THE ORMATION OF VACUOLES DUE TO BACILLUS TYPHOSUS IN THE CELLS OF TISSUE CULTURES OF THE INTESTINE OF THE CHICK EMBRYO.

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PLATES 18 AND 19.

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Lewis (1919, *b*) has shown that the formation of vacuoles in the cells of tissue culture is a phenomenon frequently associated with degeneration. It was not surprising, therefore, to find that the presence of bacteria in the culture resulted in a similar behavior of the cells. While several species of bacteria did not cause the vacuolation of the cells, others did bring about this reaction. None of these, however, caused the process to take place so rapidly or so extensively as did the typhoid bacillus.

Technique.

The cultures of bacteria were grown on agar slants.[1] The inoculation was made by touching a platinum wire against the growth of organisms and then inserting it into the hanging drop of the tissue culture. The results of the experiments were extremely complicated and only one phase, the formation of vacuoles, will be discussed in the present paper.

Pieces of the intestine of chick embryos (7 to 9 days incubation) were explanted into Locke-Lewis solution (sodium chloride 0.9 per cent plus potassium chloride 0.042 per cent plus calcium chloride 0.025

[1] Transplants from the stock cultures of the Department of Pathology of the Johns Hopkins University were obtained through the kindness of Dr. L. D. Felton. All cultures of bacteria were prepared by Mr. D. T. Smith, who kindly tested out every culture of typhoid used in these experiments.

per cent plus sodium bicarbonate 0.02 per cent plus dextrose 0.25 per cent plus chicken bouillon 15 per cent) by means of the usual tissue culture technique. The growth of this particular series of cultures was among the best so far obtained in the laboratory.[2] Practically every culture grew luxuriantly. Not only was there abundant migration of connective tissue cells, but large membranes extended out from the endodermal lining of the intestine and also from the mesothelium. Frequently an extensive plexus of sympathetic nerve fibers was present in the new growth. These cultures, unlike those described by Lewis (1919, *b*), lived from 4 to 6 days without signs of degeneration, and only in the oldest cultures (8 to 10 days) did the number of vacuoles become marked.

The cultures of the intestine were carefully examined after 24 or 48 hours, not only in regard to the size and extent of the growth, but also as to the condition of the individual cells, especially concerning the presence of vacuoles. Any culture containing cells in which vacuoles had already appeared was discarded. This happened in only a few instances. After the preparation had been thus examined the cover-slip was removed and the hanging drop was inoculated with *Bacillus typhosus*. The number of organisms carried over was necessarily large in proportion to the amount of tissue. The culture was then resealed, turned upside down for a few seconds in order to distribute the organism, and returned to the warm box (39°C.). The controls were prepared in the same manner except that the platinum point, instead of carrying bacteria, was sterilized by passing it through the flame.

RESULTS.

As shown by Table I, it is evident that the introduction of *Bacillus typhosus* into the hanging drop of the tissue cultures resulted in rapid vacuolation of the cells of the growth (Figs. 1 to 4). These vacuoles appeared as minute round bodies which rapidly grew larger and in certain instances fused together to form large vacuoles (Fig. 4). In the ordinary degeneration of the cultures, such as described by Lewis

[2] Dr. Mary J. Hogue kindly made for me a series of cultures of the intestine of the chick embryo.

(1919, *b*), not all the cells became vacuolated at the same time or to an equal extent, but in these infected cultures all the cells of the cultures suffered this change and all were in the same stage of vacuolation at the same time (Figs. 1 and 2). This phenomenon was delayed when the culture was bathed with fresh solution before inoculation. It was also delayed if, instead of touching the hanging drop with bacteria, one suspended the organisms in fresh medium and placed a drop of this upon the cultures. Vacuolation, however, took place within a short time after inoculation (10 to 12 hours), even in the fresh solution, regardless of the age of the culture, so that it seemed as though the typhoid bacilli acted in such a way as to hasten markedly some process which resulted in the vacuolation of the cell.

