THE EFFECT OF HYPOTONIC AND HYPERTONIC SOLUTIONS ON FIBROBLASTS OF THE EMBRYONIC CHICK HEART IN VITRO.

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Plate 56.

(Received for publication, August 19, 1919.)

In the literature on tissue culture are reported, more or less completely, experiments testing the effect of hypotonic and hypertonic solutions upon growth of cells and upon individual cell structures. It was with a view, therefore, of studying more fully the effect of solutions of different concentrations that the present work was undertaken. The experiments were performed in April, May, June, and July, when the weather was at summer heat and there was consequently little danger of chilling the delicate new growths, which are susceptible to any sudden change of temperature. This point is important, because several investigators have stated definitely that growth is much better in the spring and summer than in the fall and winter.

Carrel and Burrows were the first to try the effect of dilution and concentration of the medium upon tissue cultures, using the spleen of adult chicks and of 14 and 16 day chick embryos. Distilled water was added to the plasma to make it hypotonic, and sodium chloride to make it hypertonic. They found that growth in hypertonic plasma was always less than normal, while growth in the hypotonic solution (three volumes of plasma to two volumes of distilled water) was greatly accelerated. In fact, this medium gave the greatest amount of growth, much more than that obtained in normal plasma, a medium which these writers admit is not the best for this purpose.

Ruth found that by adding distilled water to plasma in which epithelial cells of frog skin were planted the growth was greatly increased.

Later, Lambert tried diluting the plasma with serum. He got the most extensive migration in plasma diluted with two parts of serum. Dilution with serum, Ringer's solution, and water gave about the same results. He therefore

concluded that "the most active migration took place in the plasma diluted with the largest quantity of fluid, and not in the medium made hypotonic by the addition of distilled water." This agrees with the work of Burrows (1913), who used spleen and diluted the plasma with serum. Both these observers attribute the increased migration to a diminution in the amount of fibrin in the clots; this makes a coarser meshwork which offers less resistance to the progressive movement of the cells. Lambert also finds that diluting the plasma affects differently the various types of cells. Actively motile cells, such as those of bone marrow and spleen, show increased migration; cells with limited power of locomotion, like those of intestinal epithelium, are not affected by the dilution of the medium; while cells of moderate motility, such as connective tissue cells, are only slightly influenced by it. Lambert concludes that this dilution of the medium has likewise no effect upon cell division.

Ebeling used hypotonic, hypertonic, and diluted media prepared by adding Ringer's solution to normal chick plasma and embryonic chick extract. Each of these media at first stimulated cell proliferation, but after a few days this decreased and ended in death unless measures were taken to revive the cultures.

Sundwall also grew tissues in hypotonic solutions, using plasma diluted with distilled water. He found the growth to be more prolific, but the difference between this and growth in plasma was not so marked as that obtained by Carrel and Burrows.

Cash used hypotonic salt solution on 3 day chick cultures and found that at once numerous vesicles appear, change shape characteristically for a short time, and then flow back into the cells. He states that it is difficult to produce these changes in younger cells; *i.e.*, in growths 1 to 2 days old.

Goldschmidt found that the direction of growth of the sex cells of the moth (*Samia cecropia* L.) was affected by hypertonic and hypotonic solutions. In the former the cells grew out against the follicle wall. The extent of outgrowth depended upon two factors—the age of the cell and the hypertonicity of the medium. The whole process was reversible without change of medium, as were also the slight changes produced by hypotonic media. Goldschmidt therefore concludes that the follicle membrane has active control of osmotic conditions inside the follicle.

Lewis and Lewis (1914-15) note a definite effect of hypotonic and hypertonic solutions upon the mitochondria; in the first they swell, while in the second they shrink. Cowdry states that very dilute solutions of formaldehyde cause swelling of the mitochondria, while a concentrated solution results in shrinkage.

Material.

For these experiments hearts of 6, 7, 8, and 9 day chick embryos were used. The medium was a modification of Locke's, known as Locke-Lewis solution. The Locke formula was as follows:

	gm.
Sodium chloride	0.9
Calcium chloride	0.024
Potassium chloride	0.042
Sodium bicarbonate	0.02

This was modified, according to Lewis' method (1915), by the addition of chick bouillon (15 cc. of bouillon and 0.25 gm. of dextrose to 85 cc. of Locke's solution), sterilized in clean test-tubes in an Arnold sterilizer, and kept in a moist chamber to avoid evaporation. In order to make it hypertonic the solution was boiled down one-fourth, one-half, etc., as required, and the amount of sodium chloride present was calculated. The hypotonic solutions were made by diluting the Locke-Lewis solution with distilled water, and in these also the amount of sodium chloride was determined. Since chick bouillon contains practically the same amount of sodium chloride as the Locke solution, the calculation of this content in the Locke-Lewis solution was made upon the same basis. It will be noted that only the amount of sodium chloride has been considered, although the other salts were present in definite amounts and always in the same proportion.

Method.

