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The nutrition of *Flabellula mira* Schaeffer [1].

By

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(With 3 figures in the text.)

Introduction.

The earliest studies on the nutritional requirements of protozoa resulted in information which was secured by observation of the feeding habits of these organisms in wild cultures. The first attempts to obtain cultures of protozoa on pure strains of bacteria were made by Beyerinck (1896). Shortly afterward and even to the present time in successful work dead bacteria, sterile solutions (organic and inorganic), and various particulate media served as a source of nutriment for protozoa (Tsujitani, 1898; Oehler, 1916, 1920, 1920 a, 1921, 1924; Glaser and Coria, 1930, 1933; Reich, 1933; Mast and Pace, 1933), and in one instance (Dawson and Belkin, 1928) substances were injected into the protoplasm. Such diets have been adequate in many cases. On the other hand the inadequacy of dead bacteria, sterile solutions, and particulate media as sources of nourishment may be only apparent; it is very probable that faulty technique was in some cases the cause of failure of protozoa to utilize such foods. Again, it seems that some protozoa have a very restricted diet (Frosch, 1897). The excellent researches of Oehler

[1]) Presented to the Graduate School of Arts and Sciences, Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. To Dr. D. L. Hopkins who suggested and directed the problem the writer desires to express his sincere appreciation. He is also indebted and grateful to Dr. A. S. Pearse, Dr. F. A. Wolf, Dr. H. L. Blomquist and others of the Departments of Zoology and Botany for their cooperation and criticism during the course of the work.

(1916, 1920, 1920 a, 1921, 1924, 1924 a) have shown that certain amoebae were rather selective in their diet of living and dead bacteria, that ciliates which he cultured utilized gram-negative and gram-positive bacteria equally well in contrast to the amoebae which grew much better on a diet of gram-negative bacteria, and that certain amoebae and ciliates could be cultured on sterile media.

A review of the literature, which is superfluous here since LUCK, SHEETS and THOMAS (1931) and PHELPS (1934) have discussed it adequately, will emphasize the variability which exists in the ability of protozoa to utilize a variety of foodstuffs. It seems that protozoa require food factors (vitamins?) other than carbohydrates, fats, and proteins.

The investigation of the nutritional needs of protozoa is fundamental to the advance of protozoology. While many factors operative in the culture of protozoa, such as p_H and salt concentration, can be controlled, very little attempt has been made to control the diet qualitatively and quantitatively. It is possible that many of the reactions and results of experimentation on protozoa may be radically influenced by a variable diet in experimental cultures.

It is true beyond question that there are several things which must be accomplished in order to study more complex problems of nutrition among protozoa. These animals must be cultured (1) on pure strains of known living or dead organisms, (2) in the presence of sterile organic substances of known chemical composition, or (3) in a purely inorganic medium. The culture medium will depend on the organism which is to be studied. The qualitative and quantitative aspects of nutritional studies on protozoa should be emphasized strongly as fundamental to other experimental studies, such as those carried out on reproduction.

Investigation of a marine amoeba, *Flabellula mira* SCHAEFFER, in the Zoological Laboratory of Duke University for the past four years has proved it a suitable animal for experimental purposes. *Flabellula* grows and reproduces well under a rather wide range of p_H, salt concentration, and temperature. Because of the admirable adaptability to external factors and ease of culture in pure lines it was decided to investigate the nutritional requirements of this animal.

Materials and Methods.

Flabellula mira was secured at Tortugas, Florida by Dr. D. L. HOPKINS. It is a marine amoeba but has a rather wide tolerance to various concentrations of hypertonic and hypotonic sea water.

It is rather small, about 25 μ in length, and fan shaped. The anterior border consists of hyaline protoplasm and progresses forward by a sort of wave-like motion. A diagnosis for the genus and species has been given by SCHAEFFER (1926).

The stock cultures were derived from a single ancestor. This stock supplied inoculum for cultures of the animal in an artificial sea water medium based upon an analysis of sea water by MCCLENDON, GAULT, and MULHOLLAND (1917). About 15 cc. of artificial sea water and five grains of wheat were sterilized in a Petri dish by autoclaving. Amoebae were transferred from the pedigreed stock to this medium. Several cultures were started in the same manner and kept in constant subculture at regular intervals. All experiments were performed with descendants of amoebae from these cultures. The cultures were kept in a warm chamber (30° to 34° C.).