The vacuoles were usually collected at one side of the nucleus around a central mass of cytoplasm (Fig. 3) so as to suggest that this central part corresponds to the centrosphere described by Lewis (1919, *a*). The vacuoles frequently appeared to be free from granules or to contain only minute ones. In other instances one or more granules were present.

About an hour after inoculation one or two organisms were sometimes observed within a single vacuole in one of the cells of the growth. After a longer interval, not only did more vacuoles contain bacilli but the number of these to a single vacuole was greater (Fig. 3). The vacuoles were confined to the cytoplasm of the cell and neither vacuoles nor bacteria were observed within the nucleus. While the proportion of cells containing organisms was always small, such cells were not limited to any one type of tissue, appearing in the connective tissue as well as in the mesothelium and even in the endoderm from the lining of the intestine (Figs. 1 to 3). Jones and Rous carried out observations in regard to the ability of isolated connective tissue cells from either rat or chicken cultures to ingest *Staphylococcus pyogenes albus*. They claim that such isolated connective tissue cells rarely take up carmine granules and that the phagocytosis of the bacteria occurred only in the presence of serum (antistaphylococcus). No mention is made of vacuoles in these cells.

In almost all cases in which the inoculated cultures were kept for several hours the typhoid bacilli remained motile and increased in number. Smyth performed an extensive series of experiments in

TABLE I.

Culture.	Bacteria.	Observations.				
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	Later observations.
1. I_8 48 hrs.*	12 hr. culture of No. 80 B.	Several very small vacuoles appear in all cells of growth. Some mitotic figures.	Many vacuoles present in all cells. No mitotic figures.	Many vacuoles in all cells; larger and fewer in connective tissue cells than in other cells.	In 5 of the cells one vacuole in each contains bacteria. Vacuoles large and few in number in connective tissue. Many small vacuoles in each cell of endodermal membrane and of mesothelium. No mitosis.	Fixed and stained at end of 2½ hrs. (Figs. 3 and 4).
2. M_8 72 hrs.	3 day culture of No. 80 B.		Many vacuoles.			
3. M_8 96 hrs.	" "	Many vacuoles. Fixed and stained.				
4. M_8 48 hrs.	" "	A few vacuoles in each cell. A number of mitotic figures.	A number of vacuoles.		No more vacuoles than were present at end of half hour.	
5. I_8 96 hrs.	" "	A number of small vacuoles in each cell.				

6. I_8 7 days.	3 day culture of No. 80 B.		A number of vacuoles.	Full of vacuoles.		Culture dead after 20 hrs.
7. I_8 48 hrs.	6 day culture of No. 80 B.	A few vacuoles in each cell.			Number of vacuoles not greatly increased.	
8. " "	" "		A few vacuoles in each cell.			At end of 4 hrs. a few small vacuoles in each cell.
9. " "	No bacteria.		No vacuoles in the cells.			At end of 4 days growth large and only occasional cell had vacuoles.
10. I_9 48 hrs.	1 drop of 10 day culture of No. 80 B in Locke-Lewis solution.		A very few small vacuoles in each cell.			At end of 20 hrs. all cells dead. Cytoplasm is composed largely of a network with here and there the walls of the vacuoles still present.
11. " "	" "		" "			" "

* I_8 48 hrs. indicates a culture of the intestine of an 8 day chick embryo, 48 hours after explantation; M_8 72 hrs., a culture of the muscle of an 8 day chick embryo, 72 hours after explantation, etc. 80 B, etc., is the departmental number of the typhoid culture. Cultures 20 to 24 were bathed with a fresh solution before inoculation. Nos. 37 to 40 and 59 to 66 were washed with Locke-Lewis solution in which neutral red (1: 50,000) had been dissolved, and later inoculated. In Nos. 10 to 18 the organism was suspended in Locke-Lewis solution and a drop of this placed on the culture. Nos. 35 and 41 to 45 were stained with neutral red in Locke-Lewis solution some time after inoculation.