All the instruments and glassware used in the experiments were sterilized; the instruments were sterilized in a flame continually during the preparation of the cultures, so that infection of the cultures was rare. The solution was warmed and poured into small Petri dishes. The heart was aseptically removed from the chick embryo in Locke-Lewis solution and transferred to another Petri dish containing about 10 cc. of the medium. Here it was cut into small pieces and transferred by means of a pipette to a cover-glass, which was immediately inverted over a hard vaseline ring on a depression slide. The chance of evaporation by this method of the Lewises is very small, as the tissue can be placed on the cover-glass and sealed in a few seconds. When the tissue was to be planted in hypertonic or hypotonic solution the heart was placed in the liquid immediately after its removal from the embryo; the tissue was therefore never in contact with other solutions that might possibly dilute the medium.

The cultures were incubated in a constant temperature box at 39°C. Many of the slides were treated with neutral red which stains certain granules and vacuoles (Lewis, 1919). Janus black No. 2 was added when the mitochondria were to be studied, but only when early results were obtained from the experiment, as the cells do not live much more than an hour after being stained with it.

Normal Growth of Heart Tissue.

The normal growth of heart tissue is characteristic. Two types of connective tissue cells appear—fibroblasts and mesothelial cells. The fibroblasts (Fig. 1, *a*) are of more frequent occurrence and show two methods of growth: (1) migratory (Fig. 1), where the cells are scattered along the transplant and out into the medium; and (2) reticular, where the cells form a close reticular network. The latter is usually the more vigorous growth. These cells have long, branching processes, often so thin that it is impossible to follow them in their farthest wanderings and anastomoses with other cells. Hence, in most of the drawings of cells these processes are omitted, as they could not be seen with the camera lucida.

The mesothelial cells (Fig. 1, *b*) are of less frequent occurrence. They always appear in membrane formation (Lewis and Lewis, 1912), sometimes as small inclusions among the fibroblasts, and again as large growths completely surrounding the transplant. They are much larger and hardier, and withstood the action of the hypotonic and hypertonic solutions much better than the sensitive fibroblasts, with their fine, branching processes, which usually reacted at once to the new medium. For that reason, and also because they were the more common form, the fibroblasts were always used in the experiments.

As controls to the other experiments 282 cultures were planted in normal Locke-Lewis solution, and of these 188 (66.6 per cent) grew. The length of life in this medium varied. The oldest growth was 19 days old, and had been made from a 7 day chick embryo. It lived to this age without any change of the medium; in fact it was sealed with a vaseline ring during the entire time. The cells contained very few fat globules and towards the end of the experiment they withdrew their processes, only blunt ends remaining. They then accumulated in groups of five or six all over the slide.

Experiments with Hypotonic Solutions.

For these experiments three different solutions were employed: (1) three volumes of Locke-Lewis solution to two volumes of distilled water; this had a sodium chloride content of 0.540 per cent; (2) one volume of Locke-Lewis solution to one of distilled water; sodium chloride content 0.45 per cent; and (3) one volume of Locke-Lewis solution to three volumes of distilled water; sodium chloride content 0.225 per cent.

The first solution, which was the proportion used by Carrel and Burrows in their work on the spleen, was employed in two experiments with hearts of chick embryos 8 and 9 days old respectively. Out of twenty-three cultures seven gave reticular growth, thirteen migration, and three showed no signs of growth. In one experiment all were dead on the 4th day, while in the other some of the cultures lived for 7 days. Migration of cells was at first rapid as compared with the controls, but the latter lived a little longer. The percentage of growths in this medium is high (86.9), but only two experiments were performed. On these 2 days the percentage of growth of cultures in normal Locke-Lewis solution was 81.8—only slightly less. The number of reticular growths in the two media was practically the same—35 per cent in hypotonic and 30 per cent in Locke-Lewis solution, although in the former the extent of growth was greater.

Burrows (1913) and Lambert attribute the increased migration to a lessening of fibrin in the diluted plasma and consequent decrease in the resistance offered to migration. Here, however, this was not the case, as there was no fibrin in any of the cultures grown in the Locke-Lewis solution. The real cause seems to me to be the search for food which acts as a stimulus, as will be seen later. Burrows (1913) has also observed that tissues planted in plasma diluted with water suffer early death. This he thinks is due to the hypotonicity. He says: "They die apparently in the plasma diluted with salt solutions from an early exhaustion of food materials." This I believe is true, although when they die after being treated with (not grown in) hypotonic solution, it is possible that the cells have taken up too much water, which of itself is known to act as a poison,

causing death. When the individual cells were examined, a few were found to be long and fusiform and often small, though many were in a vigorous condition. When neutral red and Janus black No. 2 were added to the drop of culture fluid, both stains being made up in the hypotonic fluid used for the experiment, the neutral red granules were found to be arranged around the centriole near the nucleus, just as in the cells grown in Locke-Lewis solution. The mitochondria were long, sometimes branching, and often formed a network. Sometimes they extended down the processes to a considerable length. All these forms are as one usually finds them in normal growths.

