Contamination by Air-borne Amoebae in Bacteriophage Typing[1]

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DURING routine bacteriophage typing of *Eberthella typhosa* in this laboratory, confluent lysis was obtained with one of the cultures when tested with Type H phage. As it was the first time that this type was encountered on the Island, the test was repeated. However, a second trial performed under the same experimental conditions produced no lysis with the same phage.

Looking for an explanation for these inconsistent results, a careful examination was made of the original plate. Macroscopical examination revealed that the area of lysis did not differ, to any marked extent, from a typical area of phage lysis. However, when the plate was observed under low power, a large number of round bodies were noticed. These covered the whole lysed area, except at the borders, where they merged gradually into a mass of closely packed structures of irregular outline.

Some of this material was mounted in saline and observed under higher magnification. It was found to consist of sluggishly motile amoebae and a large number of round cysts.

Other material from the cleared area was streaked on nutrient agar and blood agar plates, but no growth was obtained after two days of incubation at 37° and 26°C. The amoeba was inoculated on the surface of 24-hour-old agar cultures of *E. typhosa*. After 24 hours of incubation at the above-mentioned temperatures, a clear area 10 to 20 mm. in diameter was noted in both the nutrient and blood agar plates. This area was exactly like the one seen during the typing process, demonstrating that the clearing of the culture was due to the activity of the growing amoebae and that the amoebae, apparently, were parasites of the bacterial cultures.

Suspecting that the clearing of the cultures might be the result of an enzyme, an attempt was made to extract this substance from the organisms. The amoebae were inoculated on typhoid cultures in agar slants and incubated at 26°C. until the whole culture was cleared away. This occurred in about four days. The amoebic growth was then suspended in saline and centrifuged. The mass of

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packed amoebae was macerated in a small amount of saline, centrifuged, and filtered through a Seitz filter. Both the sterile filtrate and the supernatant were tested for bacteriolytic and bacteriostatic action against typhoid bacilli, but with negative results.

An amoeba with a similar action, parasitic on cultures of a yeast-like fungus, was found by Castellani.[2] Douglas[3] reported this amoeba to be a new species and called it *Hartmanella castellanii*, and Van Rooyen[4] studied in detail its effect upon bacterial cultures. Later Shinn and Hardley[5] found an amoeba resembling *H. castellanii* which was a spontaneous contaminant of a Friedlander's bacillus in culture. However, this amoeba differed from *H. castellanii* in the polygonal shape of the cysts, most of which had a triangular form. A third investigator to isolate a similar amoeba was Hewitt,[6] who found it as a contaminant of Rhizobium cultures. He studied its natural habitat and was able to isolate it from the soil and under the surface of damp logs but never from plates exposed to the air. Nevertheless, due to the resistance of the dried cysts, which could be transported by air currents, he considered the possibility of their transmission by such means. More recently Negroni and Fischer[7] described an amoeba isolated from an apple that was found to be parasitic on the yeast, *Pichia belgica*, and classified it as *Vahlkampfia debilis* (Jollos, 1917). In our case, amoebic contamination seemed to be air-borne for, after repeated tests, both the original typhoid culture and the phage were found free from amoebae.

When typhoid cultures on nutrient agar plates were exposed to the air, a second amoeba was isolated. It closely resembled the first one, but it took more time to produce any clearing action, which was not evident until after 48 hours of incubation. By inoculating similar plates with dust from the floor of the room, two other amoebae were isolated with clearing action upon typhoid cultures.

It is thus evident that amoebae capable of clearing bacterial cul-

2. A. Castellani, An amoeba found in cultures of yeast. J.Trop.Med.and Hyg., **33**:160, 188, 221, 237, 1930.

3. M. Douglas, Notes on the classification of the amoeba found by Castellani in cultures of a yeast-like fungus. J.Trop.Med.and Hyg., **33**:258, 1930.