TABLE I—*Continued.*

Culture.	Bacteria.	Observations.				
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	Later observations.
12. I_9 48 hrs.	1 drop of 10 day culture of No. 80 B in Locke-Lewis solution.			A few vacuoles in the cells.		At end of 20 hrs. all cells dead. Cytoplasm is composed largely of a network with here and there the walls of the vacuoles still present.
13. " "	" "			" "		" "
14. " "	" "		A very few small vacuoles in each cell.			" "
15. " "	" "		" "			" "
16. " "	" "		" "			" "
17. " "	" "		" "			" "
18. " "	" "			A few vacuoles in the cells.		" "

19. I_9 48 hrs.	Drop of Locke-Lewis solution.				No vacuoles in cells.	At end of 4 days good growth. No vacuoles.
20. 1_9 72 hrs. Cultures washed with Locke-Lewis solution.	18 hr. culture of No. 80 B M.		A few vacuoles in each cell.		No increase in vacuoles. Bacteria very motile.	At end of 20 hrs. partly dead. Cells full of vacuoles. Bacteria increased and motile.
21. " "	" "		" "		" "	" "
22. " "	" "		" "		" "	" "
23. " "	" "		" "		" "	" "
24. " "	No bacteria					At end of 20 hrs. good growth. Many mitotic figures. Very few vacuoles.
25. I_9 72 hrs.	12 hr. culture of No. 80 B M.		Many vacuoles in all cells.			
26. I_8 48 hrs.	24 hr. culture of No. 80 B M.	Small vacuoles in all cells.	Many vacuoles. Fixed and stained.			

TABLE I—*Continued.*

Culture.	Bacteria.	Observations.				
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	Later observations.
27. I_s 48 hrs.	24 hr. culture of No. 80 B M.	Small vacuoles in all cells.	Many vacuoles.	Fixed and stained.		
28. " "	" "	" "	" "		Fixed and stained.	
29. " "	" "	" "	" "			
30. I_s 72 hrs.	60 hr. culture of No. 80 B M.	Few vacuoles.			A number of vacuoles in the cells. Clasmatocytes have taken up the bacteria.	At end of 3 hrs. fixed and stained.
31. " "	Bacteria frequently long threads.	" "			" "	" "
32. " "	" "	" "			" "	" "
33. " "	" "	" "			" "	" "
34. " "	" "	" "			" "	" "

35. I_9 48 hrs.	24 hr. culture of No. 80 B M_2.	Few vacuoles.				At end of 4 hrs. stained with neutral red in Locke-Lewis solution. Many various shaped and sized red vacuoles, some containing one or more bacteria. Clasmatocytes have red vacuoles containing unstained bacteria. At end of 20 hrs. almost all growth dead. Some cells have unstained vacuoles containing active bacteria. A few cells had red vacuoles.
36. " " (3 cultures).	No bacteria.	No "				Lived 7 days. Good growth. Few vacuoles.

TABLE I—*Continued.*

Culture.	Bacteria.	Observations.				
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	Later observations.
37. I_8 48 hrs. Culture washed with Locke-Lewis solution + neutral red.	24 hr. culture of No. 80 B M.		Few vacuoles.		Many red vacuoles. No bacteria in vacuoles even in clasmatocytes.	At end of 20 hrs. growth largely dead; diffuse red. Vacuoles unstained. A few cells still alive have many large red vacuoles.
38. " "	" "		" "		" "	" "
39. " "	24 hr. culture of No. 80 B S_2.		" "		" "	
40. " "	" "		" "		" "	
41. I_8 48 hrs.	" "					At end of 4 hrs. stained with neutral red in Locke-Lewis solution. A number of red vacuoles. Some red vacuoles have unstained bacteria in them. Some bacteria in red vacuoles motile; some outlined with red in the cell.

42. I_8 48 hrs.	24 hr. culture of No. 80 B S_2.					At end of 4 hrs. stained with neutral red in Locke-Lewis solution. A number of red vacuoles. Some red vacuoles have unstained bacteria in them. Some bacteria in red vacuoles motile; some outlined with red in the cell.
43. I_8 72 hrs.	" "					" "
44. " "	" "					" "
45. I_8 48 hrs.	" "		A number of vacuoles in each cell.		Vacuoles with very motile bacteria in them. Many vacuoles in each cell.	At end of 3 hrs. stained with neutral red. A number of red vacuoles have quiet bacteria in them. Many red vacuoles in each cell.
46. " "	No bacteria.					At end of 4 hrs. no vacuoles. Lived 7 days. At end of this time there were a number of vacuoles in some cells.