The stock bacteria, *Pseudomonas striata* (RAVENEL) CHESTER, *Flavobacterium denitrificans* (LEHMANN and NEUMANN) BERGEY et al., *Achromobacter* sp. BERGEY et al., and *Serratia rubropertincta* (GRASSBERGER) BERGEY et al., were isolated from the stock amoeba cultures, identified by standard bacteriological tests with the same genera and species described in BERGEY'S Manual of Determinative Bacteriology (1925), and checked by repetition of the tests. Stock cultures of the bacteria were kept on agar slants. The medium was made up as follows: One hundred grams of wheat were added to 1000 cc. of artificial sea water in a liter flask which was placed in the autoclave for twenty minutes (15 lb., 125° C.). The boiling mixture was strained through a clean cheese cloth and subsequently filtered through coarse filter paper. Fifteen grams of Bacto agar and sufficient artificial sea water to make a liter were added, the flask was plugged with cotton, and autoclaved to dissolve the agar. The resulting medium was tubed, sterilized, and slanted. The slants were inoculated with the previously mentioned pure strains of bacteria in such a way that a set of stock and experimental cultures was secured.

In the course of the work the silica gel plate was used. A method modified after WAKSMAN und CAREY (1926) was necessary in the preparation of the plates. After dialyzing to remove chlorides, the plates were permitted to dialyze in artificial sea water for an hour. They were dried in a warm chamber (30° to 34° C.) from sixteen to twenty-four hours, sterilized, and stored until needed.

The three monobacterial cultures and the dibacterial culture of amoebae which were obtained were kept on agar slants. One set served for stock, another for use in experiments.

The amino acids used in the experiments were those of the EASTMAN KODAK Company, the sucrose, dextrose, and soluble starch were MERCK "white label" products, the lactose was "BAKER'S Analyzed", and the maltose was manufactured by PFANNSTIEHL. All salts used were "BAKER'S Analyzed" C. P.

Whenever amino acids and carbohydrates were used in solid media, they were added to a basic agar medium made up with artificial sea water; in liquid media the agar was omitted. Amino acids whether singly or in mixtures had a concentration of 0.2 per cent, carbohydrates singly or in mixtures 1.0 per cent; the concentration in the mixture of dl-leucine, glycine, l-tyrosine, and maltose was 0.4 per cent.

All inoculations were made with sterile pipettes or platinum loops under the strictest aseptic conditions. All experiments were tested for contamination by the dilution-plate method. Contaminated cultures were discarded. If liquid media were tested, about one cubic centimeter of the medium, shaken well, was used; if colonies of bacteria or cultures of amoebae on pure strains of bacteria were tested, samples were taken with a platinum loop and plated.

All glassware was thoroughly washed with soap and warm water, rinsed well with tap water, immersed in sulfuric acid-potassium dichromate cleaning solution for several minutes, and then rinsed five times with distilled water.

Experiments and Results.

Agar cultures of amoebae. Several drops of medium from the stock cultures of amoebae were pipetted aseptically onto the surface of 1.5 per cent artificial sea-water-wheat-extract-agar plates (basic medium, wheat extract, and 1.5 per cent agar as described under materials and methods). In the course of several days the surface of each plate at the point of inoculation had absorbed the liquid so that a culture of amoebae and bacteria on a "dry" agar surface resulted.

A study of such agar cultures revealed the following characteristics. The number of amoebae present at any point was directly related to the number of bacteria. Reproduction appeared to be directly related to the food supply. The rates of locomotion, however,

were inversely related to the number of bacteria present. Where great numbers of bacteria were present amoebae reproduced so rapidly that locomotion was entirely inhibited. As a result "tissue-like" layers of amoeba cells were formed.

Locomoting amoebae migrated radially from the point of inoculation onto the sterile portions of the agar surface. As a result of paucity of food the amoebae failed to reproduce, became smaller in size, and finally encysted, sometimes clumping in the process. During the migration the amoebae left refringent microscopic paths or trails which appeared as slight depressions in the agar surface. The fact that the paths could be brought into focus only slightly above or below the focus of the agar surface supported this view.

The anterior rim of locomoting amoebae exhibited a small number of large refractile bodies which were not stained by iodine, neutral red, or DELAFIELD'S haematoxylin; amoebae locomoting in artificial sea water lacked these refractile bodies.

From time to time abnormally large amoebae, "giants", were observed, as many as twenty-five in one culture. They ranged from four to seven times as large as normal amoebae.

Cultures kept at 30° to 34° C. usually completed their cycle in a week. The amoeba cysts were easily activated by transferal to a fresh agar plate, excystation occurring after several hours with subsequent growth and reproduction. Amoeba cysts from agar slants have been activated after a period of a month and healthy cultures obtained. Cysts kept at 0° to 1.1° C. for two months were not viable.