4. E. E. Van Rooyen, The effect of amoeba (*Hartmanella castellanii*) on bacterial cultures. J.Trop.Med.and Hyg., **35**:118, 1932; Observations on the clearing effect of amoeba (*Hartmanella castellanii*) on bacterial cultures; a phenomenon simulating bacteriophagy. *Idem*, **35**:259, 1932

5. L. Shinn and P. Hardley, Note on the spontaneous contamination of a bacterial culture by an organism resembling *Hartmanella castellanii*. J.Infect.Dis., **59**:23, 1936.

6. R. Hewitt, The natural habitat and distribution of *Hartmanella castellanii* (Douglas), a reported contaminant of bacterial cultures. J.Parasitol., **23**:491, 1937.

7. P. Negroni and I. Fischer, Biology of *Vahlkampfia debilis* (Jollos, 1917) and blastomycetic and bacterial lysis similar to bacteriophage. Rev.d.Inst.Bact., Buenos Aires, **10**:66–96, 1941.

tures are widely distributed and may contaminate bacterial cultures when their cysts are transported by air currents. During phage-typing procedures, the plates are usually exposed to this sort of contamination. It is convenient, therefore, for those engaged in bacteriophage typing of staphylococci, *Salmonella*, *Shigella*, and so forth, to keep present this possible source of error in their work, since the type of clearing produced by these amoebae has a very close superficial resemblance to phage lysis (Fig. 9).

As we are not too well versed in classification, we shall not make any attempt to assign these amoebae to any particular species. We shall merely limit ourselves to a description of what we saw and to pointing out some differences and similarities between these amoebae and *H. castellanii* (Douglas).

Most of the organisms herein described are similar in that they have the type of nucleus usually found in the so-called *Amoeba limax* group, consisting of a large, dark-staining, karyosome surrounded by a clear halo, the latter being enclosed in a nuclear membrane. They differ from the *limax* types in their small size and in their parasitic habits upon bacterial cultures. They also differ among themselves in several other aspects, but we cannot say whether these mark them as different species or as variants of the same species.

Morphology of the amoebae

Amoeba No. 1. In its vegetative state, this amoeba had an irregular outline, but many rounded forms were observed, probably representing initial stages of encystment. Motility was slow by the emission of one pseudopodium at a time; micropseudopodia were not observed. Its dimensions varied between 15.7 and 21.0 u. in diameter (Fig. 5).

The cysts were round with a double contoured membrane, their dimensions varying from 10 to 12 u. in diameter (Fig. 11).

Preparations stained in phosphotungstic acid hematoxylin showed a single, well-defined nucleus surrounded by a delicate nuclear membrane and followed by a clear space located between it and the large, round or oval, central karyosome. This nucleus was usually about 3.4 u. and similar to that of the vegetative amoeba; it was usually found in a central or slightly excentrical position. The karyosome was about 1.7 u. in diameter. The cysts were mononuclear with an outer double contoured wall.

Amoeba No. 2 (isolated from the air). This amoeba was slightly smaller than amoeba No. 1, said size being maintained throughout the experiments, and varying, in its vegetative state, from 11.3 to 17.1 u. in diameter. Motility was sluggish.

The cysts ranged from 6.7 to 8.6 u. They were roundish with a double contoured membrane. The outer wall was smooth. In the majority of cysts, the nucleus was usually found excentrically located (Fig. 6).

Amoeba No. 3 (isolated from dust). This was a very small amoeba, actively motile, varying from 5.1 to 7.5 u. in diameter. Its cysts varied from 3.4 to 4.1 u. Because of their small size, the nuclear structure could not be studied in detail. An outer contoured membrane was present, the outer wall of which was polygonal in shape with eight sides, usually. It was best observed by suspending the amoeba in a dilute solution of nigrosine or methylene blue. The inner membrane was round (Fig. 7).

Amoeba No. 4 (isolated from dust). A rather large amoeba whose diameter ranged from 28 to 52 u. Its cytoplasm was coarsely granular; its nucleus very conspicuous in the vegetative state. Motility was sluggish and by means of a single pseudopodium at a time; micropseudopodia were seen in saline mounts.

