments performed by the author indicated that E. coli "B" did not support the growth of E. invadens in monobacterial cultures. B. megaterium as a monobacterial associate of the ameba apparently did not inhibit the multiplication of the ameba, but it also did not noticeably increase it. Both B. megaterium and E. coli have been reported as supporting the growth of E. invadens as noted previously. Considering the differences in media used by other authors and the Locke's egg medium used for these experiments, these differences are not considered unusual. However, for comparison a different strain of E. coli was obtained and additional studies performed. The new strain of E. coli was used in the standard Locke's egg medium plus rice starch, and both E. coli and B. megaterium were inoculated into Locke's egg medium plus serum (instead of the rice starch). This strain of E. coli was found to definitely support the growth of E. invadens both with and without the presence of blood serum. The role of B. megaterium, on the other hand, still remains in doubt, as one of the Bacillus cultures apparently supported multiplication greatly, whereas the other culture

did not.

Several difficulties, which may have caused variations in the results, were encountered as the above studies were carried out. The first difficulty in the initial studies was encountered in adding the same amount of rice starch to each culture tube. Balamuth in 1962 reported that this factor caused his results to vary. He was able to eliminate this difficulty by adding the rice starch to the cultures from carefully stirred sterile suspensions. This technique was found by the author to involve an excessive use of large bore sterile pipets and was discontinued for this reason. The rice starch dispenser described in MATERIALS and METHODS was adopted as being the most suitable for these studies. Since E. invadens is a strict anaerobe, the resultant aeration of the cultures from shaking may have resulted in variations in the cultures and possibly inhibition of amebic growth. It was for this reason that most of the cultures were enumerated every other day rather than every day as was done initially.

Several axenic cultures of E. invadens were studied by means of their optical absorbance. Four different fluid media were used, to which sterile suspensions of bacteria had been added. Locke's solution was the simplest and the first medium used. The next two studies were carried out using thioglycollate broth (Difco 0432-01) which was diluted one-half with Locke's solution. For the third study 0.1 ml of a minimum vitamin supplement containing 0.01 mg of folic acid, nicotinamide, pantothenate, pyridoxal and thiamine, 0.02 mg inositol and 0.001 mg

riboflavin was added to each tube. The fourth study used Diamond's axenic medium without blood serum. All of the above studies were carried out in screw cap test tubes.

The optical density curves for each of the three species of bacteria studied, B. brevis, B. megaterium and E. coli and two media are reported in the appendix.

The results of the optical absorbance studies are reported in PLATES XXVIII-XXX. The curves reported for Locke's solution and thioglycollate broth are the average of the optical densities of four separate cultures, each of which contained one of the three species of bacterial cells. The other two studies are the average of the optical densities of each of three separate cultures.

The optical density of all of the Locke's solution cultures decreased during the first two days after inoculation and then became almost constant for the next nine days. Microscopic examination of these cultures at the end of 11 days failed to reveal any live ameba trophozoites, and the studies were discontinued. These results would seem to indicate that the amebae remained active for a few days and phagocytized or lysed a considerable number of the bacterial cells during this period, as was indicated by the decrease in optical density. It is also apparent that the dead bacterial cells do not adequately meet all of the nutritional requirements of the amebae.

The optical absorbance curves of the thioglycollate broth cultures containing the three species of bacterial cells are strikingly different. In the cultures to which vitamins had been

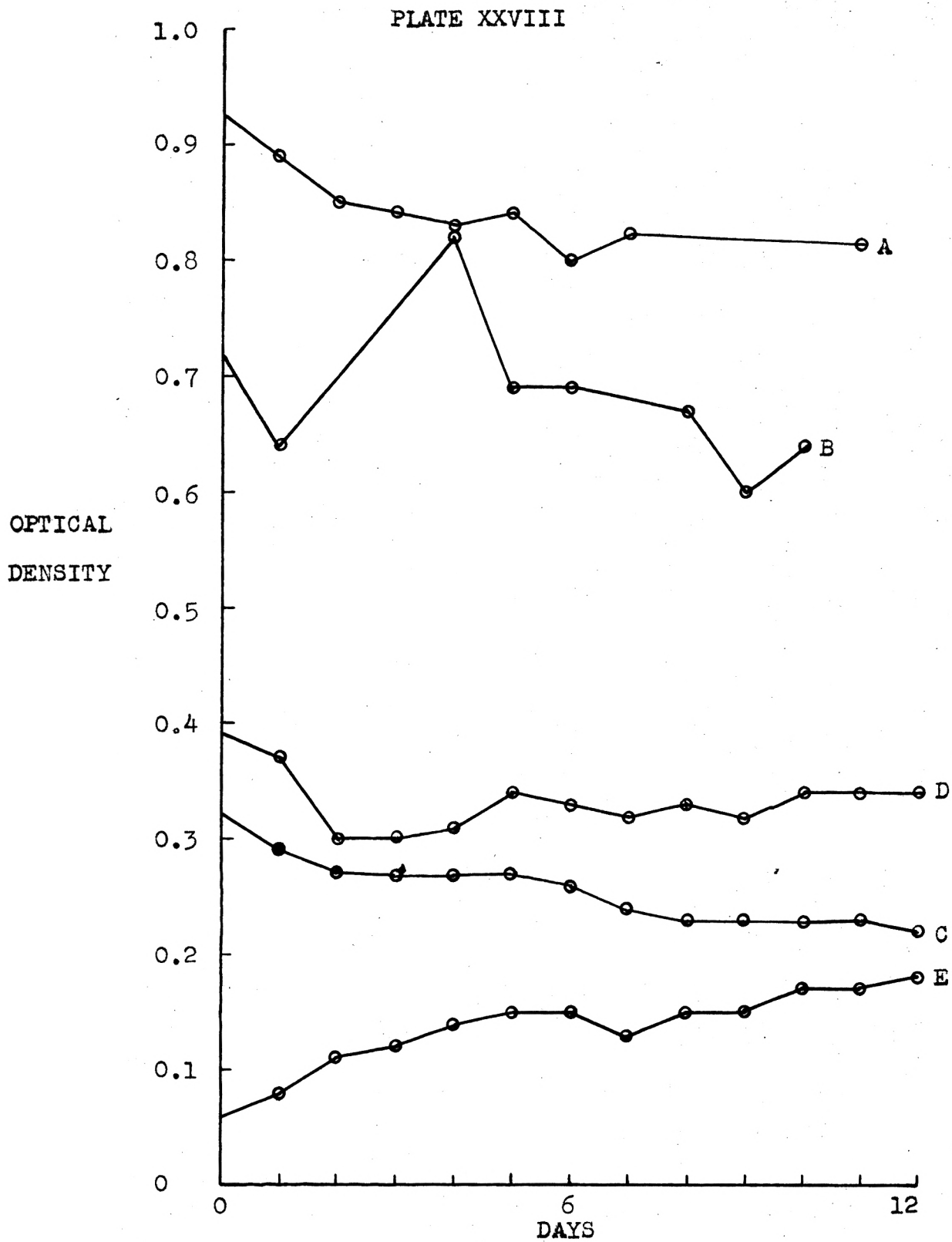
added, the ameba exhibited slightly better survival characteristics, but did not apparently multiply at a greater rate. Microscopic examination revealed no active trophozoites after ten days in the cultures without vitamins, but there were still a few ameba trophozoites alive in the other cultures after 12 days. In the cultures containing B. brevis, both cultures apparently multiplied at a rate exceeding the rate at which bacteria were being phagocytized. This is shown by the increased optical density during the first five days which decreased to approximately the initial level for the remainder of the period of study.

The optical density decreased slowly in the thioglycollate broth vitamin culture which contained either B. megaterium or E. coli cells. Essentially the same decrease occurred in the thioglycollate broth culture containing E. coli cells. These results are interpreted as indicating that the bacterial cells were being phagocytized by the amebae with little if any multiplication. The peak in the optical density curves for the thioglycollate cultures at four days may be an anomaly, since all three culture groups exhibited it. If the increase in optical density of the B. megaterium thioglycollate broth vitamin cultures is taken at face value, it would seem to indicate a considerable amount of multiplication having taken place; a fact not borne out by microscopic examination of the cultures. It is surmised from comparison to the almost identical cultures containing vitamins that there was probably some multiplication during the first two days and later phagocytosis or lysis of the

### EXPLANATION OF PLATE XXVIII

The optical density curves of Entamoeba invadens in several media. Curve E represents the optical density of a culture of Entamoeba invadens in Diamond's axenic medium without bacteria or blood serum. Curves A-D were obtained in media containing heat killed Escherichia coli cells. Curve A is for cultures of the ameba in Locke's solution. Curve B is for cultures of the ameba in thioglycollate broth. Curve C is for cultures of the ameba in thioglycollate broth which contained a vitamin supplement. Curve D is for cultures of the ameba in Diamond's axenic medium without blood serum.