Pure monobacterial cultures of amoebae. It was considered desirable to study the adequacy of monobacterial cultures as a diet for *Flabellula mira*. The bacteria used were those isolated from the sea water cultures previously mentioned. In order to exclude all bacteria except one from the diet, the method of BEYERINCK (1896), slightly modified, was used with success. A platinum loop with a diameter of 6 mm. was brought in contact with a pure strain colony of bacteria, *e. g. Pseudomonas striata*, in such a way that the entire loop on one side was covered with organisms. The loop was then aseptically brought in contact with a sterile agar (wheat extract) surface. The growth resulting from this inoculation was in the shape of a ring. After incubation at 32° C. for twenty-four hours the ring was readily visible. Amoebae with contaminating bacteria were brought to the center of the bacterial ring by means of a very small platinum loop. After several days, amoebae which had migrated radially from the center, had literally digested their

way through the ring of pure strain organisms. Amoebae outside the ring were transferred to the center of a fresh ring. Six repetitions of this process were necessary to secure a monobacterial culture. The amoebae lose their contaminating bacteria during migration, pure strain bacteria are substituted for contaminants, and the contaminating bacteria are limited in spreading by the ring of pure strain bacteria. By this method the following cultures were obtained: 1. *Flabellula mira* and *Pseudomonas striata*, 2. *Flabellula mira* and *Flavobacterium denitrificans*, 3. *Flabellula mira* and *Achromobacter* sp., 4. *Flabellula mira* and *Achromobacter* sp. and *Serratia rubropertincta*. These cultures have been kept in constant subculture for six months without loss in vitality. The same combinations of organisms were cultured in a liquid medium, artificial sea-water-wheat-extract, for a month. They were then discarded because agar slant cultures were found easier to handle and to keep free of contaminating organisms. All cultures were plated regularly, and if contaminated, were discarded. These experiments demonstrated that pure strains of bacteria and a mixture of two strains were adequate diets for *Flabellula*.

Experiments with amino acids and carbohydrates. These substances served as nutrients for the bacteria; the amoebae lived on the bacteria. In the first series of experiments liquid media were used, in the second series solid media. These were made up as described under materials and methods. The following isolated substances and mixtures of substances were used: The amino acids, dl-leucine, glycine, l-tyrosine, dl-alanine, a mixture of the first three, and a mixture of all four; the carbohydrates, sucrose, dextrose, lactose, maltose, soluble starch, a mixture of sucrose, dextrose, and lactose, a mixture of dextrose, lactose, and maltose, and a mixture of lactose, maltose, and starch. Ten cubic centimeters of each medium was introduced into each of fifteen test tubes, the tubes were plugged with cotton and sterilized in the autoclave. Five tubes of each set of fifteen were inoculated with *Flabellula mira* and *Pseudomonas striata*, five with *Flabellula mira* and *Flavobacterium denitrificans*, and five with *Flabellula mira* and *Achromobacter* sp. The cultures were examined on the fourth and seventh days after inoculation. In most cases growth of amoebae was found, but the extent of growth was variable. A number of cultures showed no growth. An explanation of this is found in the observation that amoebae were so firmly attached to the walls of the test tubes containing stock cultures that it was impossible to dislodge them by shaking without bringing

the medium in contact with the cotton plug, a procedure which might lead to contamination. Consequently, inocula for the experimental cultures contained too few amoebae, so that a number of cultures failed to develop.

The second series of experiments confirmed this view. Agar media containing dl-leucine, glycine, l-tyrosine, dl-alanine, a mixture of the first three, a mixture of all four, sucrose, starch, and a mixture of dl-leucine, glycine, l-tyrosine, and maltose were prepared. A control medium of 1.5 per cent agar in artificial sea water was used. Sixteen plates of each medium, including the control medium, were poured. Four were inoculated with *Flabellula mira* and *Pseudomonas striata*, four with *Flabellula mira* and *Flavobacterium denitrificans*, four with *Flabellula mira* and *Achromobacter* sp., and four with *Flabellula mira* and *Achromobacter* sp. and *Serratia rubropertincta*.

Amoebae developed in all cultures. Growth and reproduction were rapid, the cycle of the culture being completed in about a week. Thus the experiments proved that pure strain bacteria and a mixture of two bacteria which had been nourished on the various isolated amino acids, carbohydrates, and mixtures were an adequate diet for *Flabellula*. They also showed that the results obtained in the series of experiments with liquid media were influenced by the number of amoebae inoculated from stock cultures.