TABLE I—*Continued.*

Culture.	Bacteria.	Observations.				
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	Later observations.
47. I_7 48 hrs.	24 hr. culture of No. 80 B S_4.		A number of vacuoles.			At end of 20 hrs. cells almost all dead. Bacteria grew and are motile.
48. " "	" "		A few vacuoles in each cell.	No increase in vacuoles.		At end of 20 hrs. good growth. Many vacuoles in each cell. Bacteria increased and are motile.
49. " "	" "		A number of vacuoles in each cell.			At end of 20 hrs. all dead. Bacteria grew and are motile.
50. " "	No bacteria.				An occasional vacuole.	At end of 20 hrs. good growth. Many figures of division. A few vacuoles in a few cells.
51. " "	24 hr. culture of No. 80 B S_5.		A number of vacuoles.	Many vacuoles; some large.		
52. " "	" "		A number of vacuoles. Some dead cells.	Many vacuoles; some large. Fixed and stained.		

53. I_7 48 hrs.	24 hr. culture of No. 80 B S_8.		Many vacuoles.	Many vacuoles. Some large ones have one motile bacillus.		At end of 20 hrs. few cells survive; all full of vacuoles. Only one vacuole seen containing a bacillus.
54. " "	" "	No vacuoles.			No vacuoles.	At end of 20 hrs. no vacuoles.
55. " "	24 hr. culture of No. 80 C.		Many vacuoles. Many dead cells.	Many vacuoles. No bacteria seen in vacuoles.		At end of 20 hrs. all dead. Bacteria grew.
56. " "	" "		Many vacuoles. No dead cells.			At end of 20 hrs. a few spindle cells survive. These are full of vacuoles. Bacteria grew.
57. " "	" "		" "			Dead at end of 20 hrs. Bacteria grew.
58. " "	" "		Many vacuoles; some large.			" "
59. " " Washed with neutral red in Locke-Lewis solution.	" "	A few small red vacuoles.		A number of small red vacuoles.		At end of 20 hrs. all cells dead. Bacteria grew and are motile.
60. " "	" "			" "		" "

TABLE I—*Concluded.*

Culture.	Bacteria.	Observations.				
		After 10 min.	After 30 min.	After 1 hr.	After 2 hrs.	Later observations.
61. I_7 48 hrs. Washed with neutral red in Locke-Lewis solution.	24 hr. culture of No. 80 C.					At end of 4 hrs. many red vacuoles of different shapes, some containing an unstained granule. Dead at end of 20 hrs.
62. " "	" "			A number of small red vacuoles.		At end of 4 hrs. many cells dying. A number of unstained vacuoles. Many red vacuoles. No bacteria seen in vacuoles. At end of 20 hrs. cytoplasm of cells has become a network and is full of unstained vacuoles. Cell is diffuse red. Bacteria grew and are motile.
63. " "	" "			" "		" "

64. I_7 48 hrs. Washed with neutral red in Locke-Lewis solution.	48 hr. culture of No. 80 B S_4.			A number of small red vacuoles.		At end of 20 hrs. a number of cells still alive; full of bright red vacuoles. Bacteria motile.
65. " "	" "			" "		Dead at end of 20 hrs. Bacteria motile.
66. " "	" "			" "		At end of 20 hrs. some of growth still alive with cells full of large red vacuoles.
67. I_7 24 hrs.	48 hr. culture of No. 80 B S_5.			Many vacuoles.		
68. " "	" "			" "		
69. " "	" "			" "		
70. " "	No bacteria.			No "		At end of 20 hrs. good growth. Many mitotic figures.

regard to the effect of bacteria upon plasma cultures. He states that *Bacillus typhosus* never grew in plasma alone. However, when the plasma had been incubated to destroy its bactericidal action, the bacteria developed freely with especial affinity for the tissue cells. According to Smyth the typhoid bacteria had no toxic action on the tissue cells. It is difficult to understand this statement in the light of the present experiments, as in every culture in which the organism grew, there resulted a rapid degeneration of the tissue. It is possible that the strain of typhoid bacillus employed by Smyth was different from that used in these experiments.