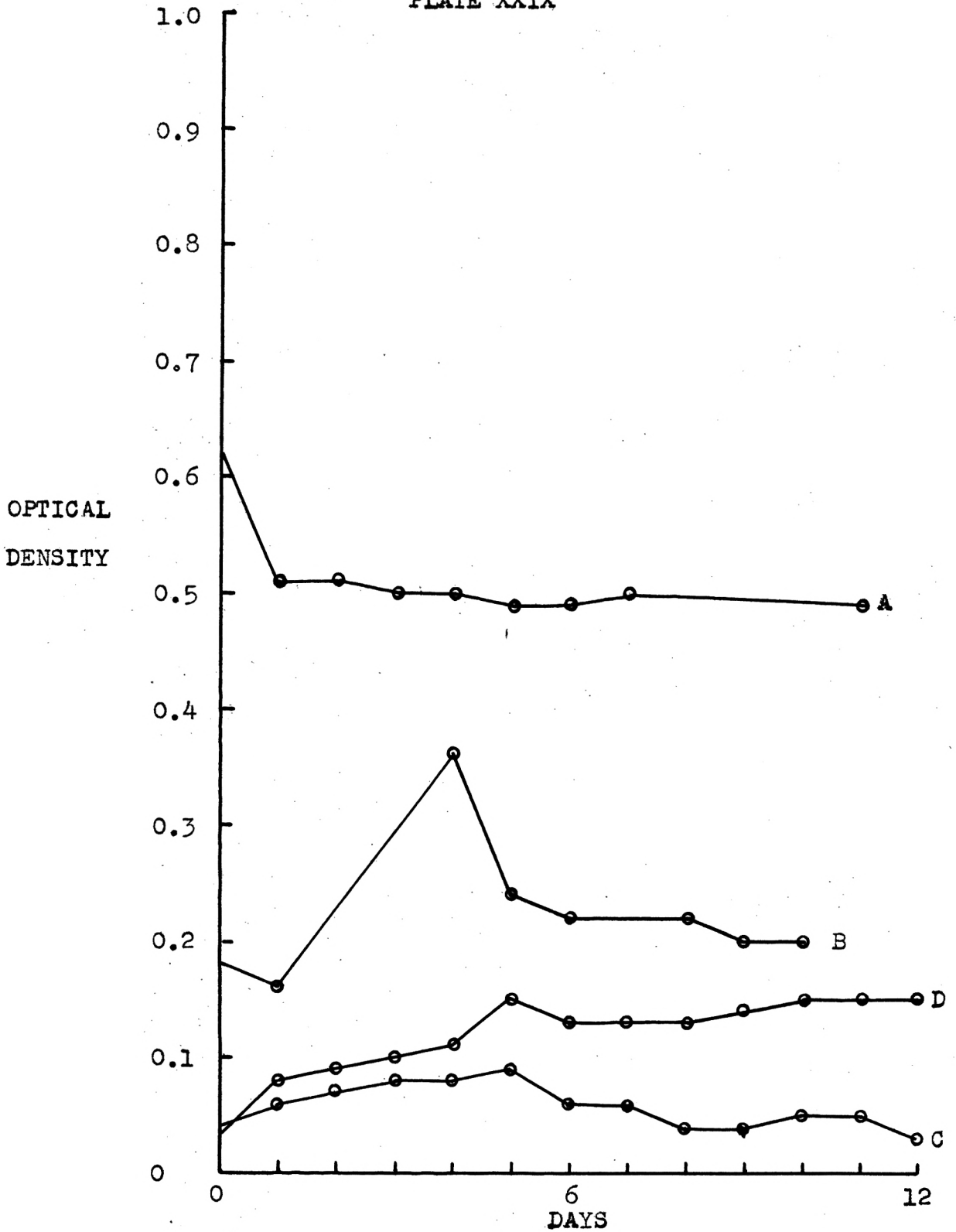




#### EXPLANATION OF PLATE XXIX

The optical density curves of cultures of Entamoeba invadens in various media containing heat killed Bacillus brevis cells. Curve A represents the optical density of cultures of the ameba in Locke's solution. Curve B represents the optical density of cultures of the ameba in thioglycollate broth. Curve C represents the optical density of cultures of the ameba in thioglycollate broth containing a vitamin supplement. Curve D represents the optical density of cultures of the ameba in Diamond's axenic medium without blood serum.

PLATE XXIX



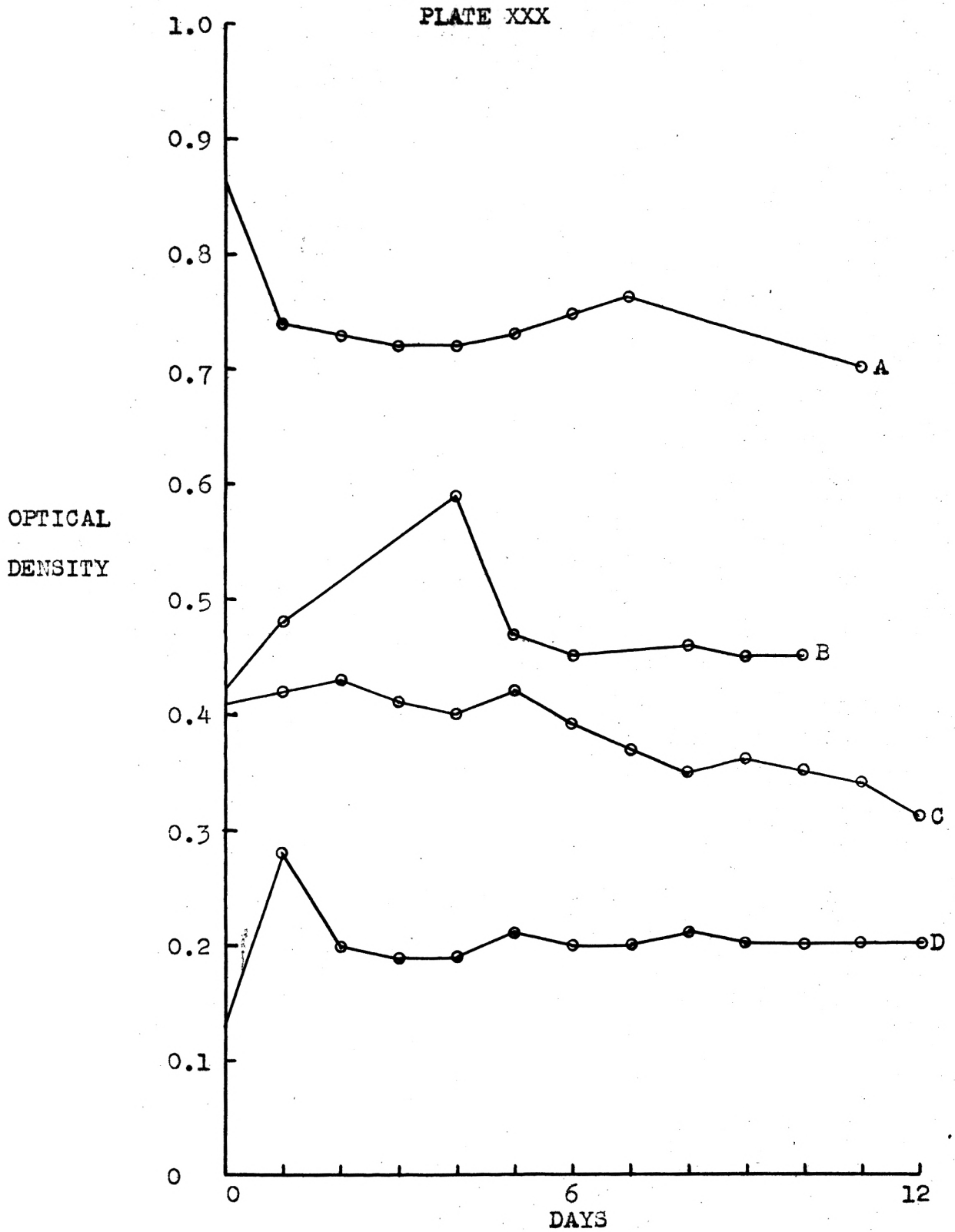
#### EXPLANATION OF PLATE XXX

The optical density curves of cultures of Entamoeba invadens in various media containing heat killed Bacillus megaterium cells.

Curve A represents the optical density of cultures of the ameba in Locke's solution.

Curve B represents the optical density of cultures of the ameba in thioglycollate broth.

Curve C represents the optical density of cultures of the ameba in thioglycollate broth containing a vitamin supplement. Curve D represents the optical density of cultures of the ameba in Diamond's axenic medium without blood serum.



bacterial cells caused the optical density to decrease to the level it reached on the sixth day.

Of the four studies carried out, the cultures prepared with Diamond's axenic medium without serum proved to be the most informative. On Plate XXVII, in addition to the bacterial cultural curves, curve E presents the increase in optical density produced by the multiplication of E. invadens in the medium without added bacteria. The number of active trophozoites in the cultures increased over the twelve day period to approximately four times their original number. The rate of multiplication, though not as great as the rate in the normal axenic stock culture containing serum and over twice as much medium, was sufficient to be measured by the optical density of the cultures. It is thus exceptionally interesting to compare the optical density curves of the cultures in the same medium which contained bacteria cells.

In the cultures containing E. coli cells the optical density of the cultures dropped sharply during the first two days and never reached the original level again. Microscopic examination of these cultures revealed that the number of trophozoites at the end of twelve days had only increased to about twice their original number. In consideration of the latter, it is surmised that initially the amebae phagocytized the bacteria rapidly but were then apparently somewhat inhibited from multiplying by these same bacteria. Autolysis of the bacterial cells may have also taken place.

In the culture containing B. brevis cells within Diamond's

axenic medium, the results are very striking. On first examination, the optical density curve is almost identical to that produced in Diamond's axenic medium without bacteria, but in the B. brevis cultures there were six times as many ameba trophozoites after 12 days as there were initially. This difference in the number of trophozoites should be measurable but is not apparent in the curve of the B. brevis cultures. The theoretical increase in optical density after 12 days can be easily calculated on the basis of the increase in optical density versus the increase in population of the cultures without bacterial cells. For a six-fold increase in the number of trophozoites (within the same general optical density range) the increase in optical density should have been approximately 0.18; it was actually only 0.09 units. The difference of 0.09 units apparently represents the change in optical density produced by the phagocytosis or autolysis of the B. brevis cells. The observation that there was greater multiplication in the cultures containing B. brevis would seem to indicate that the dead B. brevis cells are in some way beneficial to the amebae.

The optical density curve for the culture of E. invadens in Diamond's axenic medium containing B. megaterium cells does not match the other curves in Diamond's medium. The curve levels off into almost a horizontal line after the second day. The optical density increased sharply during the first day and then decreased sharply again on the second day. The overall pattern is that of unchanging optical density after the second day, but between the inoculation with amebae and the twelfth day the number

of ameba trophozoites increased 3.6 times. Thus the curve apparently indicates that the bacteria were, after the second day, being phagocytized or autolysed at a rate which held the optical density at the same level. If this optical density curve is examined on the same basis as the B. brevis curve, it is apparent that the phagocytosis or autolysis of the bacterial cells lowered the optical density approximately 0.04 units. The increase in the number of trophozoites was less than in the cultures without bacterial cells apparently indicating some inhibition of multiplication by the presence of the killed B. megaterium cells.

#### Electrophoretic Studies

The electrophoretic studies are reported in Table 3 and Table 4. The primary aim in studying the electrophoretic properties of E. invadens was to discover if there were any demonstrable relationship between these properties and the phagocytosis of bacteria by the ameba. All of the initial measurements of the electrophoretic mobility were carried out in phosphate buffer of the same ionic strength as that used in Diamond's axenic medium for the ameba. At pH 6.90 in 0.009 M phosphate buffer, the charge exhibited by the ameba was negative, and the mobility was  $0.096 \mu/\text{sec}/\text{v}/\text{cm}$ .<sup>1</sup> At pH 6.75 in 0.008 M phosphate buffer, the electrophoretic mobility was found to be  $1.35 \mu/\text{sec}/\text{v}/\text{cm}$ . These initial results were based on 25 and 45 measurements respectively. They were sufficient to indicate

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<sup>1</sup>All electrophoretic mobilities were corrected to 30 C.



Table 3. Electrophoretic mobility comparisons of Entamoeba invadens and bacteria.

Buffer	Age in days <sup>1</sup>	Sign of charge	Molarity	pH	Electrophoretic mobility <sup>2</sup>
<u>Entamoeba invadens</u>					
Phosphate <sup>3</sup>	7	neg.	0.009	6.90	0.96
Phosphate	7	neg.	0.008	6.75	1.35
Phosphate	7	neg.	0.013	8.93	1.43
Phosphate	7	neg.	0.013	7.00	1.14
Phosphate	7	neg.	0.013	6.00	1.25
Phosphate	7	neg.	0.013	4.60	0.43
Sodium Acetate	7	pos.	0.020	4.00	0.50
Phosphate	30	neg.	0.013	7.00	1.05
Phosphate	60	neg.	0.013	7.00	2.06
<u>Bacillus brevis</u>					
Phosphate	1	neg.	0.013	7.00	4.29
<u>Bacillus megaterium</u>					
Phosphate	1	neg.	0.013	7.00	2.36
Phosphate	1	neg.	0.008	6.75	2.44
<u>Escherichia coli "B"</u>					
Phosphate	1	neg.	0.008	6.75	10.1
<u>Entamoeba invadens with Bacillus brevis</u> in Locke's egg medium for amebae					
Phosphate	30	neg.	0.013	7.00	2.86

1 Age refers to the age of culture.

2 Mobilities are reported as  $\mu$ /sec./v/cm.

3 The phosphate buffer consisted of mixtures of  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ .

Table 4. Electrophoretic mobility comparisons of Entamoeba invadens and human erythrocytes in buffers containing carboxy-methyl-cellulose.