The most striking point about the growths in this solution, and indeed in all hypotonic solutions, was that the cells nearest the explant died first (Fig. 2). This is exactly the reverse of the condition prevailing in all Locke-Lewis cultures and in the hypertonic growths, where the cells at the outer edge of the growth die first, until finally only those next to the transplanted tissue are left. These frequently live on for days after the main part of the growth has died. In the hypertonic solutions the migration is slower. There is a greater amount of food material (salts, etc., from the Locke solution and chicken bouillon) in the medium, so that the cells do not need to migrate so rapidly and consequently do not give off so much in the form of waste products. Hence the medium is not altered so rapidly (Burrows, 1913) and migration is retarded. In short, all depends upon the amount of food in the medium. When this is small, as in hypotonic solutions, the cells must migrate more rapidly to obtain food. They then give off more waste products which alter the medium, thus further stimulating movement. In a short time the accumulation of waste products becomes so great and the supply of food near the explant so small that the cells in that vicinity die. The same phenomenon was observed in *Amœba limax* cultures grown on agar (Hogue). The amebæ nearest the old point of inoculation on the Petri dish die before those which have wandered out over the dish, where they are free from waste products and can get more food and oxygen.

In the second hypotonic solution, made with one part of Locke-Lewis solution to one part of distilled water, 90 out of 202 explants grew (44.5 per cent). The migration was not so rapid as in the former

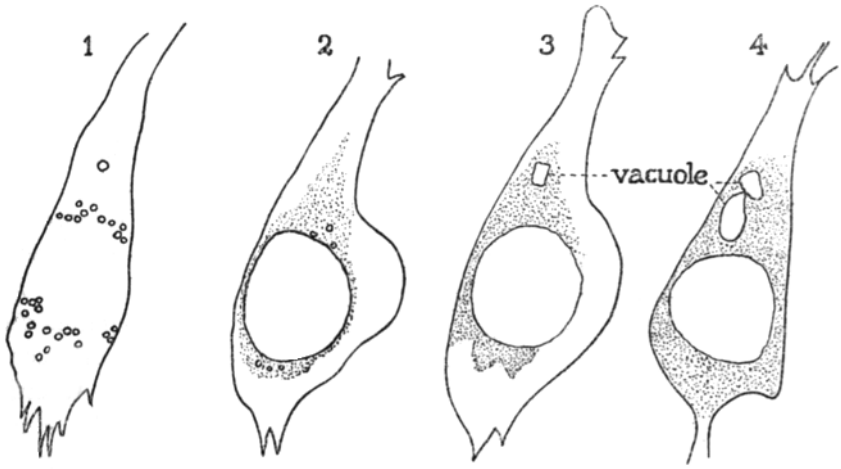
medium (0.54 per cent sodium chloride), and consequently the cells survived longer. On one slide they lived for 12 days without a change of the solution. As a rule, the cells appeared normal, with large growths in both reticular and migratory formation. The individual cells were normal in shape and the neutral red granules were usually the same as those in tissue grown in Locke-Lewis solution. Even the mitochondria presented a normal appearance. The cells seemed to have completely adapted themselves to their hypotonic environment. In this medium, as in the former one, the first cells to die were those next to the tissue explant.

The third hypotonic solution (one part of Locke-Lewis solution to three parts of distilled water, with a sodium chloride content of 0.225 per cent) gave no growth whatever after 48 hours of incubation. One drop of Locke-Lewis solution was added to some of the slides and the tissue again incubated in the hope of inducing migration, but the cells appeared to be dead.

Hypotonic Plus Hypotonic Solution.—The fact that excellent growths had been obtained on some slides of the Locke-Lewis 0.45 per cent sodium chloride medium while there was none on others planted the same day, in the same medium, and from the same heart, suggested the possibility that the particular explants that grew so well may have contained sufficient plasma of their own to change the medium. Accordingly, to the 24 and 48 hour growths in this medium was added some of the same fresh solution. On many slides the cells were all dead on the following day. On others the cells at the edge of the growth, which were very flat and thin with a large surface exposed, had been killed by the new hypotonic solution, while others had migrated out over them and were still in good condition (Fig. 3), showing that they retained the power of adapting themselves to the medium. It is not possible to determine here whether the explant had taken sufficient plasma with it to modify the medium, or whether the resistance of the individual cells was greater on some slides than on others. This individual difference in the cells is a factor always to be considered. It makes experimenting with tissue cultures a difficult problem, and one must perform a large number of experiments in order to draw any trustworthy conclusions.

Effect of Hypotonic Solutions on Normal Growth.

Hypotonic Solution, 0.225 Per Cent Sodium Chloride.—When a normal growth of 48 hours was treated with hypotonic Locke-Lewis solution with 0.225 per cent sodium chloride content, it was killed almost at once. The cells could therefore be stained with Janus black No. 2 without danger of interfering with the results of the experiment. After the growths had been stained with neutral red and Janus black, drawings were made of certain cells and the hypotonic solution was added.