The normal cultures contained many cells in the process of division. When these cultures were inoculated, the cells undergoing mitosis, as well as the resting cells, became vacuolated. The dividing cells completed the process but no new mitotic figures appeared in the growth, except in rare instances. While the normal culture of the series usually exhibited eight to ten dividing cells, even when the culture was more than 4 days old, the infected cultures seldom showed any.

When the cultures were stained with neutral red before inoculation the vacuoles were red from their first appearance. This color faded upon the death of the cell. The vacuoles were stained at various times during their formation and always became bright red, regardless of whether they contained bacteria or not. The motility of the organism in the vacuoles usually decreased when the preparation was stained with neutral red.

These vacuoles cannot be regarded as the result of the ingestion of the bacteria, as they appeared very rapidly in all the cells at the same time, being small at first but later increasing in size and even fusing to form exceedingly large vacuoles. The bacteria were never found in the cells until some time after the vacuoles had appeared and then only in a few of the many hundred cells comprising the growth. Even when present they were not found in all the vacuoles of a cell but usually in only one. The organisms were very motile in the unstained vacuole, occasionally dashing from side to side, but more frequently circling rapidly round and round the boundary of the vacuole. If the vacuoles are the result of the ingestion of some substance by the cell, as has been claimed by some writers, one

would not expect to find any difference in results, whether the original hanging drop were used or had been replaced by a fresh one. However, the utilization of a fresh drop did markedly alter the results; so that, instead of vacuoles appearing at once and rapidly increasing in size, it was some time before they became noticeable in numbers. The results obtained in regard to phagocytosis will not be given here, as they are at present inseparable from many other complicated factors.

Metchnikoff, who not only performed many experiments himself but discussed the work of innumerable other investigators upon the subject of phagocytosis, gives only a meager description of vacuoles in cells other than protozoa. Metchnikoff and his followers seemed to take it for granted that the leucocyte contained digestive vacuoles; this, however, is not the case. Normal leucocytes do not contain vacuoles, although they rapidly become vacuolated under abnormal conditions. Plato[3] claims that there are no bodies that stain with neutral red in the leucocytes of the freshly drawn blood. Miss R. Prigosen in a number of observations upon the blood of the hen and also upon human blood in this laboratory found no vacuoles of any kind in leucocytes immediately upon their withdrawal from the body. Frequently writers describe the ingestion of bacteria without mentioning vacuoles, and other authors, even with the use of neutral red, claim that the ingested particles stain with neutral red, but they often either fail to describe the stained vacuoles or definitely state that the vacuoles remain unstained.

Barber inoculated *Nitella*, *Saprolegnia*, and *Achyla* with *B. typhosus*. In nearly very instance the organism grew luxuriantly in the cells. Barber does not mention vacuoles in the cytoplasm other than the vacuole which was a definite structure of the cell. He states that the bacteria apparently met with no resistance in passing from the cell vacuole into the protoplasmic layer. It was not possible to find any harmful action of the protoplasm upon the bacteria. The cell finally died as a result of parasitism.

A number of writers discussing necrosis, degeneration, or old age frequently describe vacuolation of the tissue. Calkins[4] mentions this in regard to paramecium. Dr. W. Gary told me of certain experiments on autolysis of protozoa in which vacuoles appeared in the cytoplasm of the organism. Miss Prigosen, in the studies previously mentioned, cites a number of cases in which lack of oxygen caused the formation of vacuoles in different sorts of cells. Symmers shows vacuolation of the cells in multiple non-inflammatory necrosis of the liver. Lewis (1919, *b*) describes the vacuolation of tissue cultures associated with degeneration of these growths.

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[3] Plato, p. 913.

[4] Calkins, p. 127.