The nuclear structure of this amoeba was very similar to that of amoeba No. 1. The cysts measured 14 to 21 u. and were either roundish or polygonal in shape. The inner membrane was also polygonal with four or five angles that gave them a star-like appearance; it was thinner at the points where it met the double contoured wall. When the cysts were suspended in a dilute solution of methylene blue, numerous deeply staining round granules were seen; these seemed to be located inside the double contoured wall but always towards one side of the cyst. No pores were observed in the cyst walls (Figs. 8 and 12).

The activity of all four amoebae upon different bacterial cultures was investigated. Nutrient agar plates were swabbed with 24-hour-old broth cultures of the different organisms, inoculated in the center with a loopful of the amoebae, and incubated at 26°C.

The results, shown in Table 1, indicated that amoeba No.1 was much more active against bacterial cultures than *H. castellanii*, not only with regard to the variety of species cleared but also with regard to the rapidity with which the different cultures were cleared, being active toward most cultures after 24 hours of incubation (Fig. 1). It was similar to *H. castellanii* in failing to attack *P. pyocyanea*, *S. citreus*, *B. subtilis*, and *B. anthracis*. It differed in being able to attack *S. albus*, *S. aureus*, *B. mycoides*, *A. fecalis*, *C. xerosis*, and *C. hoffmanii*.

Action of Amoebae upon Different Bacterial Cultures

Incubation Period in Hours	Hartmanella castellanii			Amoeba No. 1			Amoeba No. 2			Amoeba No. 3			Amoeba No. 4		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
Staphylococcus albus	—	—	—	10	23	35	—	—	—	—	7	9	—	—	—
Staphylococcus aureus	—	—	—	10	25	32	—	—	—	—	18	30	12	18	20
Staphylococcus citreus	—	—	—	7	20	30	—	—	—	—	4	8	—	—	—
Sarcina lutea		—	—	—	—	—	—	—	—	—	—	12	10	35	45
B. subtilis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B. megatherium	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B. anthracis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B. mycoides	—	—	—	3	15	22	—	—	—	—	—	—	—	—	—
E. typhosa	+[a]	++		12[b]	35	80	3	6	42	—	10	18	13	40	80
S. schottmuelleri	—	—	+	12	27	40	—	23	40	—	8	15	10	25	50
S. pullorum	—	++		6	40	60	—	20	30	—	15	25	8	50	75
S. typhimurium				12	30	52	—	12	40	—	10	15	—	30	40
S. enteritidis	—	—	+	12	35	70	3	17	50	—	8	15	12	35	60
Sh. sonnei				15	35	60	—	15	40						
Sh. dispar				12	31	60	—	10	55	—	10	20	7	23	53
Sh. alkalescens				8	24	50	—	13	45	—	10	26	15	38	45
Sh. paradysenteriae (Flexner Z)				10	35	75	—	15	30	—	5	25	20	45	65
A. aerogenes				5	8	22	—	6	25	—	5	12	—	—	—
E. coli	—	+		—	22	38	3	12	30	—	15	25	12	40	63
A. fecalis	—	—	—	15	40	80	—	15	30	—	7	20	15	50	60
P. vulgaris				8	16	32	—	10	22	—	15	25	10	35	60
B. friedlander	+			7	35	55	—	14	25	—	10	22	—	—	—
Ps. pyocyanea	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N. catarrhalis	—	++		15	55	80	—	26	55	—	—	15	8	45	65
C. xerosis	—	—	—	—	25	35	—	—	—	—	—	—	—	—	—
C. hoffmanii	—	—	—	8	15	22	—	—	—	—	—	—	—	—	—

[a] Van Rooyen denoted the presence or absence of clearing as + or —. A distinct clearing was recorded as ++.
[b] Diameter of cleared area in millimeters.

When duplicate plates of some of the cultures were incubated at 37°C., the same results were obtained. The clear area produced in *E. typhosa* after 24 hours of incubation at 37°C. was 10 to 12 mm. in diameter, which was about the same area of lysis produced by the standard amount of phage used in the typing technique (Fig. 9).