Buffer	Molarity	Viscosity (centi- poise)	pH	Sign of charge	Mobility corrected for temp. to 30 C <sup>1</sup>	Mobility corrected for vis- cosity <sup>1</sup>
<u>Entamoeba invadens</u> <sup>2</sup>						
Phosphate	0.009	36.6	6.80	neg.	3.72	17.0
Phosphate	0.013	36.6	7.00	neg.	3.47	15.9
Sodium acetate	0.020	40.2	4.55	neg.	3.30	16.6
Human Erythrocytes <sup>3</sup>						
Phosphate	0.067	36.6	7.35	neg.	1.66	7.57

1 Mobilities are reported as  $\mu$ /sec./v/cm.

2 The Entamoeba invadens trophozoites used in these studies were from cultures which were seven days old.

3 Indicates that the mobility values for the erythrocytes were corrected to 25 C in order that they may be compared to the value reported by Abramson (1929) of 1.31  $\mu$ /sec./v/cm.

that the ameba was negatively charged, and it is therefore doubtful that the surface charge of the ameba is involved in the phagocytosis of bacteria since bacteria are known to be negatively charged. The apparent mobility of the amebae was found to be very much more variable than that of bacteria. This variability is perhaps to be expected due to the fact that the live and active trophozoites being studied varied from  $4 \mu$  to  $30 \mu$  with an average of  $15 \mu$ . The larger amebae, especially, considerably overlapped the stationary level in the microelectrophoresis cell and would thus be affected by the electroosmotic movement of the buffer in the cell. Moreover, several of the larger amebae were capable of reaching the bottom of the microelectrophoresis cell with their pseudopodia from the stationary level and often did so. Although the initial studies indicated that there probably was no relationship between the electrokinetic properties of the Entamoeba and phagocytosis of bacteria, a number of studies were carried out to establish the electrokinetic properties more completely.

In 0.013 M phosphate buffer, the electrophoretic mobility of the ameba was determined at pH values ranging from 8.93 to 4.60. As can be seen in Table 3, the mobility decreased with decreasing pH reaching a minimum at pH 4.60. In sodium acetate buffer at pH 4.00, the ameba became positively charged indicating that the isoelectric point for the ameba was between 4.00 and 4.60. The above results were based on 50 or more determinations for pH 8.93, 7.00 and 6.00. At pH 4.60, some reversal of charge was noted, and only 19 values were determined for this reason.

At pH 4.00, all of the amebae were killed by the low pH, and 39 determinations were made.

At pH 7.00 in the 0.013 M phosphate buffer, a detailed study of the electrokinetic properties were carried out. The average electrophoretic mobility was found to be  $1.18 \mu/\text{sec}/\text{v}/\text{cm}$ . The mean deviation was  $\pm 0.39 \mu/\text{sec}/\text{v}/\text{cm}$ , and the standard error was  $\pm 0.56 \mu/\text{sec}/\text{v}/\text{cm}$ . The probable error was  $\pm 0.38 \mu/\text{sec}/\text{v}/\text{cm}$ .

The Zeta potential was found to be  $1.49 \times 10^{-4}$  millivolts. These results indicate the extreme variability of the electrophoretic mobility of this ameba.

The results obtained for the amebae may be compared to those obtained for bacteria in Table 3. In the 0.013 M phosphate buffer at pH 7.00, live, 24 hour old cells of B. brevis and B. megaterium grown on nutrient agar were found to have mobilities of 4.29 and  $2.36 \mu/\text{sec}/\text{v}/\text{cm}$ , respectively. Both of the above results were based on over one hundred determinations.

Autoclaved cells of B. megaterium had a mobility of  $2.44 \mu/\text{sec}/\text{v}/\text{cm}$  at pH 6.75. Live cells of E. coli grown in nutrient broth had a much higher mobility  $10.12 \mu/\text{sec}/\text{v}/\text{cm}$  at pH 6.75. Thus it is evident that bacterial cells generally have higher electrophoretic mobilities and surface charges than the Entamoeba in the same buffer systems.

The effect of culture age on E. invadens was studied. When the Entamoeba cultures were 30 days old, the electrophoretic mobilities of the amebae were not significantly different from those from one week old cultures. When 60 days old, however,

the mobilities were considerably higher and also more variable. Many of the amebae were inactive in the 60 day old culture.

The effect of culture medium and cultural associates on the ameba was studied using cultures containing E. invadens in monobacterial culture with B. brevis in Locke's egg medium. It was possible to make only nine determinations due to the small number of amebae available from these cultures. The average electrophoretic mobility was  $2.86 \mu/\text{sec}/\text{v}/\text{cm}$ . This information indicates that the amebae have a considerably higher mobility in such monobacterial egg cultures.

The variability of electrophoretic results for the ameba made it desirable that methods be studied which might retard the settling of the amebae during the measurement of their electrophoretic mobility. The first method tried was an effort to develop a buffer with approximately the same specific gravity as the amebae. Since an electrolyte could not be used, sugar was selected as a suitable non-electrolyte and was added to the buffer. It was found, however, that concentrations sufficient to retard the settling were also sufficient to cause plasmolysis of the amebae.

Since increasing the specific gravity was apparently impractical, the viscosity of the buffer was increased by the addition of 0.5% by weight of carboxy-methyl-cellulose (CMC). This product was neutral and increased the viscosity of the buffers markedly. It was used in three different buffers to study the effect on the ease and reliability of the measurement of the electrophoretic mobility of the amebae. The results of these

studies are reported in detail in Table 4. At pH 6.80 in 0.009 M phosphate buffer, the electrophoretic mobility was found to be  $3.72 \mu/\text{sec}/\text{v}/\text{cm}$  corrected to 30 C. At pH 7.00, the mobility was  $3.47 \mu/\text{sec}/\text{v}/\text{cm}$ , and at pH 4.55 in sodium acetate buffer, the mobility was  $3.30 \mu/\text{sec}/\text{v}/\text{cm}$ .

The increase in mobility of E. invadens in each of the buffers containing CMC was very nearly the same. The mean deviations were numerically about the same but less on a percentage basis, indicating increased reliability in results. However, the biggest improvement was that it was much easier to make the determinations of electrophoretic mobility. For example, 65 measurements were made at pH 6.9 and 100 at pH 7.00 and at pH 4.55 in approximately the same amount of time required previously to make 50 measurements without the CMC in the buffer.

The addition of carboxy-methyl-cellulose to the buffers resulted in an apparent increase in the electrophoretic mobility of the ameba. This increase in mobility occurred despite the greatly increased viscosity of the buffers. This is readily apparent from a comparison of the mobilities reported in Table 4 to those for equivalent buffers in Table 3. It may be noted that the electrophoretic mobility of E. invadens when not corrected for the increased viscosity of the buffers had almost the same value in the three buffer systems. The increase in mobility is not the same in the three buffers. The increase in electrophoretic mobility is even more apparent when the mobility values are corrected for the viscosity of the medium. These corrections were applied by multiplying the temperature corrected values by the

ratio of the viscosity of the buffer containing CMC to that of water.

The effect on the electrophoretic mobility of human erythrocytes in buffer containing CMC was also studied. Since the mobility of human erythrocytes is known to be  $1.31 \mu/\text{sec}/\text{v}/\text{cm}$  in the same buffer without CMC, it is apparent that their mobility was effected only slightly by the CMC. When this mobility is corrected for the viscosity of the medium, the increase was still only about one-half of that which occurred for the amebae. The effect on the electrophoretic mobility of particles by CMC thus seems to depend upon the nature of the surface of the particle. Although none of the amebae appeared to be harmed by the addition of CMC to the buffer, many of the erythrocytes were crenated in the buffer containing CMC.

Buffers containing carboxy-methyl-cellulose are most valuable for the study of heavy particles at or near neutrality. Since the effect is not the same on surfaces of different types, CMC should be used only for studies of particles having similar surface characteristics.

## Photographic Studies



Fig. 1. A phase-contrast photograph of a normal trophozoite of Entamoeba invadens in Diamond's axenic medium with unknown bacteria.

Figure 1 shows the normal active shape of an E. invadens trophozoite. The amoeba moves toward the blunt end frequently with clear ectoplasm showing in the anterior end of the amoeba as is shown here. At the posterior of the amoeba can be seen the uropod of the amoeba. The magnification of this amoeba is approximately 1,800 times its natural size. The posterior three fourths of the amoeba is densely granulated which is normal for the Entamoeba trophozoites. There may be bacteria within this trophozoite, but it is impossible to be sure due to its density.



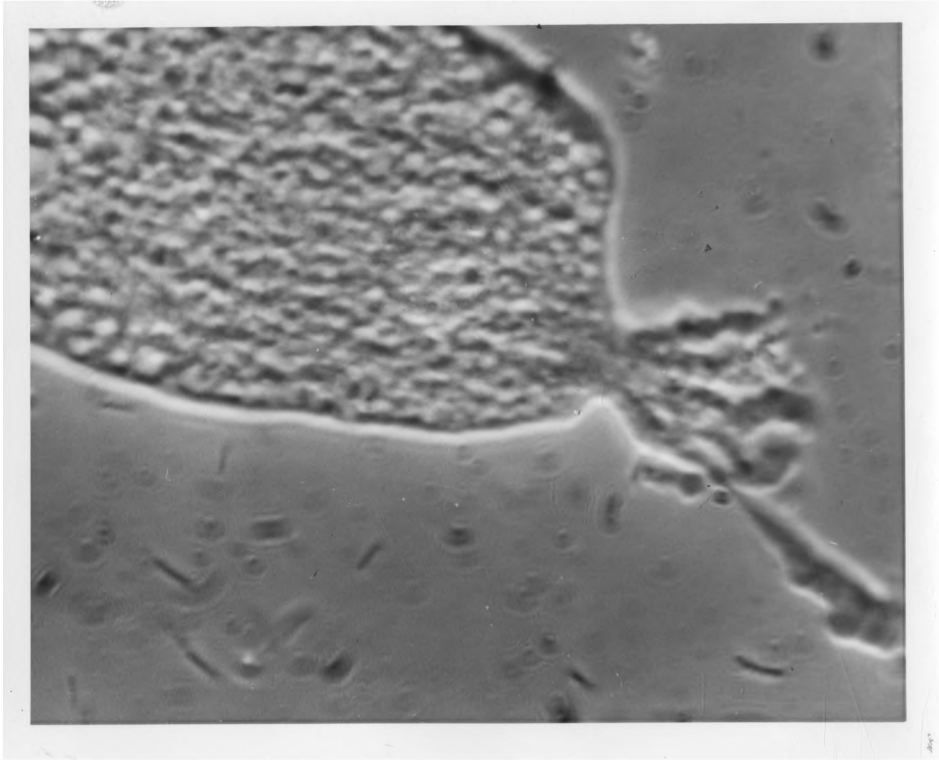


Fig. 2. Phase-contrast photograph of uriopod of Entamoeba invadens.