Many substances have been introduced into the hanging drop of tissue cultures in an effort to obtain information as to what factor in the life history of the cell is chiefly concerned in the formation of vacuoles. Food substances such as egg albumin, blood serum, dextrose, glycogen, aleuronat, etc., when added to the medium did not cause a marked or rapid increase in the number of vacuoles. Some of these substances caused a slight increase in the number of neutral red granules. Other substances such as phosphorus, carbon dioxide, urea, and ammonia caused all sorts of distortions of the cells, and often death ensued, but they failed to result in a constant vacuolation of the cytoplasm. As far as has been determined the effect of the typhoid bacteria upon the culture seems to be one that sets up some disturbance of the normal behavior of the cell in such a way as to result rapidly in a type of degeneration which is not uncommon in old cultures under usual conditions; *i.e.*, such a degeneration as has been described by Lewis (1919, *b*) in uninoculated cultures. It would be interesting to determine whether cloudy swelling common in acute infection is a form of vacuolation of the cells.

CONCLUSION.

The introduction of *Bacillus typhosus* into the hanging drop of a tissue culture of the intestine of a chick embryo leads to the rapid vacuolation of the cells of the tissues which comprise the growth of the cultures. The cells of the connective tissue, the endodermal membrane, the mesothelium, and also the clasmatocytes exhibit the ability to ingest *Bacillus typhosus* in the cultures.

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EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. 48 hour growth of mesothelium from the intestine of an 8 day chick embryo, 2½ hours after inoculation with *B. typhosus*.

FIG. 2. 48 hour culture of the intestine of an 8 day chick embryo. Vacuolated endodermal membrane due to the presence of *B. typhosus* in the hanging drop for 2½ hours.

PLATE 19.

FIG. 3. 48 hour culture of the intestine of an 8 day chick embryo. Bacilli were moving rapidly in a vacuole in the elongated binucleate cell near the center of the field. The other organisms seen in the photograph became fastened to the growth upon fixation. They were not within the living cells but moved freely in and out of the field of vision. The cells show the vacuoles arranged around the centrosphere at one side of the nucleus as described by Lewis (1919, *a*).

FIG. 4. 48 hour growth of the connective tissue from the intestine of an 8 day chick embryo after inoculation with *B. typhosus*. 1 hour after the introduction of the organism the cells were full of small vacuoles. These later became larger and fewer in number.

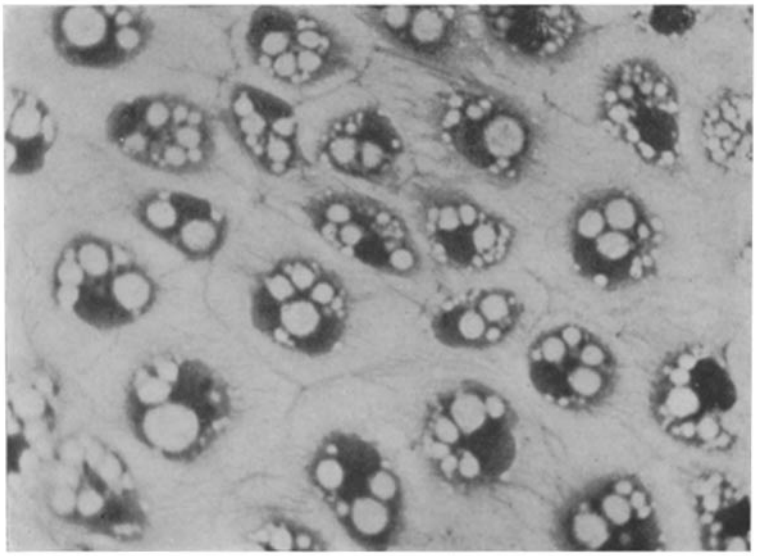

FIG. 1.