The amoebae which wandered from the point of inoculation onto the sterile portions of the agar became smaller and smaller and finally encysted on both experimental and control plates, but on the control plates this was much more marked. This indicated that the vitality of the amoebae was dependent on the adequacy of the medium as a diet for the bacteria which were used for food, or that nutrients were utilized by amoebae directly from the experimental plates. Decrease in size with ultimate encystment of amoebae on the sterile agar surface demonstrated that the amoebae were unable to utilize the dissolved nutrients to support continuous growth and reproduction. If the nutrients had been adequate, amoebae should have multiplied in number and covered the sterile agar surface. However, it is not denied that dissolved nutrients may have supplemented the bacterial diet.

Experiments on the utilization of dead bacteria by amoebae. A drop of culture medium containing a heavy suspension of dead organisms, e. g. *Pseudomonas striata*, was placed aseptically on an agar surface. After twelve hours or slightly longer time the liquid had been absorbed or evaporated, and a

bacterial spot or area was evident. Some 4 to 5 mm. distant from the bacterial spot amoebae with living bacteria (*Pseudomonas striata*) were inoculated. In about twenty-four hours a few amoebae had migrated across the sterile intervening space and entered the area of dead bacteria in a sterile condition or comparatively so. The agar bearing the amoeba colony (point of inoculation) and the agar between it and the area of dead bacteria was aseptically cut out and removed with a sterile needle. The progress of the amoebae in the area of dead bacteria was observed and in no case was growth and reproduction observed. If cytoplasmic division occurred at all, it soon ceased; amoebae became smaller and smaller in size and encysted. The same results were obtained when dead bacteria of the species *Flavobacterium denitrificans*, *Achromobacter* sp., and *Serratia rubropertincta* were used. The bacterial spots on plates were tested for sterility by sampling the area with a sterile platinum loop and then streaking it on sterile agar. Sterility was evident in all cases by the failure of bacterial colonies to develop from the streaks.

The bacteria in these experiments were killed by autoclaving heavy suspensions of them at 125° C. (15 lbs.) for one hour. There is a possibility that this treatment was responsible for chemical changes in the bacteria which made them inadequate as a diet for the amoebae.

Attempts were made to kill the bacteria by exposing them to ultra-violet light without destroying the substances so necessary for the growth and reproduction of amoebae. Eighty plates were prepared from stock media of the various amino acids, carbohydrates, and mixtures. Eight plates of each type of medium (amino acids, carbohydrates, and mixtures) were secured, of which two received a drop each of a heavy suspension of *Pseudomonas striata*, two *Flavobacterium denitrificans*, two *Achromobacter* sp., and two *Serratia rubropertincta*. After loss of the liquid over night each plate showed a circular area of living bacteria. The plates were exposed to ultra-violet light for five and ten minute periods, this length of exposure having been found sufficient to kill all bacteria. The circular bacterial areas were then tested for sterility by streaking samples from several parts of each area. Sixty of the plates were sterile, twenty still showed living bacteria. Amoebae and pure strain bacteria (similar in species to the dead strains) were inoculated at a point 5 mm distant from the bacterial area on each sterile plate, cutouts of agar were made after migration of several

amoebae into the area of dead bacteria, and the progress of the amoebae observed. With exception of the twenty contaminated plates none of the areas of dead bacteria showed growth and reproduction of amoebae. Amoebae always became smaller and finally encysted. Cases of growth and reproduction were found among the twenty plates which tests demonstrated to contain living bacteria, thus proving that living bacteria are necessary for continuous growth and reproduction.

The different media in these experiments were used because it was thought that the nutrition of the bacteria before killing might have some influence on their value as food for amoebae. No influence was observed.

Experiments with carbon particles. It was found in earlier experiments that amoebae were unable to utilize amino acids, carbohydrates, and mixtures of these dissolved in agar. It is possible that these substances were not taken through the cell membrane. If such substances could be introduced into the protoplasm of amoebae, perhaps growth and reproduction would result.

Carbon particles (lamp black) of very small size, ranging from to 10 μ in diameter, were placed in 0.2 per cent solutions of a leucine-glycine-tyrosine mixture, 0.4 per cent solutions of a leucine-glycine-tyrosine-maltose mixture, and 1.0 per cent solutions of maltose. The resultant suspensions of carbon particles were sterilized by autoclaving. Drops of these suspensions were pipitted aseptically onto the surfaces of thirty agar plates so that ten received the first suspension, ten the second, and ten the third. After loss of water a circular area of carbon particles appeared on each plate. The particles presumably carried some of the organic substances on their surfaces either as a solution film or as crystals. Amoebae and *Achromobacter* sp. were inoculated at a point 5 mm. distant from the area of carbon particles. After the migration of several amoebae into this area cutouts of agar were made as in preceding experiments.