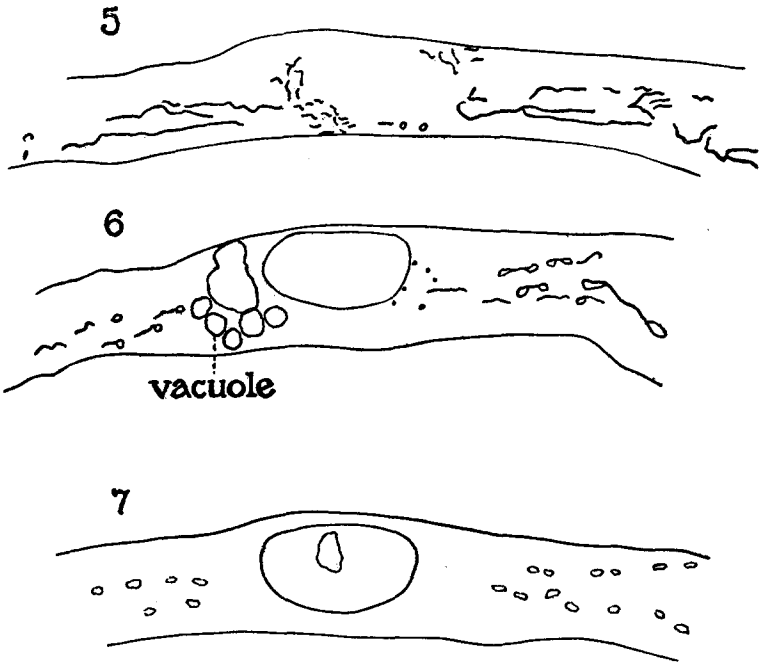
All the text-figures are camera lucida drawings made from living cells which have been stained with neutral red and often also with Janus black No. 2. The neutral red granules are represented as small circles, the mitochondria as solid lines or solid rods.

TEXT-FIG. 1. A normal fibroblast from a 48 hour growth explanted from a 9 day chick embryo heart and stained to show the neutral red granules.

TEXT-FIGS. 2 to 4. The same cell after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). Text-fig. 2 shows the cytoplasm much enlarged by the intake of water and divided into a granular and a clear area. The nucleus is swollen and has a distinct nuclear wall. A few neutral red granules are still visible. In Text-figs. 3 and 4 vacuoles are appearing. In Text-fig. 4 the cell is becoming smaller. Death changes have set in.

Many of the cells immediately took up a large amount of water. Text-fig. 1 shows a cell stained with neutral red, in which the gran-

ules are grouped together around the centriole. Text-fig. 2 represents the same cell 8 minutes after treatment with the Locke-Lewis sodium chloride 0.225 per cent solution. Here we have two kinds of cytoplasm, a granular part around the much swollen nucleus, and a clear outer area which has taken up water in the process of equalizing the osmotic pressure within the cell incident to the new environ-



TEXT-FIG. 5. Normal fibroblast from a 48 hour growth explanted from a 9 day chick embryo heart.

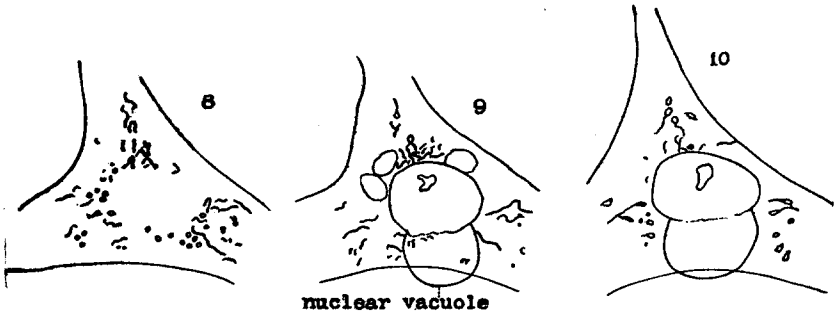
TEXT-FIG. 6. The same cell 5 minutes after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). The mitochondria are forming vesicles at their ends. Vacuoles are appearing in the cytoplasm.

TEXT-FIG. 7. The same cell 55 minutes after treatment. Only faintly staining vesicles remain where the mitochondria were.

ment. The processes have become more distinct as they, too, are full of the absorbed water. In Text-fig. 3 the neutral red color has disappeared from the granules and the nuclear wall is very distinct—both indications of cell death. In Text-figs. 3 and 4 vacuoles are appearing. Text-fig. 4 was drawn 4 hours, 25 minutes after the cell

was treated. Again the cell is small and most of the clear cytoplasm has been filled up with fine granules. This slide was later stained with neutral red, but the new vacuoles did not take up the stain, having undergone some death changes.

When the reaction of the cell to the hypotonic solution was not too sudden the effect on the mitochondria could be followed. Text-fig. 5 shows a cell with long branching mitochondria, drawn at 3.17 p.m. It was treated with hypotonic Locke-Lewis 0.225 per cent sodium chloride solution at 3.20 p.m.; by 3.25 p.m. (Text-fig. 6) no neutral red granules could be seen, the nucleus had a distinct membrane, vacuoles were appearing, and the mitochondria were beginning



TEXT-FIG. 8. Normal fibroblast from a 9 day chick embryo heart; 48 hours growth.

TEXT-FIGS. 9 and 10. The same cell 30 and 50 minutes after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). All the neutral red granules have disappeared. The mitochondria are forming vesicles. Vacuoles have appeared in the cytoplasm and a nuclear vacuole has formed.

to degenerate. In some, either one extremity or both seemed to swell (Text-fig. 6). Gradually the connection between these two small vesicles became fainter, then disappeared, and finally there remained only a faintly staining pale blue vesicle of irregular outline where a large mitochondrium had been (Text-fig. 7). The small mitochondria had disappeared entirely.