Figure 2 is a phase-contrast photograph of E. invadens in Locke's egg medium with B. brevis. The oil immersion study (approximately 3,500 X) clearly shows several features of the trophozoite. The uriopod at the posterior of the trophozoite appears to consist of a group of streamers of ectoplasm. Several of these may contain bacterial cells. The nucleus is very evident a short distance to the anterior of the uriopod. The cytoplasm of this ameba is very dense, but nevertheless, some of the bacteria in this ameba are apparent in the photograph.

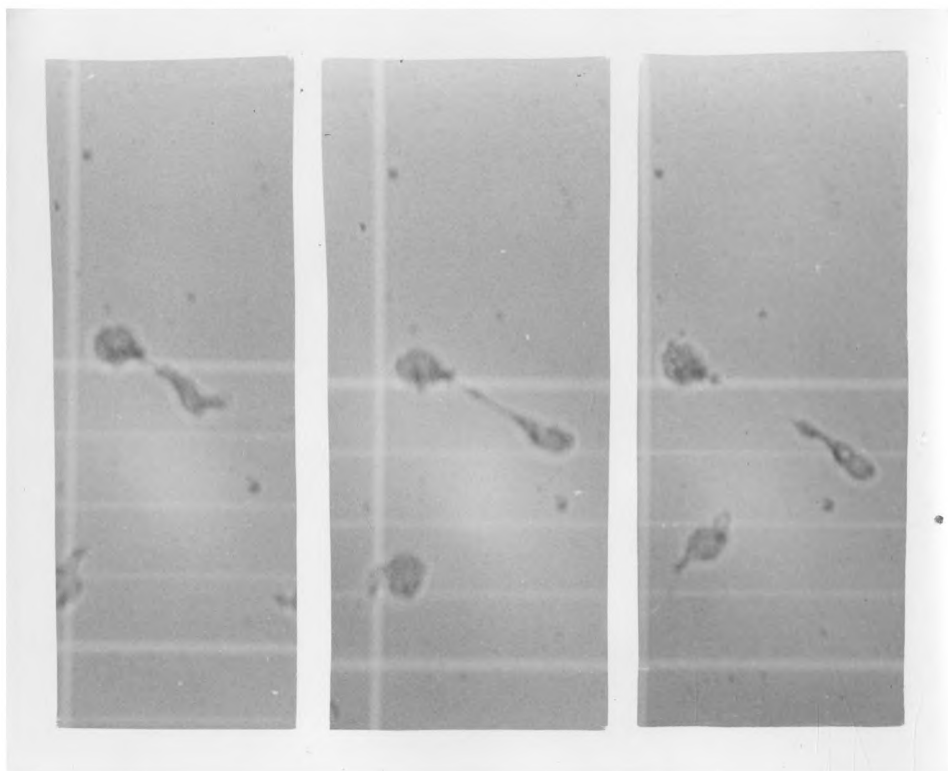


Fig. 3. Time lapse photographs of Entamoeba invadens undergoing division on hemacytometer grid.

This photograph is not in exact focus, because it was photographed on a hemacytometer. However, it clearly shows the division of the ameba. The size of the trophozoites involved can be judged by comparison to the divisions on the hemacytometer. The trophozoite undergoing division separated into two large masses of protoplasm which tend to move in opposite directions as is shown by the photograph on the left. In the center photograph, the trophozoite has stretched out, and only a thin strand of protoplasm connects the two parts. In the third photograph, the strand between the sections has just parted, and the two trophozoites are proceeding on their separate ways.

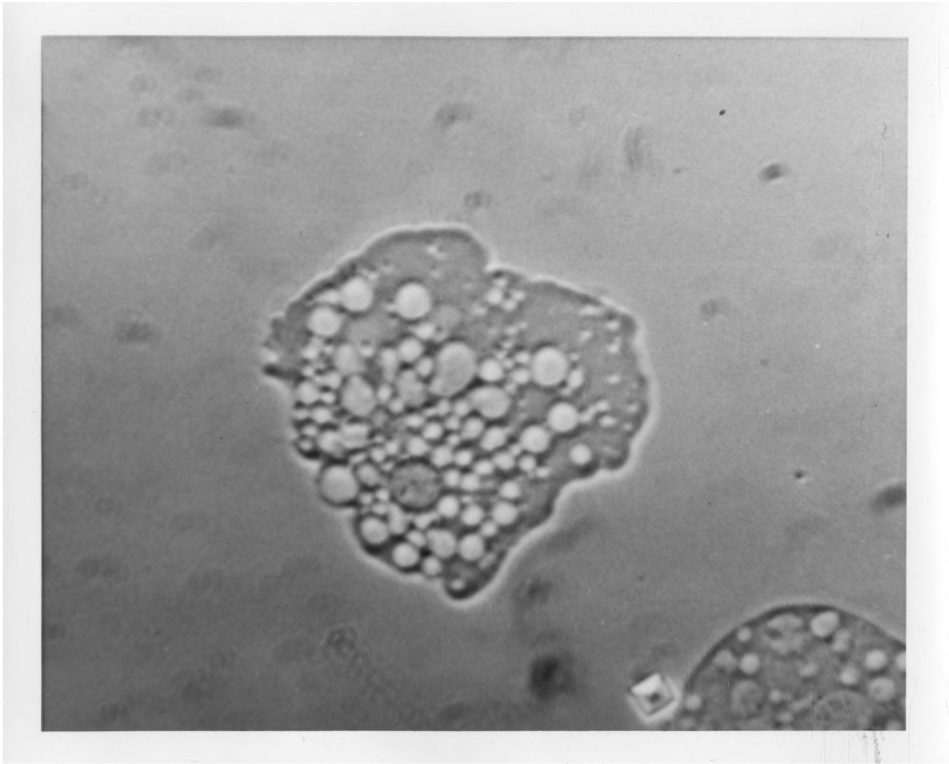


Fig. 4. Phase-contrast photograph of Entamoeba invadens in axenic culture.

In Diamond's axenic medium, some of the Entamoeba trophozoites became highly vacuolated. This vacuolation is readily seen in Fig. 4 at approximately 2,150 times its actual size. The nucleus of the trophozoite is clearly shown in the photograph. The crystal in the lower right hand corner of the photograph is apparently cysteine. On numerous occasions these crystals were observed within the trophozoites and in the culture medium.

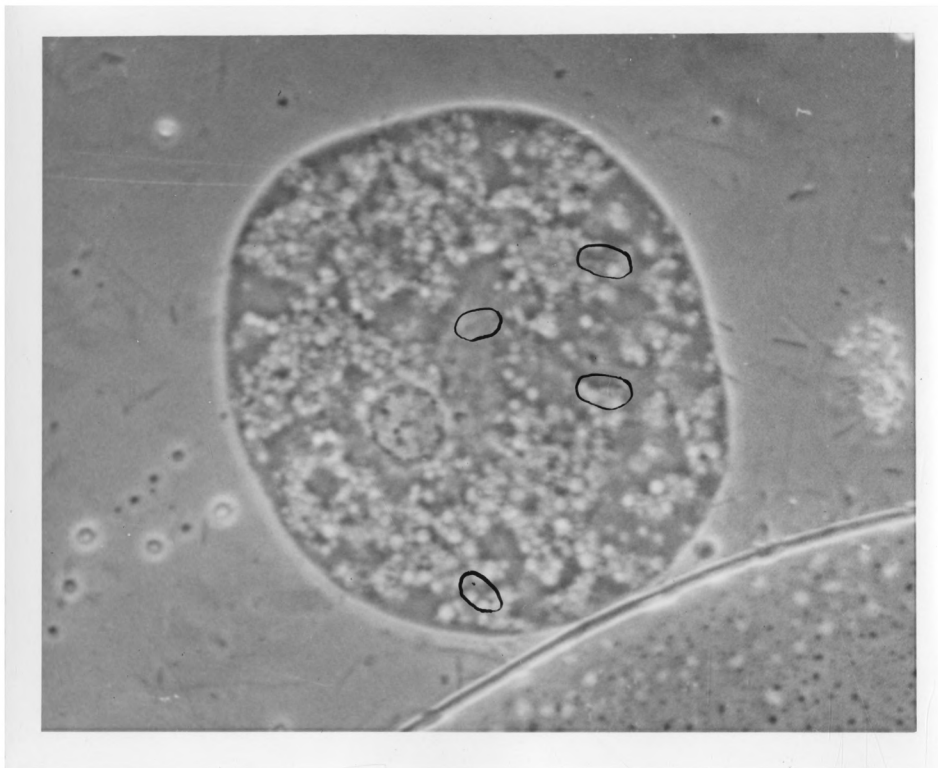


Fig. 5. Phase-contrast photograph of Entamoeba invadens in Locke's egg medium with Bacillus brevis.

The ameba shown in Fig. 5 was very large and exhibited sluggish movements. The ameba is magnified approximately 3,900 times its actual size. B. brevis cells within the trophozoite are indicated by circles. The chromatin granules around the edge of the nucleus are clearly evident even though this was an unstained active trophozoite.

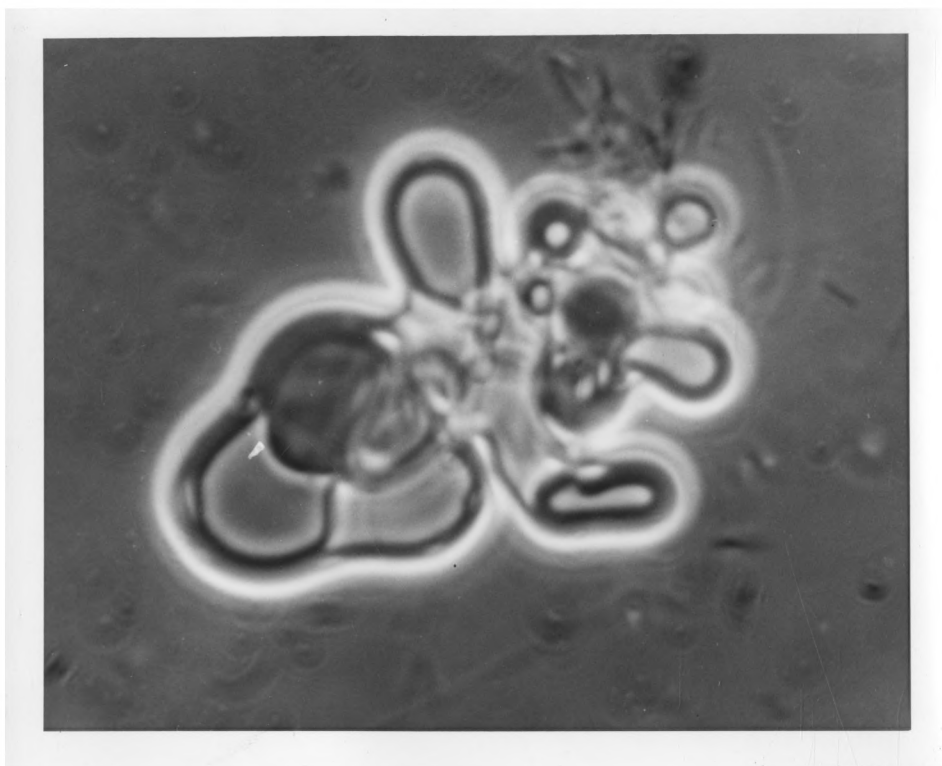


Fig. 6. A phase-contrast photograph of a particle of egg from a Locke's egg medium culture of Entamoeba invadens with Bacillus brevis.