Amoebas Nos. 2 and 3 took about 48 hours to produce the same clear area and are, therefore, less liable to cause confusion in phage-typing. Furthermore, the attack of amoeba No. 3 upon bacterial cultures seemed to be more feeble and diffused (Figs. 2 and 3). Sometimes it was difficult to detect at first glance, but when the cleared area was observed by transmitted light, a color spectrum was seen, probably attributable to the polyhedral shape of the cysts. Areas of amoebic growth 50 to 70 mm. in diameter were suspended in 10 cc. volumes of water, alcohol, and ether, and centrifuged at high speed. The clear supernatants with the corresponding blanks were tested in the fluorophotometer. The results gave no indication of the presence of a fluorescent substance in the extracts.

The type of clearing produced by amoeba No. 3 was in contrast with that produced by amoeba No. 4. Its attack upon the cultures was vigorous. The large size of its cysts produced an opaque clear area, which is not liable to be confused with phage lysis by the experienced technician. The border of this clear area was well-defined, usually having a whitish elevated ring 2 to 3 mm. in diameter (Figs. 4 and 10).

The "antibacterial spectrum" of the four amoebae were different in themselves and differed also from that of *H. castellanii*. In this respect, amoeba No. 2 was the one that most closely resembled *H. castellanii*. However, as our amoebae were not tested with the same bacterial strains used by Van Rooyen we cannot make an adequate comparison. The latter demonstrated that, in some cases, amoebic activity will vary according to the type of the species under consideration. For example, pneumococcus Type II was attacked while pneumococcus Type III was not.

We have noticed that in some of the aerobic spore formers, which were apparently not attacked by the amoebae, some multiplication of the protozoan still occurred. The bacterial colony, however, soon sporulated, but the spores did not seem to be eaten up by the amoebae. Multiplication of the amoebae then slowed up, yet they would wander for long distances among the spores, apparently feeding upon the remains of the vegetative bacilli.

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B. megatherium	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B. anthracis	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
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Biochemical activity. Douglas considered the ability of *H. castellanii* to liquefy coagulated serum and gelatine as one of its most

important physiological characteristics. To our knowledge, the presence of this proteolytic enzyme has not been reported in connection with other amoebae.

Each one of the four amoebae was inoculated into a gelatine tube together with a small amount of an *E. typhosa* culture. Another gelatine tube was inoculated with a pure culture of *E. typhosa* as control. After nine days of incubation at 26°C., liquefaction of the gelatine was evident in the tubes inoculated with amoebae 1, 2 and 4. The tubes inoculated with amoeba 3, as well as the control, did not show any liquefaction. It will be noted that the inability of amoeba 3 to liquefy gelatine is correlated to the weakness of its action upon bacterial cultures.

Further observations on amoeba 1. In his study of *H. castellanii*, Van Rooyen noticed that a culture of an S variant of the typhoid bacillus showed areas of clearing 24 hours before the corresponding R variant. We decided to try out the action of the amoeba on the V-W variation.

Five organisms were selected out of our typhoid stock, which gave a four plus agglutination with "Vi" antiserum and a negative agglutination with an "O" antiserum. Five other organisms, which were partly or wholly degraded to the W form, were also selected. They gave a 4+ agglutination with an "O" antiserum and a "V" agglutination ranging from negative to 1+.

Six-hour-broth cultures of these ten organisms were adjusted to approximately the same turbidity, and 0.1 ml. was smeared on the surface of agar plates. The idea was to obtain areas of growth of approximately the same thickness, for the rate of destruction of the bacterial growth by the amoeba was considered to be proportional to its thickness. The plates were dried for 30 minutes in the incubator and were then seeded in the center with a loopful of the amoebae. The diameter of the clear area was measured in millimeters after 16 and 41 hours of incubation at 26°C. The results shown in Table 2 indicate that the amoebae did not discriminate between typhoid bacilli on the basis of the presence or absence of the "Vi" antigen.

Van Rooyen noticed that, occasionally, "secondary" colonies appeared in the zone of clearing if the plates were observed long enough. He subcultured some of these colonies and found that they did not differ from the parent strain as to any degree of susceptibility to the amoebae.