Observation of the amoebae which had migrated showed that they readily ingested the carbon particles; these could be seen in the protoplasm. However, in no case did growth and reproduction occur. The usual decrease in size followed by encystment resulted. The experiments support the conclusion reached in earlier experiments in which amino acids, carbohydrates, and mixtures were dissolved in agar, that these dissolved nutrients were inadequate as food for amoebae.

Experiments with living dormant bacteria as food for amoebae. In all of the experiments in which bacteria served as a source of nourishment for amoebae a nutrient medium was present. It was found that wheat extract, amino acids, carbohydrates, and various mixtures would not in themselves support growth and reproduction. Emphasis was laid on the bacteria as the important source of energy for growth and reproduction. Several questions arose. 1. Are bacteria alone sufficient in the nutrition of *Flabellula* or are certain nutrients in the medium essential? 2. Are bacteria which are growing and reproducing the only source of nutrients or can living dormant bacteria (resting cells) serve as an adequate diet?

To each of four chemically clean 250 cc. flasks 200 cc. of nutrient bouillon consisting of 0.3 per cent beef extract, 0.5 per cent peptone, and 1.0 per cent dextrose in artificial sea water were added. The bouillon was sterilized in the autoclave. The first flask was inoculated with *Pseudomonas striata*, the second with *Flavobacterium denitrificans*, the third with *Achromobacter* sp., and the fourth with *Serratia rubropertincta*. The cultures were incubated for several days at 30° to 34° C., that is, until a heavy growth had been obtained. The organisms were then concentrated separately by alternately centrifuging and washing them with artificial sea water; six washings were considered sufficient to rid the bacteria of all organic matter. The sedimentation of organisms in the centrifuge tubes was uniform, no variation in color from top to bottom being apparent.

Four silica gel plates were prepared. The first received a heavy suspension of *Pseudomonas striata*, the second *Flavobacterium denitrificans*, the third *Achromobacter* sp., and the fourth *Serratia rubropertincta*. The organisms were carried to the surface of the plates in sterile pipettes and placed so as to form a circular area with a diameter of some 10 to 15 mm. After evaporation and absorption of excess water the plates were inoculated with monobacterial cultures of *Flabellula mira* at a point about 5 mm. distant from the circular area of washed organisms. The inoculations were made so that the bacteria accompanying the amoebae corresponded to the washed bacteria as to species.

The amoebae soon migrated across the sterile portion of the silica gel surface into the area of washed bacteria. In this area growth and reproduction were as good apparently as in any cultures in which nutrient media had been used. The cultures on silica gel plates were kept in subculture for a month at the end

of which time they were discarded. There is little doubt, however, that they could have been continued indefinitely.

In these experiments an answer is found to the questions raised earlier. 1. Bacteria alone may serve as an adequate diet for *Flabellula*; dissolved nutrients are not needed to supplement the diet. 2. Living dormant bacteria may serve as an adequate diet demonstrating that those substances essential as food for *Flabellula* are present in bacteria which are not growing or reproducing.

Appearance of amoebae on silica gel plates. The appearance of amoebae on silica gel plates did not differ from the appearance of these animals on agar plates. Refractile granules or bodies were present in the anterior rim, trails were apparent in the gel surface, and giant amoebae were observed. The presence of trails on silica gel plates disposes of any assumption that such trails were the result of enzymes excreted by the amoebae, inasmuch as there is no known enzyme which will act on both agar and silicic acid.

An experiment was suggested to test whether giant amoebae found on agar plates were the result of the presence of lactic acid produced by bacteria. Eight silica gel plates were prepared. Two were supplied with circular areas of washed (six times) *Pseudomonas striata*, two with *Flavobacterium denitrificans*, two with *Achromobacter* sp., and two with *Serratia rubropertincta*. All were inoculated with amoebae and the corresponding bacteria except in the case of *Serratia rubropertincta* which plate received a mixture of this organism and *Achromobacter* sp. Inoculations were made some 5 mm. distant from the circular areas of washed bacteria. One set of four plates served as controls, the other set was used experimentally in the following way: To each circular area of washed organisms two large drops of lactic acid solution (in artificial sea water) of p_H 5.4 were added. If lactic acid was the direct cause of giant amoebae, these should be produced in fairly large numbers on experimental plates as compared with control plates which should exhibit few or none. The only lactic acid that could possibly be present in control plates would be contained in bacterial cells or produced by amoebae.