Figure 6 is included in order to demonstrate one of the difficulties encountered in enumerating cultures of E. invadens in Locke's egg medium. This photograph shows a particle of egg which could easily be confused with an ameba at low magnification. Such particles as this were typical of most of the cultures in this medium. They became extremely numerous toward the end of the experiments and greatly increased the difficulty in enumerating the amebae.

## SUMMARY

In monoxenic culture with E. invadens in Locke's egg medium, the following bacteria were found to support the growth and multiplication of the ameba: B. brevis, B. subtilis, B. subtilis var. niger, E. coli, A. aerogenes, S. epidermidis, M. lysodeikticus, P. rettgeri, C. perfringens and P. aeurginosa. B. brevis, E. coli in LES medium, A. aerogenes, M. lysodeikticus, P. rettgeri and P. aeurginosa had the greatest beneficial effect on the survival of the ameba. Two species of A. cloacae and S. marcescens affected the ameba adversely. The other species of bacteria studied did not noticeably affect the growth of the ameba.

The study of the growth supporting characteristics of heat killed cells of B. megaterium, B. brevis and E. coli demonstrated that the dead bacterial cell alone was sufficient to support the growth of E. invadens in Locke's solution. In thioglycollate broth, B. brevis cells apparently enhanced the multiplication of the ameba. In Diamond's axenic medium without blood serum, multiplication was enhanced by B. brevis cells and apparently somewhat inhibited by the presence of either B. megaterium or E. coli cells. The results with the B. brevis cells indicated that they were being actively phagocytized, as demonstrated by microscopic and photographic examination of the amebae.

Electrophoretic studies of E. invadens have demonstrated that the trophozoites carry a negative charge at pH above 4.0. The isoelectric point of the trophozoite lies between pH 4.6 and

pH 4.0. The nature of the surface charge of this ameba is such that it is unlikely that it plays any part in the ability of the trophozoites to phagocytize bacterial cells. It is also, however, sufficiently low that it is also unlikely that there is any significant repulsion of the bacterial cells from the amebae due to their having the same charge.

The electrophoretic studies carried out with the addition of carboxy-methyl-cellulose (CMC) to the buffers have indicated that it may be of considerable value in micro-electrophoresis studies of heavy particles. The CMC (0.5%) noticeably retarded the settling of the trophozoites during the electrophoretic mobility measurements which allowed the measurements to be made with greater ease. The CMC greatly increased the mobility of the E. invadens trophozoites, even though the viscosity of the buffer was much higher. Since the CMC apparently has some type of active cell surface reaction, it should be used only with cells of the same general type.

Photomicrography with the phase-contrast microscope has demonstrated the uriopod, the nucleus, the vacuolation, the normal active shape and the phagocytosis of bacteria by live E. invadens trophozoites. Although all of these features have been described previously for E. invadens, they have not, to the knowledge of the author, been photographed in the unstained living trophozoite.

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## LITERATURE CITED

- Abramson, H. A. 1929a. Modification of the Northrop-Kuntz microcataphoresis cell. *J. Gen. Physiol.* 12:469-472.
- Abramson, H. A. 1929b. The cataphoretic velocity of mammalian red blood cells. *J. Gen. Physiol.* 12:711-725.
- Abramson, H. A. 1934. Electrokinetic phenomena and their application to biology and medicine. *The Chemical Catalog Co.*, New York. 317 p.
- Abramson, H. A. and L. Michaelis. 1929. The influence of size, shape and conductivity of microscopically visible particles on cataphoretic mobility. *J. Gen. Physiol.* 12:587-598.
- Balamuth, W. 1962. Effects of some environmental factors upon growth and encystation of Entamoeba invadens. *J. Parasitol.* 48:101-109.
- Balamuth, W. and B. Howard. 1946. Biological studies on E. histolytica. I The growth cycle of populations in a mixed bacterial flora. *Am. J. Trop. Med.* 26:771-782.
- Boeck, W. C. and J. Drbohlaw. 1925. The cultivation of Endamoeba histolytica. *Am. J. Hyg.* 5:371-407.
- Breed, R. S., E. G. D. Murray and N. R. Smith. 1957. *Bergey's manual of determinative bacteriology*. 7th ed. Baltimore. Williams and Wilkins, 1094 p.
- Burrows, R. B. 1957. Endamoeba hartmanni. *Am. J. Hyg.* 65:172-188.
- Burrows, R. B. 1961. Prevalence of amebiasis in the United States and Canada. *Am. J. Trop. Med. Hyg.* 10:172-184.
- Burrows, W. 1959. *Textbook of microbiology*. 17th ed. Saunders, Philadelphia. 954 p.
- Chandler, A. C. and C. P. Read. 1961. *Introduction to parasitology*. 10th ed. Wiley, New York. 822 p.
- Daniel, G. E. and G. H. May. 1950. Observations on the reaction of Pelomyxa carolinensis subjected to a direct current electric field. *Physiol. Zool.* 23:231-236.
- Diamond, L. S. 1960. The axenic cultivation of two reptilian parasites Entamoeba terrapinae, Sanders and Cleveland 1930, and Entamoeba invadens Rodhain 1934. *J. Parasitol.* 46:484.

- Geiman, Q. M. and H. L. Ratcliffe. 1936. Morphology and life-cycle of an amoeba producing amoebiasis in reptiles. *Parasitol.* 28:208-228.
- Gittens, G. J. and A. M. James. 1960. An improved microelectrophoresis apparatus and technique for studying biological cell surfaces. *Anal. Biochem.* 1:478-485.
- Hoare, C. A. 1952. The commensal phase of Entamoeba histolytica. *Exp. Parasitol.* 1:411-427.
- Jacobs, L. 1947. The elimination of viable bacteria from cultures of Endamoeba histolytica and the subsequent maintenance of such cultures. *Am. J. Hyg.* 46:172-176.
- Jacobs, L. 1950. Oxidation-reduction potentials in the cultivation of Endamoeba histolytica. *Am. J. Trop. Med.* 30:803-815.
- Jennings, H. S. 1904. Contributions to the study of the behavior of the lower organisms. No. 16. Carnegie Institution, Washington.
- Kahler, H., H. W. Chalkley and C. Voegtlin. 1929. The nature of the effect of a high-frequency electric field upon Paramecium. *Public Health Reports (U.S.)*. 44:339-347.
- Kudo, R. 1954. *Protozoology*. 4th ed. Thomas, Springfield. 966 p.
- Luttermoser, G. W. and B. P. Phillips. 1952. Some effects of cultural associates on the infectivity of a strain of Endamoeba histolytica for the rabbit. *Am. J. Trop. Med. Hyg.* 1:731-735.
- McConnachie, E. W. 1955. Studies on Entamoeba invadens, Rodhain, 1934 in vitro, and its relationship to some other species of Entamoeba. *Parasitol.* 45:452-481.
- McConnachie, E. W. 1956. Modification and elimination of the bacterial flora in the cultures of Entamoeba invadens, Rodhain, 1934. *Parasitol.* 46:117-129.
- McConnachie, E. W. 1958. Factors affecting the growth and behavior of axenic strains of Entamoeba invadens, Rodhain, 1934. *Parasitol.* 48:423-432.
- Meerovitch, E. 1960. Thermal barrier to infectivity of Entamoeba invadens in snakes. *Nature (Lond.)*. 185:631.
- Meerovitch, E. 1961. Infectivity and pathogenicity of polyxenic and monoxenic Entamoeba invadens to snakes kept at normal and high temperatures and the natural history of reptile amoebiasis. *J. Parasitol.* 47:791-794.
- Miller, M. J. 1953. Bacteria-free Endamoeba invadens. *Nature (Long.)*. 172:1192-1193.

- Moyer, L. S. 1936. A suggested standard method for the investigation of electrophoresis. *J. Bacteriol.* 31:531-546.
- Nakamura, M. 1953. Nutrition and physiology of Endamoeba histolytica. *Bacteriol. Rev.* 17:189-212.
- Northrop, J. H. and M. Kuntz. 1925. An improved type of microscopic electrocataphoresis cell. *J. Gen. Physiol.* 7:729-730.
- Paulson, M. 1932. An accurate method for the numerical determination of Endamoeba histolytica in vitro and its possible use with other intestinal protozoa; suggested clinical applications. *Am. J. Trop. Med.* 12:387-399.
- Phillips, B. P. 1950. Cultivation of Endamoeba histolytica with Trypanosoma cruzi. *Science.* 111:8-9.
- Phillips, B. P. and P. A. Wolfe. 1959. The use of germfree guinea pigs in studies on the microbial interrelationships in amoebiasis. *Ann. N.Y. Acad. Sci.* 78:308-314.
- Phillips, B. P., P. A. Wolfe, C. W. Rees, H. A. Gordon, H. H. Wright, and J. A. Reyniers. 1955. Studies on the ameba-bacteria relationship in amoebiasis. Comparative results of intracecal inoculation of germfree, monocontaminated, and conventional guinea pigs with Entamoeba histolytica. *Am. J. Trop. Med. Hyg.* 4:675-692.
- Porter, R. J. 1953. Amebiasis. *Ann. Rev. Microbiol.* 7:273-294.
- Reardon, L. V., and C. W. Rees. 1939. Cultivation of Endamoeba histolytica without serum. *J. Parasitol.* 25(Suppl.):13-14.
- Rees, C. W., L. V. Reardon, and L. Jacobs. 1941. The cultivation of the parasitic protozoa without bacteria. *Am. J. Trop. Med.* 21:695-716.
- Reeves, R. E., D. I. Schweinfurth, and W. W. Frye. 1960. The cultivation of Entamoeba histolytica with radiation-inactivated bacterial cells. *Am. J. Hyg.* 72:211-217.
- Rodhain, J. 1934. Entamoeba invadens n. sp. parasite de serpents. *C. R. Soc. Biol. Paris.* 117:1195. Original not seen. Article referenced in Geiman and Ratcliffe, 1936.
- Shaffer, J. G., and V. Iralu. 1961. The effect of erythrocytes on the propagation of Entamoeba histolytica in culture. I Demonstration of toxicity of several erythrocytes to a strain of Entamoeba histolytica. *Am. J. Trop. Med. Hyg.* 10:10-16.
- Stoll, N. R. 1957. Axenic culture of Entamoeba invadens in the absence of tissue. *J. Protozool.* 4:Suppl. 8.

- Uber, F. M. 1950. Biophysical research methods. Interscience, New York. 667 p.
- U. S. Department of Health, Education, and Welfare. 1948, October-December. Public Health Service. Communicable Disease Center, Atlanta. n.p.
- U. S. Department of Health, Education, and Welfare. 1955? Laboratory Diagnosis of Parasitic Diseases. Public Health Service. Communicable Disease Center, Atlanta. n.p.
- Verworn, M. 1889. Psycho-physiologische protisten-studien. Jena. Original not seen. Referenced in Kudo, 1954.