This process is also seen in Text-fig. 8 (normal), and in Text-figs. 9 and 10, drawn respectively 30 and 50 minutes after treatment.

Not all the mitochondria in a cell form these vesicles while undergoing degeneration, nor do those which do form them appear to bear any relation to the nucleus. Some are near it, some are farther away

near the cytoplasmic processes. In no case did I observe a swelling of the mitochondria as Lewis and Lewis (1914-15) and Cowdry claim occurs in hypotonic solutions. In every experiment the neutral red color disappeared from the granules almost as soon as the hypotonic solution was added. The mitochondria are much slower in disappearing.

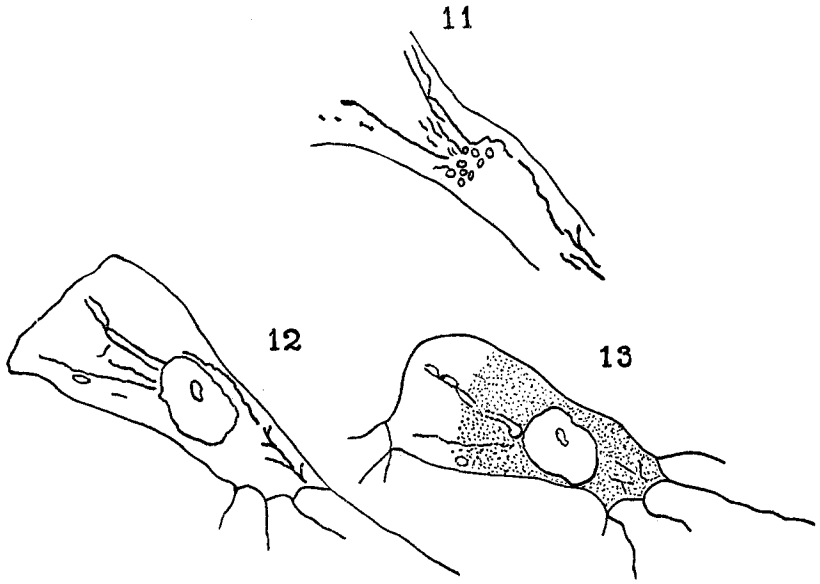
Hypotonic Solution, 0.3 Per Cent Sodium Chloride.—When the normal cells were treated with hypotonic Locke-Lewis solution with 0.3 per cent sodium chloride content, practically the same results as those recorded above were obtained. Certain mitochondria were followed and most of them simply faded from view. A few first formed a small vesicle at one end.

Hypotonic Solution, 0.45 Per Cent Sodium Chloride.—Hypotonic Locke-Lewis solution with 0.45 per cent sodium chloride content was tried on normal 48 hour growths. One cell (Text-fig. 11) with large, characteristic mitochondria was followed closely for 6 hours. Two large mitochondria in the anterior end were crossed; near them was a long, slender mitochondrium extending almost to the nucleus, and in the posterior end were some shorter ones with characteristic shapes. When treated with hypotonic solution the cell reacted at once. The color in the neutral red granules soon disappeared and the nuclear wall became distinct and irregular in outline. The mitochondria did not change shape at first but gradually became fainter (Text-fig. 12). The two peripheral ends of the long crossed mitochondria seemed to come together to form a ring (Text-fig. 13). About half way down the mitochondria there was a break and the parts nearer the nucleus became granular. About 1 p.m. it was noticed that these granular parts of the mitochondria seemed to be connected with a small clear vesicle near the nucleus, It looked as though they were forming a tubule which opened into the vesicle.

During the next 3 hours there was little change in the mitochondria, except that they grew more slender and faint. The cytoplasm around them became more granular, so that it was difficult to follow them. At 4.15 p.m. they were finely granular; this condition persisted for 3 days, when the slide was destroyed. The long mitochondrium to the right of those which were crossed did not appear so granular. It seemed, like the distal ends of those which were crossed,

to become more slender as though some substance were dissolving away from it. I could not observe, however, that any of the mitochondria became shorter.

In some of the cells treated with hypotonic Locke-Lewis solution large, clear vacuoles forming around the nucleus were frequently observed (Text-figs. 9 and 10). Others would form in the cytoplasm,



TEXT-FIG. 11. Normal fibroblast from a 9 day chick embryo heart, 48 hours growth, showing characteristic mitochondria and neutral red granules grouped around the centriole area.

TEXT-FIG. 12. The same cell 2 hours later. The neutral red granules have disappeared. The nuclear wall is distinct and the mitochondria are becoming fainter.