After migration of amoebae into the areas of washed organisms it could be see throughout the period of culture that the numbers of giant amoebae were very small (two or three per plate), and the experimental plates showed no more giant amoebae than the controls. Nor were other differences, such as inhibition of reproduction,

observed in the experimental plates. The presence of lactic acid in amoeba cultures was not the cause of giant amoebae. Giant forms appeared in cultures at times, at other times they were absent during the course of a year's work with pure monobacterial strains of amoebae. None of the bacteria used in this work belong to the lactic acid group; their lactic acid output would be rather low.

Sensitivity of amoebae to food. The amoeba trails on agar plates offered excellent opportunities for observing the reactions of amoebae to food. The trails were easily traced with the aid of a camera lucida and their relationship to isolated bacterial colonies on agar plates ascertained. The observations, which are to be recorded from definite experiments, have been made many times in the course of a year.

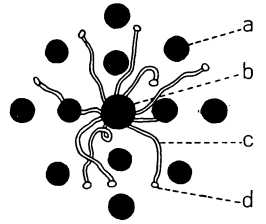

Fig. 1. Diagram of the inoculation pattern of agar plates used in sensitivity experiments. a, colony of bacteria; b, point at which amoebae and pure strain bacteria were inoculated; c, an amoeba trail; d, an amoeba.

Thirty-two artificial sea-water-wheat-extract-agar plates received by pipette small drops of bacterial suspensions to form a pattern as shown in figure 1. Eight plates received *Pseudomonas striata*, eight *Flavobacterium denitrificans*, eight *Achromobacter* sp., and eight *Serratia rubropertincta*. After loss of the excess water each set of eight plates was inoculated in the center of the pattern with amoebae and the particular bacterium which corresponded to the species of bacterium in the pattern, except in the case of plates containing patterns of *Serratia rubropertincta*, these plates receiving the combination amoebae, *Achromobacter* sp., and *Serratia rubropertincta*.

Migration of amoebae from the point of inoculation was radial in a general way. Thus some animals would move into one of the peripherally placed colonies of bacteria, others would pass between the colonies. A number of paths of migration might be mentioned, but what should be considered as important is what might happen when an amoeba approached one of the colonies. There were several possibilities. The amoeba might migrate into the colony and remain, it might migrate into the colony and shortly afterward migrate out, it might migrate in, out, and then return. etc., or it might migrate very close to the colony even to the point of barely making contact and then migrate away not to return.

The two possibilities which are important as criteria of the sensitivity of *Flabellula mira* to food are these: 1. If an amoeba approaches a colony of bacteria so that it passes within 20 μ of the colony (slightly less than the diameter of the amoeba) and does not enter, or actually establishes contact without entrance being effected, the amoeba may be considered as insensitive to the bacteria. 2. If an amoeba enters the colony to leave it shortly afterward, regardless of whether it returns, the act of departure may be considered as evidence of the insensitiveness of the amoeba to the bacteria (Textfig. 2).

The first criterion needs little qualification. On approach to the colony the amoeba may with propriety be assumed to be in need of food, because it has just completed a journey of some 5 mm. across a sterile agar surface (this only applies to the first fifteen or twenty animals completing the migration since amoebae effecting the migration subsequently will find bacteria which have been left behind by the first group).

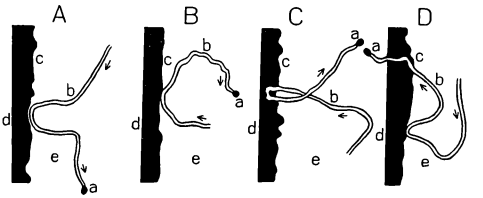

Fig. 2. Camera lucida sketches showing the relationship of amoeba paths to the edges of bacterial colonies (× 175). A, path passes within 2.7 μ of edge of colony; B, path touches edge of colony; C, path enters colony and leaves it shortly afterward; D, path touches colony and later enters; a, amoeba; b, amoeba path; c, edge of bacterial colony; d, section of edge of bacterial colony; e, sterile agar surface.

With regard to the second criterion it may be argued that the amoeba may be satiated when it leaves the colony of bacteria and hence the stimulus of food would have little effect in keeping the amoeba in the bacterial colony. This is a legitimate objection. However, on the assumption that an amoeba eats, grows, and reproduces continuously and that satiety does not occur as in higher animals, and in addition, if an amoeba is sensitive to its food, then the amoeba should never leave the bacterial colony. For when the amoeba begins to leave, certain areas of the membrane will have lost contact with the colony, and will not be stimulated as strongly by dissolved bacterial products which theoretically have a decreasing concentration gradient from the center of the bacterial colony. Therefore, the amoeba should locomote toward the side which is stimulated strongest, the side toward the colony (Textfig. 3).