APPENDIX

## The Design of a Dipping Conductance Cell.

Purpose. It was desired to construct a conductance cell having vertical rather than horizontal electrodes and which could be used with small volumes of solution.

Procedure. A dipping type cell was constructed using platinum wire coiled into flat plates for the electrodes. The electrodes were attached to copper wires, and the copper wires were sealed into glass tubing. The electrodes were positioned by forcing the glass tubing through tightly fitting holes in two rubber stoppers. Further rigidity was obtained by placing an open tube through both of the rubber stoppers. This tube also serves as an air opening for the cell, so that liquid can enter the cell freely. The electrodes and rubber stoppers were then placed in an open tube in such a position that the electrodes were centered in the tube and protected from damage, yet would require a minimum of solution to cover them. The electrodes were finally cleaned in aqua regia and then platinized as described in Daniels et al., (1956). The cell is illustrated in Plate XXXI.

#### EXPLANATION OF PLATE XXXI

The dipping conductance cell used in the electrophoresis experiments. The cell is designed to require a small volume of sample to cover the vertical electrodes.

PLATE XXXI

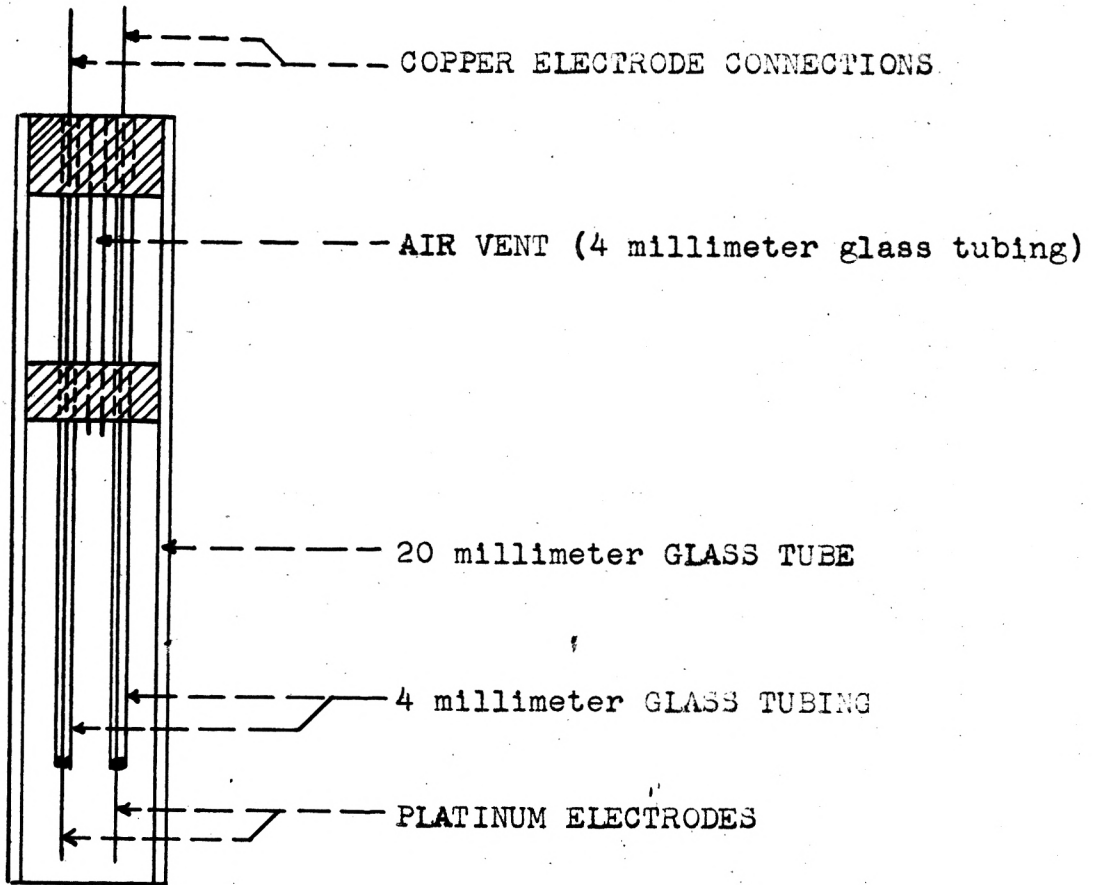


ILLUSTRATION OF  
PLATINUM ELECTRODE  
SPIRAL CONSTRUCTION



The Determination of the Absorption Spectra  
for Three Species of Bacteria

Procedure. Cultures of B. brevis, E. coli and B. megaterium were grown on nutrient agar and harvested at 24 hr. They were then washed and suspended in Locke's salt solution and autoclaved at 121 C at 15 lbs. pressure for 20 min. Suitable dilution of the suspensions for the determination of absorbency was carried out using Locke's salt solution. Optical absorbency was determined by use of the Bausch & Lomb Spectronic 20 colorimeter. Bacterial cultures were enumerated with Petroff-Hauser counting chamber.

Results. The results are plotted in Plates XXXII-XXXVII.

Discussion. Plate XXXII is the absorption spectrum for E. coli. It has maximae at 4000-4100 A and 7000-8100 A and a minimum at 6500 A. Plate XXXIII is the absorption spectrum for B. megaterium and Plate XXXIV is the absorption spectrum for B. brevis. They have approximately the same maximae and minimum as E. coli. On the basis of the above curves, 4000 A was selected, and the range of concentrations obeying Beer's law was determined for the suspension of E. coli at this wave length. Serial dilutions were prepared of the suspension and the optical absorbence determined for each dilution. The results are illustrated in Plate XXXV. The solid line indicates that the best obeyance of Beer's law is in the region of optical absorbence of 0.150 to 0.650. The dashed line indicates an area which obeys Beer's law but which is actually undesirable for use because of the high concentrations involved and narrow range of

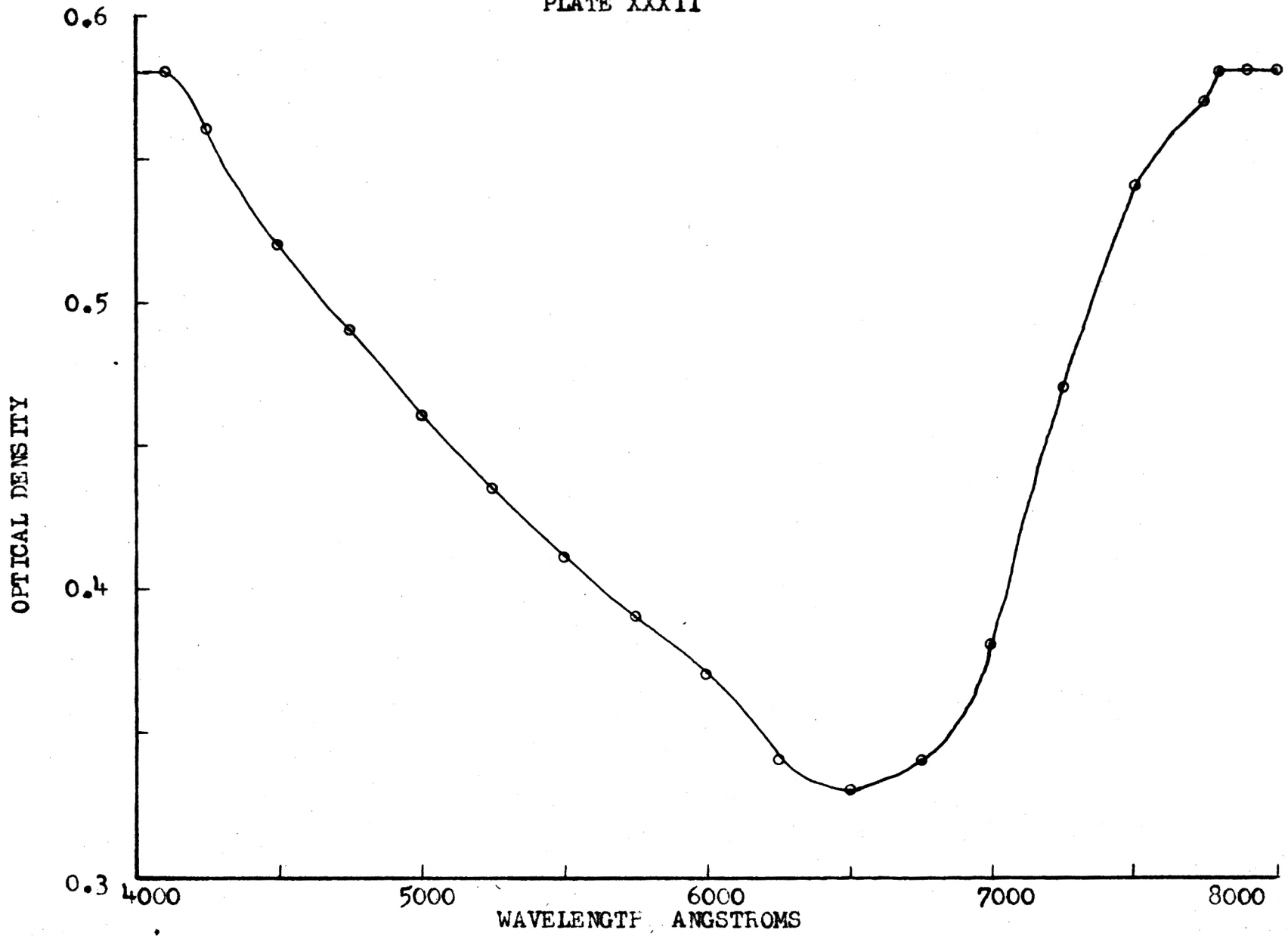
use. The concentrations indicated are in millions of bacterial cells per milliliter of solution as determined by use of the Petroff Hauser chamber. Thus, to a limited extent, for bacteria species of the same general size and shape the spectrophotometer may be used to enumerate bacterial suspensions.

For the studies which were carried out for suspensions of bacteria in thioglycollate broth or Diamond's axenic medium, it was necessary to determine the absorption spectra of these media. Plate XXXVI is the absorption spectra for thioglycollate broth diluted one-half with Locke's solution. Plate XXXVII is the absorption spectra for Diamond's axenic medium. Since these media also have absorption peaks at 4000 A, the absorption of the media were minimized by studying these cultures in either of these media at 6000 A.

EXPLANATION OF PLATE XXXII

The absorption spectrum for Escherichia coli.  
The optical density is plotted against the wave  
length in angstroms for a suspension of heat  
killed Escherichia coli cells in Locke's solution.