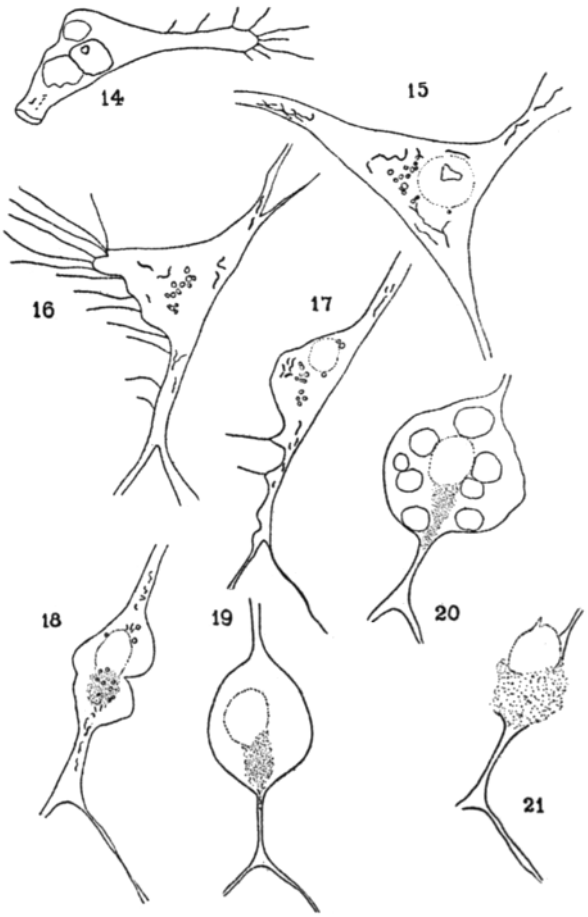
TEXT-FIG. 13. The same cell 2 hours later. The cytoplasm is granular. The two long crossed mitochondria are broken into pieces, some of which are granular. The other mitochondria are fainter or have disappeared entirely.

but these did not have the regular round or oval outlines of the nuclear vacuoles where the surface tension seemed to be very great. The nucleus, which was usually swollen in these cases, had taken up more water than it could hold and had broken out to form a vacuole. The nuclear wall near the vacuole was irregular in outline.

As already stated, the cells frequently become swollen with the intake of water as soon as they have been treated with the hypotonic solution. They remain in this condition for an hour or so, until the cell has begun to undergo the changes following death, when they show shrinkage. At times all the processes at one end are withdrawn and the end of the cell rolls back on the cell itself, as though from sudden contraction (Text-fig. 14).

A few experiments were tried with tissues which had failed to grow in Locke-Lewis 0.45 per cent sodium chloride solution. To each was added a drop of normal Locke-Lewis solution and the slides were again incubated, but no migration of cells ever appeared. A similar experiment was tried with tissue that would not grow in normal Locke-Lewis solution. Distilled water was added to the slides but the tissues did not respond, even after being in the incubator 48 hours. They appeared to have lost the power to migrate. They were not dead, for the pieces of heart frequently continued to contract, although cell migration could not be induced.

It was found that when hypotonic Locke-Lewis 0.45 per cent sodium chloride solution was added to normal growths it did not always kill the cells. A series of general experiments with this solution was tried with 24 and 48 hour growths. After thoroughly washing the cultures with the solution they were put in the constant temperature chamber at 39°C. and observed from hour to hour in a warm box. In a short time they took on a very different appearance from the normal. The cells were spider-like, their form completely changing from the flat, triangular, and fusiform shape, with long, fine processes almost invisible at the ends. Now the processes had a definite outline and seemed to anastomose, and the cell bodies had thickened into round or oval forms. Sometimes only the cells at one side of the explant would be thus affected, at other times those at one end, and again, all the cells. After 24 hours some of these cells would be dead, others would have recovered, and growth and migration would be going on beyond the dead cells. In one series of experiments pieces from the same heart were planted in hypotonic Locke-Lewis 0.45 per cent sodium chloride solution and in normal Locke-Lewis solution. Growth took place in both. When the controls were 24 and 48 hours old they were treated with hypotonic



TEXT-FIG. 14. Fibroblast half an hour after treatment with Locke-Lewis solution (0.45 per cent sodium chloride content), showing one end rolled over onto the cell as if from contraction.

TEXT-FIG. 15. Normal fibroblast from a 7 day chick embryo heart; 4 days growth.

TEXT-FIGS. 16 to 21. The same cell after treatment with Locke-Lewis solution (1.8 per cent sodium chloride content). Text-fig. 16 shows long fine processes and a condensation of the cytoplasm. In Text-figs. 17 and 18 the processes have been withdrawn and blebs are forming. The mitochondria are shorter and more condensed. In Text-fig. 19, a large bleb surrounds the nucleus and granular cytoplasm. Text-figs. 20 and 21 show death processes. Vacuoles appear and the cell wall breaks down leaving only the nucleus and granular cytoplasm.

Locke-Lewis 0.45 per cent sodium chloride solution. Some were killed while others grew.

The effects following the addition of the hypotonic 0.45 per cent sodium chloride solution were varied. On some slides all the cells were killed; on others only a few, and migration went on beyond them; on still others the solution acted as a stimulant and growth and migration were greatly accelerated. Here again the condition of the cells in the original growth must have had a great influence on the results. While I tried to take only slides which had good reticular growths, there must have been great individual differences, as even in normal Locke-Lewis solution some cultures will live much longer than others made from the same heart, in the same medium, and at the same time.

Summary of Results with Hypotonic Solutions.