No differential results were obtained by using the previously mentioned pure strains of bacteria as stimuli. The results have

been combined for the four species. The first criterion was supported by 57 cases on 32 plates, the second by 38 cases on the same number of plates. That is to say, 57 amoebae passed within 20 μ of a bacterial colony or actually established contact without entering, while 38 amoebae entered the colony only to depart shortly afterward. If *Flabellula mira* was sensitive to the presence of food, it certainly should have reacted positively in all cases under the conditions described. The conclusion may be drawn that *Flabellula mira* was insensitive to its food even when in actual contact with it. The evidence makes it very probable that the finding of food is a matter of chance. There is no reason for supposing that some of the amoebae were sensitive to food while others were not.

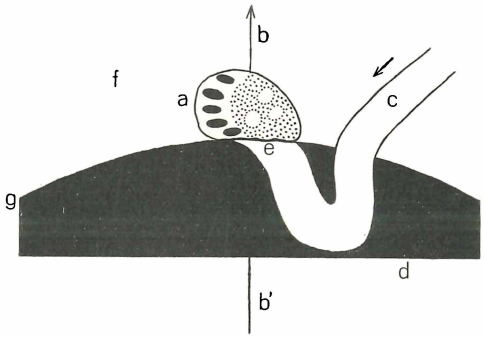

Fig. 3. Diagram of the relationship of an amoeba to the edge of a bacterial colony. a, amoeba; b-b', concentration gradient of bacterial products (decrease in direction of arrow); c, amoeba trail; d, section of bacterial colony; e, point where stimulation should be most intense; f, sterile agar surface; g, edge of bacterial colony.

Discussion.

The observations made on *Flabellula mira* as it appeared on agar plates are in agreement with those made by Mouton (1902), Wherry (1913), and Oehler (1916) on other species. Mouton described clumping of amoeba cysts and suggested that the amoebae rounded up more easily when in contact with each other. Mouton and Oehler both had observed the microscopic paths left by amoebae on agar surfaces but offered no explanation. Evidence has been presented in this paper to show that they probably resulted from the pressure of amoebae on the agar surface. Horizontal locomotion of amoebae and the force of the weight of these animals would produce either a depression in the agar or a shearing of the prominences on the agar surface. It has been shown that the paths were not the result of enzymatic action. The refractile bodies observed in *Vahlkampfia* sp. by Wherry may be identical with those found in the anterior rim of *Flabellula mira*. Wherry believed that these bodies were important in the oxidative processes of the cell. Attempts to identify the refractile bodies in *Flabellula mira* with

neutral red granules, glycogen, starch, and chromatin by staining have failed.

With reference to the utilization and adequacy of pure strains of bacteria as the sole diet of protozoa the results for rhizopods and ciliates differ. The work of BEYERINCK (1896), TSUJITANI (1898), MOUTON (1902), OEHLER (1916), and CASTELLANI (1930) shows that various amoebae are capable of utilizing pure strains of bacteria, yeasts, and several molds, and that pure strain diets are adequate. On the other hand FROSCH (1897) found that *Amoeba nitrophila* reproduced better on one kind of bacterium than any other. The findings in the present work support those of the majority of workers. Three pure strains of bacteria and a mixture of two strains were found to be adequate diets for *Flabellula mira.*

While amoebae used by OEHLER (1916) utilized gram-negative bacteria much better than gram-positive organisms, ciliates (OEHLER, 1920) seemed to utilize one type as well as the other. HARGITT and FRAY (1917) found pure strains of bacteria inadequte as food for *Paramecium* and were supported by PHILLIPS (1922). PHILPOTT (1928) obtained flourishing cultures of *Paramecium aurelia* MÜLLER, *Paramecium caudatum* EHRBG., and *Paramecium calkinsi* WOODRUFF on a pure strain culture of *Bacillus pyocyaneus* GESSARD. DAMERON (1931) was unable to culture *Paramecium* on pure strains of tubercle bacilli. HARGITT and FRAY (1917) found that *Paramecium aurelia* would not live on a pure strain culture of *Bacillus fluorescens,* which is in contradiction to the work of OEHLER (1920) who obtained good cultures of *Paramecium aurelia* on the same organism.