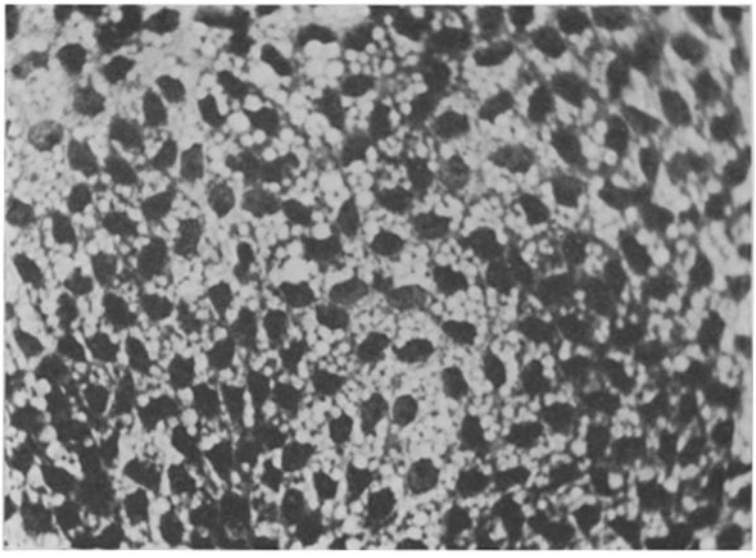

FIG. 2.

(Lewis: Vacuoles in cells of tissue cultures.)

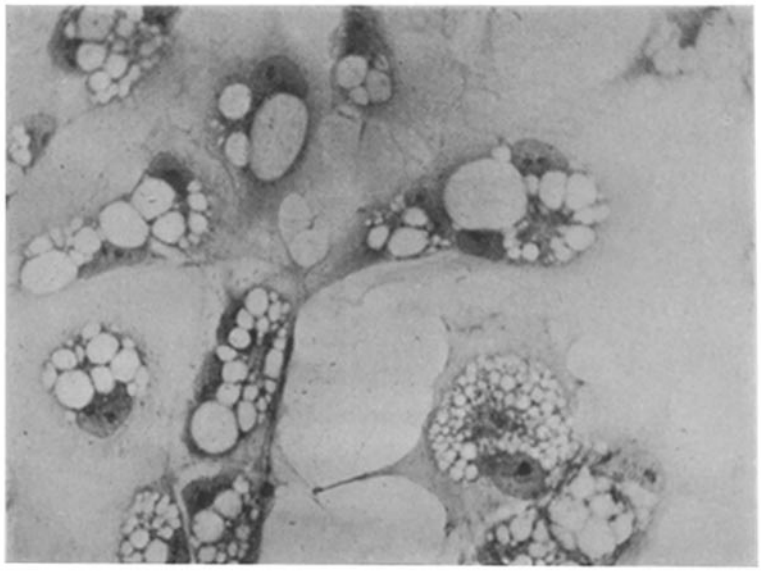

FIG. 3.

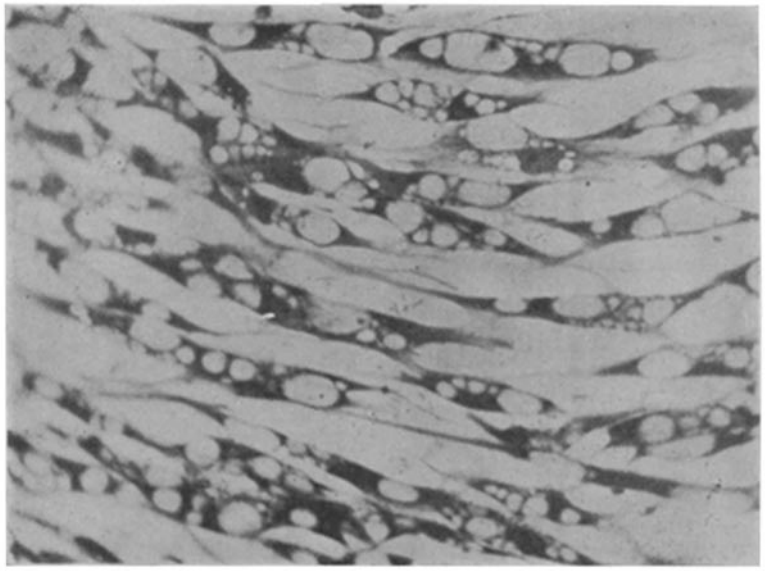

FIG. 4.

(Lewis: Vacuoles in cells of tissue cultures.)