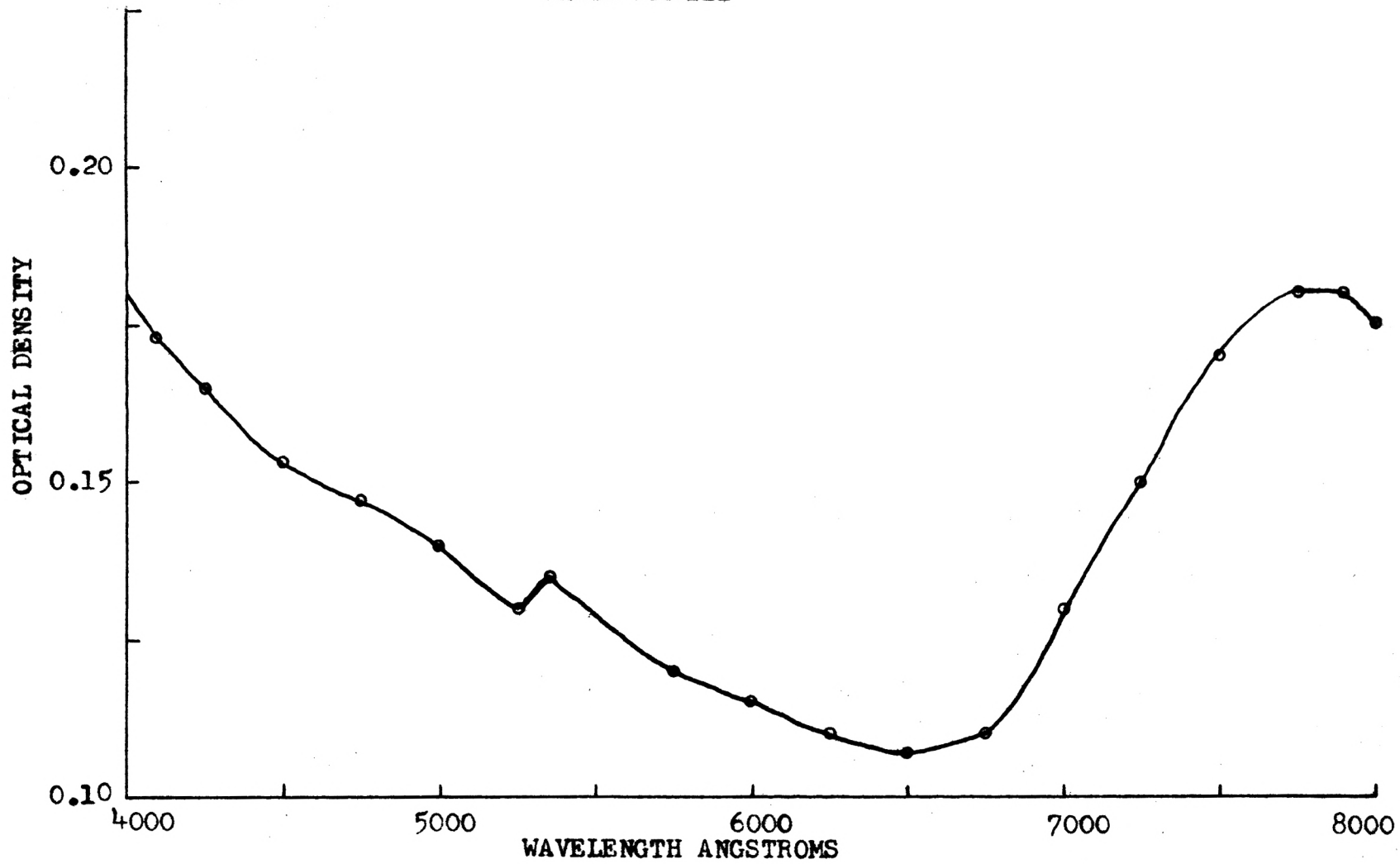
PLATE XXXII



EXPLANATION OF PLATE XXXIII

The absorption spectrum for Bacillus megaterium.  
The optical density is plotted against the wave  
length in angstroms for a suspension of heat  
killed Bacillus megaterium cells in Locke's  
solution.

PLATE XXXIII

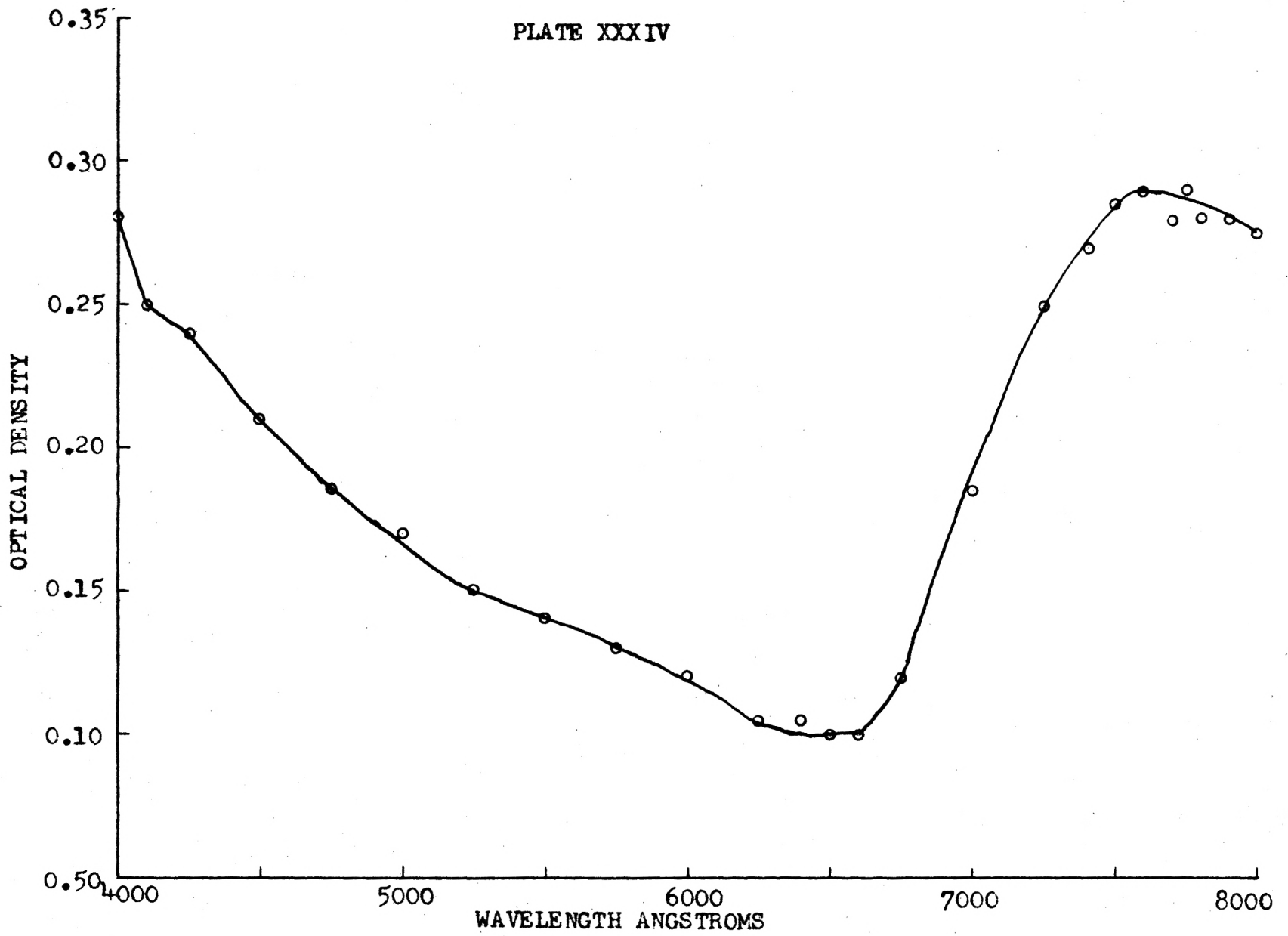


EXPLANATION OF PLATE XXXIV

The absorption spectrum for Bacillus brevis.

The optical density is plotted against the wave length in angstroms for a suspension of heat killed Bacillus brevis cells in Locke's solution.

PLATE XXXIV

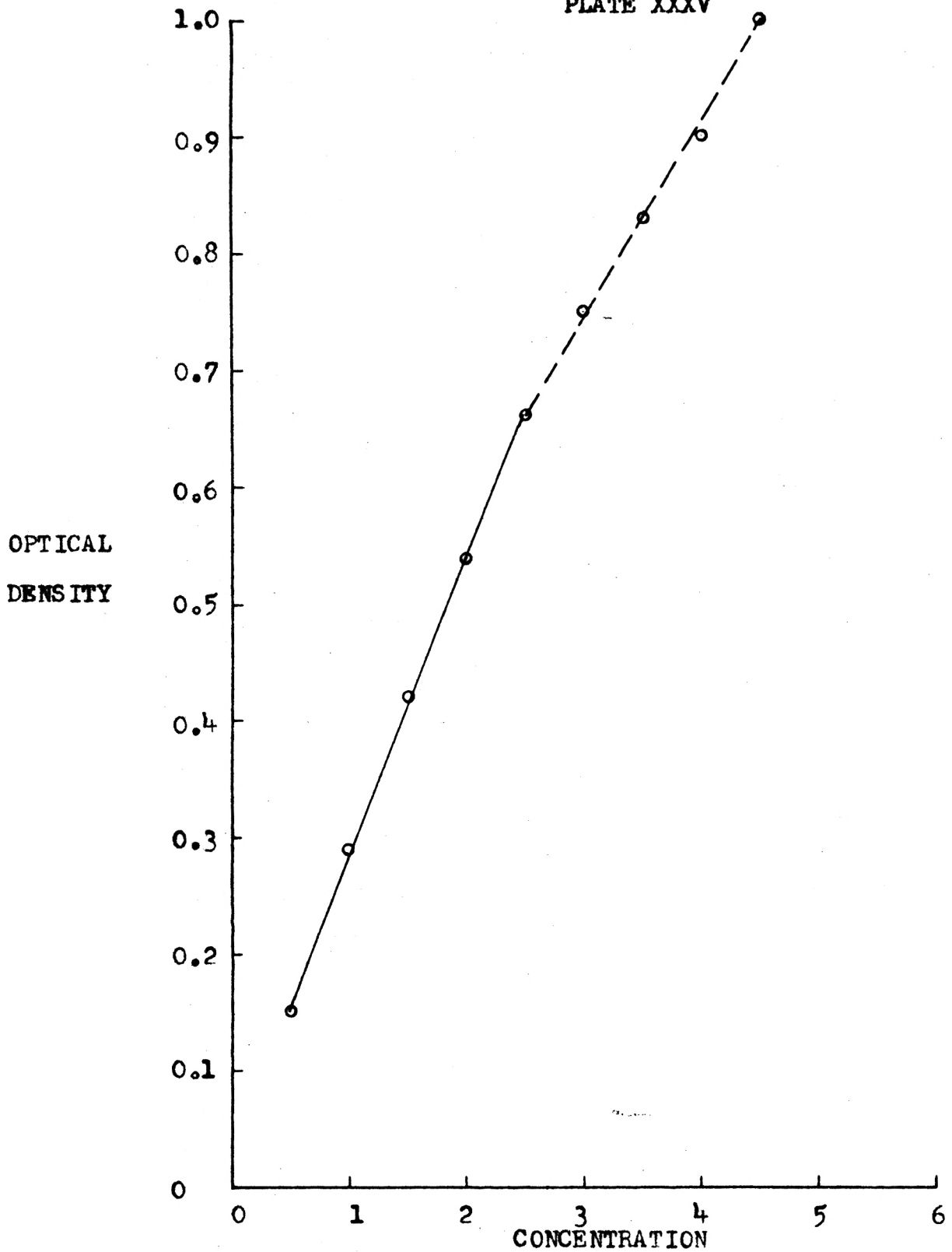




EXPLANATION OF PLATE XXXV

Obediance of Beer's law by a suspension of  
heat killed Escherichia coli cells suspended  
in Locke's solution.