1. Locke-Lewis solutions were made hypotonic by the addition of distilled water. They were used with a sodium chloride content of 0.54, 0.45, 0.3, and 0.225 per cent respectively.
2. Tissue grew in the first two solutions, which acted as a stimulus.
3. The tissues did not live so long in these media as in normal Locke-Lewis solution, but growth was more rapid.
4. The cells of normal growth were killed by treatment with hypotonic solutions with a sodium chloride content of 0.3 and 0.225 per cent respectively.
5. The cells absorbed much water, as did also the nucleus, which frequently formed a nuclear vacuole as an outlet for the extra amount of liquid absorbed.
6. Neutral red vacuoles and granules soon lost their color when the cells were treated with the hypotonic solutions that caused their death.
7. Mitochondria were not affected by the hypotonic solutions, but as the cell died vesicles formed at the extremities and persisted after the rest of the mitochondrium had disappeared; or the mitochondria broke up into granules or simply became more slender until only a faint, rough outline was visible.

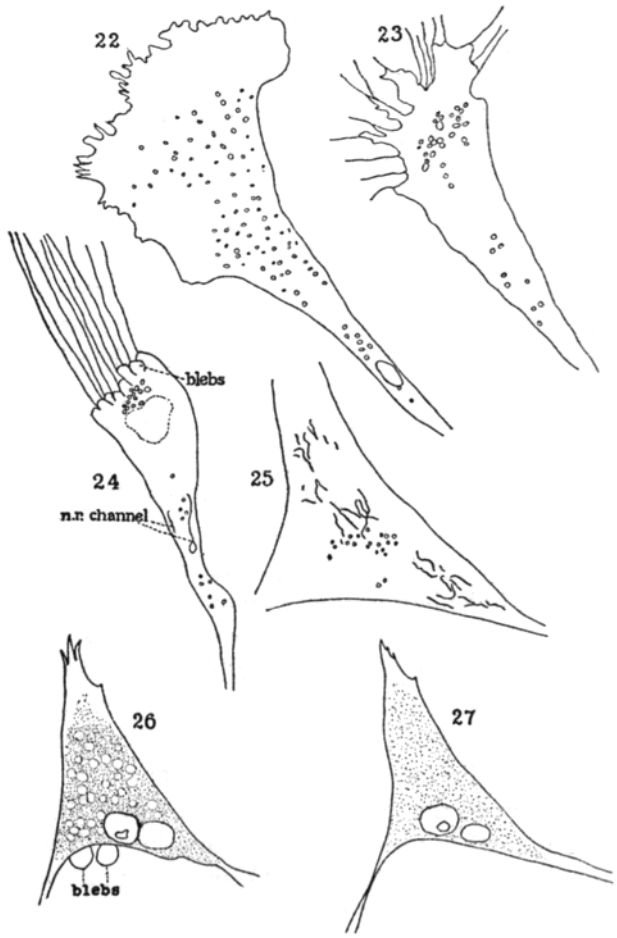
Experiments with Hypertonic Solutions.

Three grades of hypertonic Locke-Lewis solution, containing respectively 1.8, 1.5, and 1.2 per cent of sodium chloride, were used in this series of experiments.

Hypertonic Solution, 1.8 Per Cent Sodium Chloride.—Thirty-eight transplants were made, not one of which showed any signs of growth. It was evident that the fluid was too hypertonic to induce cell migration. Next the effect of this medium was tried upon tissue that had been growing well in normal Locke-Lewis solution for 2, 3, and 4 days respectively. In all the cells were killed within 3 or 4 hours, and in some within 1 hour after treatment.

The effect was followed on certain individual cells which were first stained with neutral red and Janus black No. 2. Text-fig. 15 is a cell taken from a 4 day growth of a 7 day chick heart after it had been treated with neutral red and Janus black at 11.15 a.m. At 11.20 the Locke-Lewis 1.8 per cent sodium chloride solution was added. By 11.25 the cell had begun to contract and crumple up like folds in a piece of cloth. At 11.30 (Text-fig. 16) the body of the cell was thick, and from one side and from the ends, where there had been processes, fine, thread-like structures extended out into the medium. By 11.40 (Text-fig. 17) most of these processes had been withdrawn; by 12.02 p.m. (Text-fig. 18) all the side processes had been withdrawn and the central granular part was surrounded by a clear, flat area. In Text-figs. 16 and 17 both mitochondria and neutral red granules are arranged as in normal growth. In Text-fig. 18 the mitochondria have become shorter and more concentrated in the center of the cell. At 12.15 (Text-fig. 19) the neutral red granules and mitochondria had disappeared and the cell was dead. Text-figs. 20 and 21, drawn at 12.20 and 12.40, show further death changes. In Text-fig. 20 large vacuoles are appearing in the clear areas. These later break down, leaving only the granular cytoplasm and nucleus (Text-fig. 21).

Text-figs. 22 to 24 show the contraction of the cytoplasm and the concentration of the neutral red granules at the end where the nucleus is located. The fine processes at the large end were invisible at 10.07 a.m. when the cell was treated with Locke-Lewis 1.8 per cent



TEXT-FIGS. 22 to 24. Cell from a 4 day growth of a 7 day chick embryo heart, stained with neutral red and treated with Locke-Lewis solution (1.8 per cent sodium chloride content). The processes at the anterior end have become visible in Text-fig. 22. Text-fig. 23 shows a contraction of the cytoplasm and a partial withdrawal of the anterior end to form thread-like processes. These processes are much longer in Text-fig. 24 where neutral red channels are shown opening into neutral red vacuoles.