"Secondary" colonies were also noted in some of the cleared plates. These were picked to nutrient broth and incubated at 37°C. for 24

TABLE 2

Activity of Amoeba No. 1 upon V and W Variants of E. typhosa

Culture No.	*"Vi" antiserum*	*"O" antiserum*	*Cleared Areas in Millimeters*	
			16 Hrs.	*41 Hrs.*
859 A	±	++++	8	36
242 C	+	++++	10	39
285 A	—	++++	9	39
332 A	—	++++	10	40
771 B	+	++++	5	33
147 G	++++	—	10	36
3 D	++++	+	10	38
933 A	++++	—	9	36
698 C	++++	+	8	42
2014 B	++++	—	5	35

hours. The culture was then swabbed on an agar plate and seeded with the amoebae. Several days after complete clearing of the culture, "secondary" colonies reappeared. These were picked again, and the same procedure repeated four times. In no case was any resistance to clearing developed by the "secondary" colonies, thus confirming Van Rooyen's original observations.

"Secondary" colonies of a W variant of *E. typhosa* gave negative agglutination with "Vi" antiserum and 4+ agglutination with "O" antiserum. "Secondary" colonies of *E. typhosa* in the V form gave positive "Vi" agglutination and a negative "O" agglutination. This is further proof of the inability of the amoebae to discriminate between typhoid bacilli on the basis of the presence or absence of the "Vi" antigen.

Two different strains of *E. typhosa* were seeded in duplicate nutrient agar slants. One tube of each pair was inoculated with the amoebae. The four tubes were incubated at 26°C. for one week, in which time the amoebae had completely cleared the cultures. The growth in each tube was suspended in two ml. of sterile saline, transferred to sterile tubes, and centrifuged. A precipitin test was performed with an "O" antiserum, using the supernatant fluid as antigen. A positive test was obtained with the washings from the amoebae-cleared cultures while a negative result was obtained with the washings from the typhoid cultures not inoculated with the amoebae. The results indicate that a soluble antigen was released in the culture by the activity of the amoebae. This matter is under further investigation.

SUMMARY

1. A possible source of confusion during routine bacteriophage typing of *E. typhosa*, due to contamination with an amoeba, was reported.

2. This amoeba was able to clear away an area 10 to 12 mm. in diameter from a culture of *E. typhosa* after 24 hours of incubation at 37°C.

3. The amoeba was found to be parasitic upon bacterial cultures.

4. Similar amoebae were isolated from the air and from the dust in the room.

5. Because of their wide distribution, the cysts of these amoebae may be transported by air currents with the possible hazard of contamination to phage-typing work.

6. A morphological description of four amoebae was given. Their action upon a large number of different bacterial species was recorded. Points of difference and similarity with *H. castellanii* (Douglas) were noted.

7. The inability of the amoebae to discriminate between typhoid bacilli on the basis of the presence or absence of the "Vi" antigen was demonstrated.