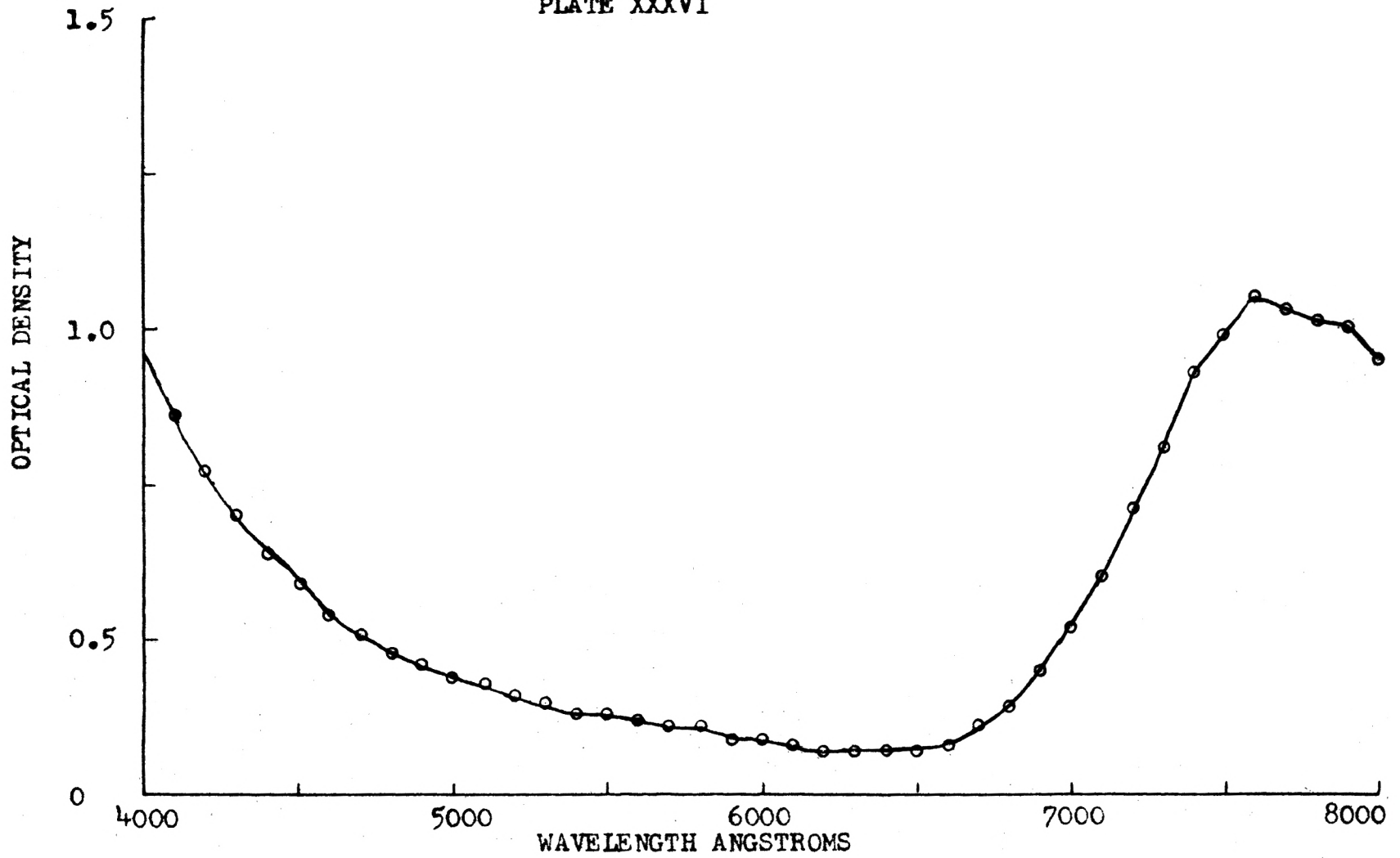
PLATE XXXV



#### EXPLANATION OF PLATE XXXVI

The absorption spectra for thioglycollate broth. The optical density is plotted against the wave length in angstroms for a sterile tube of thioglycollate broth diluted 50% with Locke's solution.

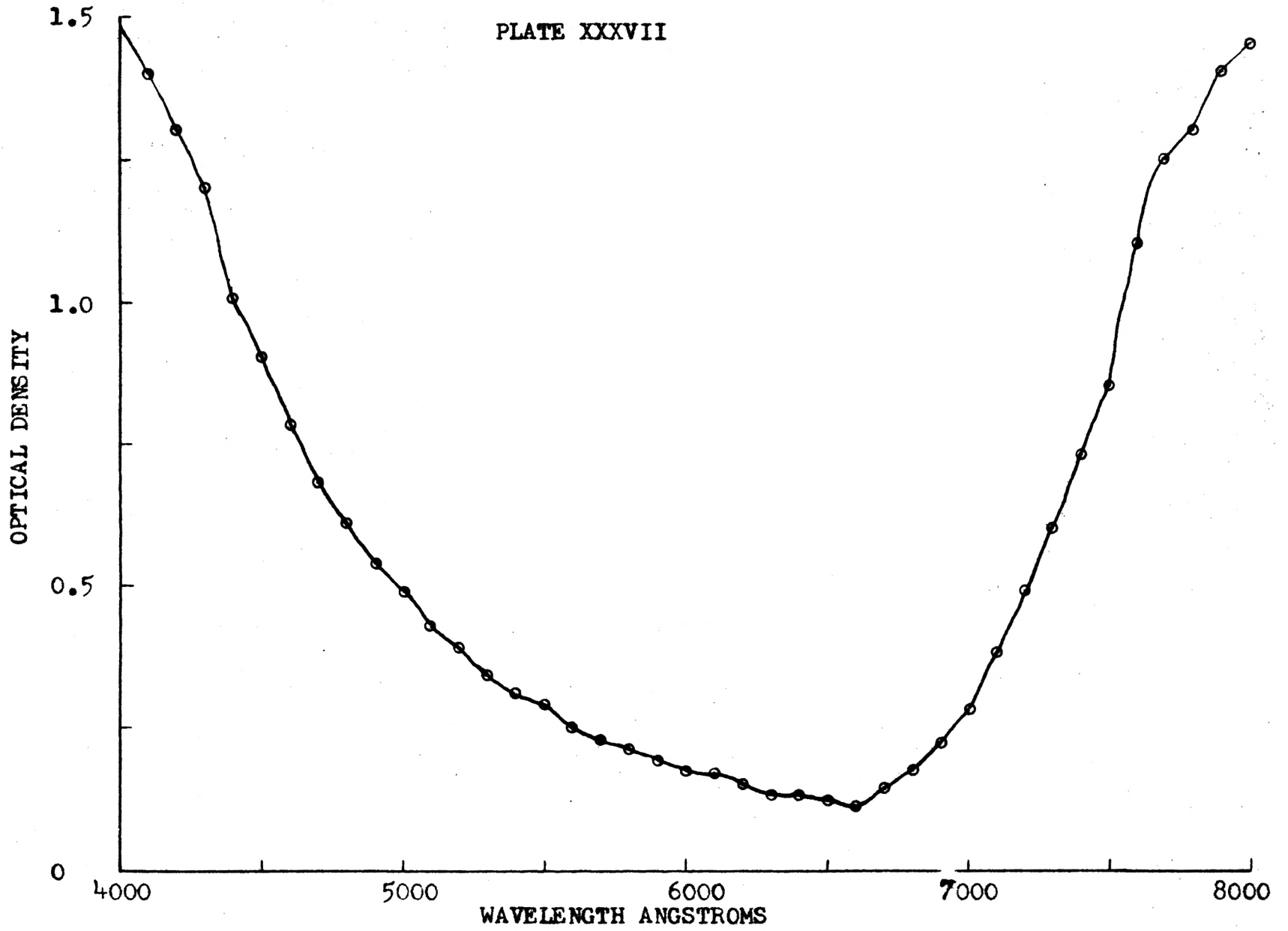
PLATE XXXVI



#### EXPLANATION OF PLATE XXXVII

The absorption spectra for Diamond's axenic medium for Entamoeba invadens. The optical density is plotted against the wave length in angstroms for a sterile tube of the medium.

PLATE XXXVII



ELECTROKINETIC PROPERTIES AND  
BACTERIAL INTERRELATIONSHIPS OF  
ENTAMOEBIA INVADENS RODHAIN, 1934

by

DONALD WAYNE THAYER  
B. S. Kansas State University, 1962

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AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

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Manhattan, Kansas

1963

This study was undertaken in an effort to provide additional information about the bacterial interrelationships of Entamoeba invadens. The investigation was divided into four parts: (a) studying the growth of E. invadens when associated with single species of living bacteria; (b) studying the multiplication of E. invadens by optical absorbence measurements when associated with single species of killed bacterial cells in various fluid media; (c) determining the electrophoretic mobility and charge of E. invadens and its relationship, if any, to the phagocytosis of bacteria by the ameba; and (d) the use of the phase contrast microscope and photomicrography to illustrate the structure of the ameba and the phagocytosis of bacteria.

In monoxenic culture with E. invadens in Locke's egg medium, the following bacteria supported the growth and multiplication of the ameba: Bacillus brevis, Bacillus subtilis, Bacillus subtilis var. niger, Escherichia coli, Aerobacter aerogenes, Staphylococcus epidermidis, Micrococcus lysodeikticus, Proteus rettgeri, Clostridium perfringens and Pseudomonas aeruginosa. The following bacterial species had either adverse effects or no noticeable effect on E. invadens: Aerobacter cloacae, Serratia marcescens, Bacillus cereus, Bacillus megaterium, Bacillus pumilus, Salmonella typhosa, Proteus mirabilis and Mycobacterium smegmatis.

Locke's solution containing heat killed bacterial cells did not support the growth and multiplication of E. invadens. The addition of B. brevis cells but not B. megaterium or E. coli cells to dilute thioglycollate medium increased the survival



and multiplication of E. invadens. The same effect was noted in Diamond's axenic medium without blood serum.

Electrophoretic studies of Entamoeba invadens have demonstrated that the living trophozoites have a negative charge. The trophozoite is killed at pH 4.0 and acquires a positive charge. The isoelectric point of the trophozoite lies between pH 4.6 and pH 4.0. The nature of the surface charge of the ameba is such that it is unlikely that it plays any part in the ability of the ameba to phagocytize bacterial cells. Because they settled rapidly, it was difficult to determine the electrophoretic mobility of the trophozoites. The addition of carboxy-methyl-cellulose to the buffer system effectively delayed the settling and increased the accuracy of the mobility measurements.

Photomicrography with the phase contrast microscope was used to demonstrate the uropod, the nucleus, the vacuolation, the normal active shape and the phagocytosis of bacteria by live E. invadens trophozoites. Although all of these features have been previously described for Entamoeba invadens, they have not, to the knowledge of the author, been photographed previously in the unstained living trophozoite.