The substrates used for the culture of amoebae and other protozoa bacteria-free have been many, but in few cases have successful cultures resulted. OEHLER (1924) obtained sterile cultures of *Amoeba radiosa* DUJARDIN and a pond amoeba on coagulated serum which had been spread on an agar plate. The same worker (1920) cultured *Bodo, Prowazekia,* and *Colpoda* on powdered egg white, powdered fish muscle, casein, and edestin in the absence of bacteria. GLASER and CORIA (1933) grew *Paramecium caudatum* on a medium of liver extract, killed yeast cells, and pieces of rabbit kidney. ELLIOTT (1933) was successful in culturing *Colpidium striatum* STOKES on several sterile media. These successful cases are few when the great number of failures are considered. In the experiments with *Flabellula mira* sterile amino acids, carbohydrates, and mixtures of these failed to support growth and reproduction even when these substances were held on carbon particles and were ingested into

the protoplasm. There is a possibility that the quantity of substance borne by the carbon particles was insufficient.

Several important questions arise with regard to the essential food substances of amoebae. 1. Are these substances found only in living bacteria? 2. Are the requirements of protozoa varied with regard to essential food substances? 3. Does the surrounding nutrient medium supply the substances in sufficient quantities to support growth and reproduction? 4. Are nutrients necessary in the medium? 5. Does the physiological state of a bacterium affect the value or presence of such substances as food for amoebae?

The first two questions may be discussed first. The substances may be found in coagulated serum since OEHLER (1924) successfully cultured two amoebae on this substance. On the other hand killed bacteria, yeasts, etc. have not always yielded successful cultures. OEHLER (1916) pointed out that the temperature at which a bacterium is killed is important. Certain amoebae would live on dead bacteria only if these were killed below certain temperatures while others were not so limited, being able to utilize bacteria which had been killed at 130° C. However, *Flabellula mira* was unable to utilize bacteria which had been killed both by heat and ultra-violet light. Whether the variation among amoebae is the result of the destruction of different substances in bacteria at different temperatures is not known.

The experiments with *Flabellula mira* showed that the surrounding nutrient medium would not support continuous growth and reproduction. There is no doubt that sufficient foodstuffs were present, and it seems very probable that the essential substances were absent. That essential substances dissolved in the medium were unnecessary was shown by the experiments in which amoebae existed entirely on washed bacteria which were given them on silica gel plates. Such a diet was shown to be adequate.

Does the physiological state of a bacterium affect the value or presence of essential nutrient substances as food for amoebae? There is no doubt that living dormant (resting) bacteria present a different physiological state than growing reproducing bacteria. When living dormant bacteria were given as food to *Flabellula mira*, the growth and reproduction of this amoeba was as good as in cultures with growing reproducing bacteria. Therefore, it may be concluded that this particular change in physiological state does not affect the value or presence of substances essential to the growth and reproduction of *Flabellula mira*.

SCHAEFFER in a series of papers (1916, 1916 a, 1917, 1917 a, 1917 b) presents evidence to show that *Amoeba proteus* PALLAS, *Amoeba dubia* SCHAEFFER, and *Amoeba discoides* SCHAEFFER are sensitive to certain test objects some of which are soluble, some insoluble, some digestible, and some indigestible. He thought that motion of the test particle or natural food particle is an important factor, perhaps the most important factor. The experiments with *Flabellula mira*, however, showed that this animal was insensitive to the presence of food even when actual contact was established with the food. It seemed to be a matter of chance whether a migrating amoeba entered a bacterial colony or passed it at very close range.

Summary.

1. *Flabellula mira*, a marine amoeba, has been cultured for six months without loss of vitality on pure strains of *Pseudomonas striata*, *Flavobacterium denitrificans*, *Achromobacter* sp., and a mixture of *Achromobacter* sp. and *Serratia rubropertincta*.

2. When the same pure strains of bacteria and the mixture were grown on media containing various amino acids, carbohydrates, and certain mixtures, these bacteria served as an adequate diet for *Flabellula mira*, but this amoeba was unable to utilize any of the substances bacteria-free.

3. *Flabellula mira* was unable to utilize pure strains of *Pseudomonas striata*, *Flavobacterium denitrificans*, *Achromobacter* sp., or *Serratia rubropertincta* after these were killed by heat or ultra-violet light.

4. *Flabellula mira* was able to obtain all of the essential substances needed for growth and reproduction from washed (living dormant) bacteria. Nutrients in the surrounding medium were unnecessary.

5. The paths left by *Flabellula mira* on agar and silica gel plates were probably the result of pressure of the animal or a shearing of the prominences of the gel surface, that is, the trails were probably depressions in the gel surface.

6. Giant forms of *Flabellula mira* found on agar plates were not a result of the presence of lactic acid.

7. *Flabellula mira* was found to be insensitive to the presence of food at a distance of 20 μ on agar plates. Sensitivity was absent even when contact with food was established.

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