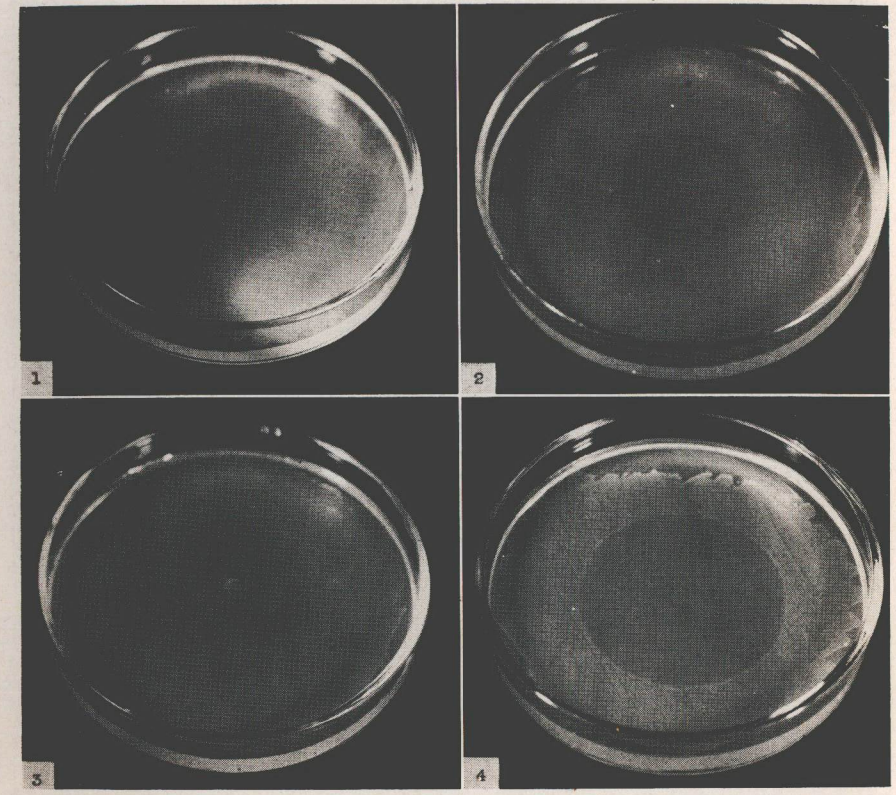

Plate 1

FIGS. 1–4. Clearing effect of the amoebae on 24-hour old cultures of *E. typhosa* after 72 hours of incubation at 26° C.

GRAB. 1–4. Esclarecimiento provocado por las amibas en un cultivo de 24 horas de *E. typhosa*, después de 72 horas de incubación a 26° C.

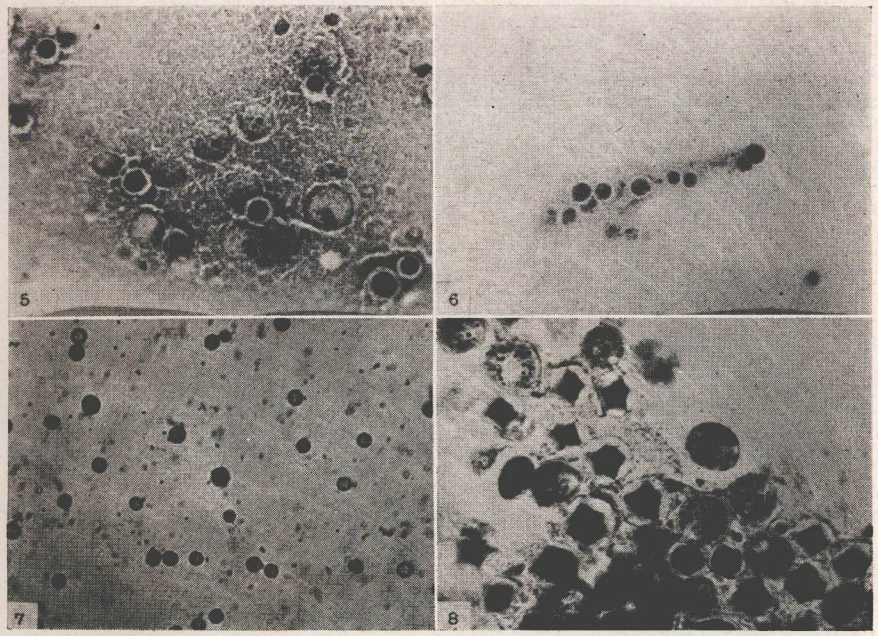

Plate II

FIG. 5. Amoeba No. 1. Resting vegetative cells.

GRAB. 5. Amiba núm. 1. Células vegetativas en reposo.

FIG. 6. Amoeba No. 2. Precystic state. Note excentrical position of the nucleus.

GRAB. 6. Amiba núm. 2. Estado prequístico. Nótese núcleo excéntrico.

FIG. 7. Amoeba No. 2. Fresh preparation in dilute methylene blue solution. Note the polyhedral shape of the cysts.

GRAB. 7. Amiba núm. 2. Preparación reciente en solución diluída de azul de metileno. Nótese la forma polihédrica de los quistes.

FIG. 8. Amoeba No. 4. Resting vegetative cells and cysts.

GRAB. 8. Amiba núm. 4. Células vegetativas y quistes.

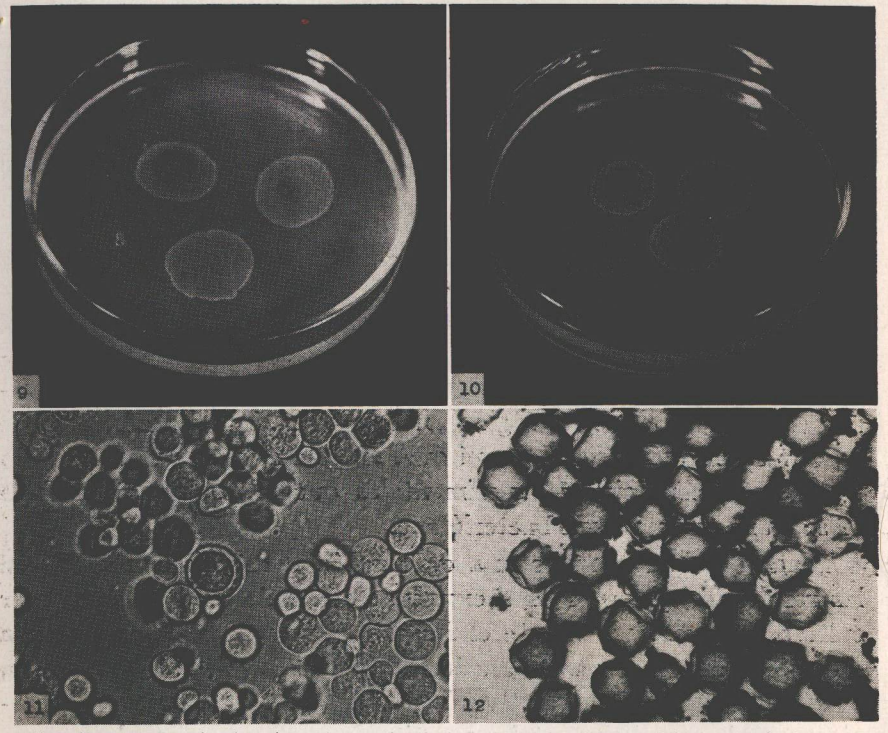

Plate III

FIG. 9. Comparison of the effect of Amoeba No. 1 and 'Vi' phage upon a culture of *E. typhosa* after 24 hours of incubation at 37° C. (Left, amoeba; right, phage).

GRAB. 9. Compárese la acción de la amiba núm. 1 y del bacteriófago 'Vi' sobre un cultivo de *E. typhosa* tras 24 horas de incubación a 37° C. (A la izquierda, la amiba; a la derecha, el fago).

FIG. 10. Comparison of the effect of Amoeba No. 4 and 'Vi' phage upon a culture of *E. typhosa* after 24 hours of incubation at 37° C. (Left, phage; right, amoeba).

GRAB. 10. Compárese la acción de la amiba núm. 4 y el bacteriófago 'Vi' sobre un cultivo de *E. typhosa* tras 24 horas de incubación a 37° C. (A la izquierda, el fago; a la derecha, la amiba).

FIG. 11. Amoeba No. 1. Fresh preparation in saline. Note double contoured membrane of cyst.

GRAB. 11. Amiba núm. 1. Preparación fresca en solución salina. Nótese el doble contorno de la membrana en un quiste.

FIG. 12. Amoeba No. 4. Fresh preparation in dilute methylene blue. Note the starlike appearance of the cysts and the deeply staining granules inside the double contoured membrane.

GRAB. 12. Amiba núm. 4. Preparación fresca en azul de metileno diluída. Nótese el aspecto estrellado de los quistes, los gránulos intensamente teñidos y situados dentro de la doble membrana del contorno.