TEXT-FIG. 25. Fibroblast from a 7 day chick heart; 4 days growth.

TEXT-FIGS. 26 and 27. The same cell after treatment with Locke-Lewis solution (1.8 per cent sodium chloride content). The alveolar structure of protoplasm is shown (Text-fig. 26), which 1 hour later (Text-fig. 27) became finely granular.

sodium chloride solution. By 10.10 (Text-fig. 22) they had contracted until they were plainly visible. Neutral red vacuoles are scattered over the cell, a very large one being in the posterior long process, only part of which was drawn. By 11.45 (Text-fig. 23) the cell had shrunken appreciably, the neutral red granules were concentrated at the anterior end, and thin processes were forming. At 12.35 p.m. (Text-fig. 24) these looked like long streamers. It appeared as though the anterior end of the cell had been attached at places to the cover-glass, and that when the cell began to shrink from loss of water the cytoplasm was pulled out into these long processes. When the pull was too great they lost hold and were rapidly withdrawn into the cell.

Coincident with the formation of these protoplasmic streamers, blebs—clear, round cytoplasmic protrusions—appeared behind them. Toward the center of the cell neutral red channels opened into the large neutral red vacuole. In many of the cells these channels were branching and anastomosing. This particular cell was dead at 1.45.

To those who still believe in the alveolar and reticular structure of protoplasm Text-figs. 25 and 26 will prove of interest. Text-fig. 25 shows a cell stained with Janus black and neutral red which bring out the mitochondria and the neutral red granules. At 11.20 a.m. the tissue was treated with Locke-Lewis 1.8 per cent sodium chloride solution. This cell did not contract so much as many of the others, nor were there formed the long, fine processes which I have found so characteristic of death in these hypertonic solutions. The cytoplasm, however, became alveolar (Text-fig. 26), small, round spaces appearing over the granular part of the cell by 12.25 p.m. The nuclear wall was very distinct, the granules no longer showed the neutral red color, and the mitochondria had disappeared—all evidences of cell death. By 1.45 (Text-fig. 27) these alveoli had also disappeared and the protoplasm had become granular. It would seem quite certain, therefore, that the alveolar structure described by the early cytologists was an artifact caused by the fixing solution, which was hypertonic to the protoplasm.

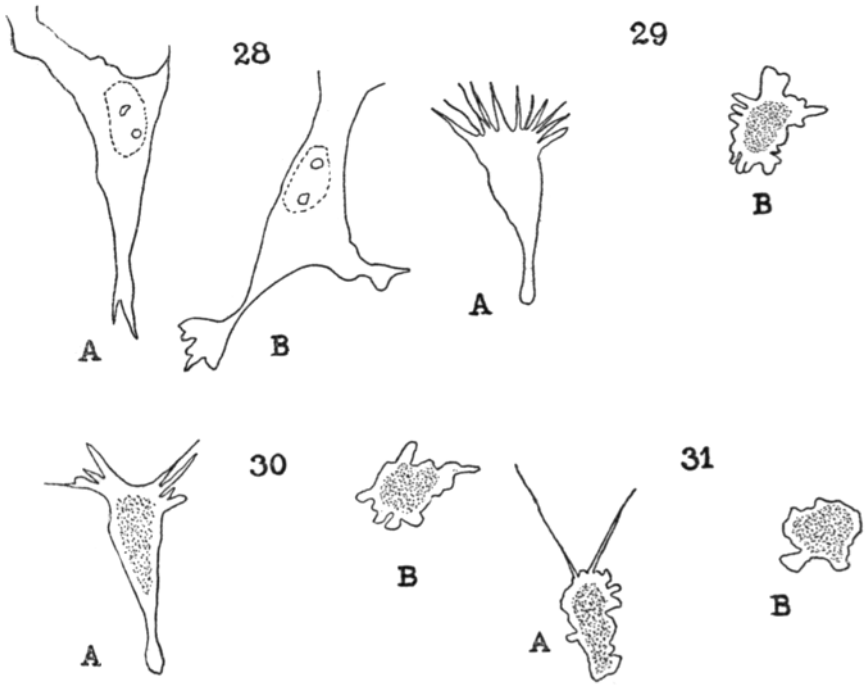
Hypertonic Solution, 1.5 Per Cent Sodium Chloride.—In spite of the fact that several authors have stated that tissues will not grow at all, or, at best, very poorly in hypertonic solutions, I have neverthe-

less grown a few in hypertonic Locke-Lewis solution with a sodium chloride content of 1.5 per cent. Out of 130 explants of heart tissue in this medium, twelve (9 per cent) showed migration and reticular formation. One of these lived 7 days. The migration was not great, and only in a few instances were there reticular growths.

I was interested in learning how the mitochondria would be affected by this new medium stained with Janus black No. 2 and neutral red. The mitochondria in many of the slides were long, irregular, and spread out normally into the processes of the cells. The neutral red granules were also arranged around the centriole and the mitochondria mingled with them as in normal cells. Occasionally the mitochondria were round or slightly oblong, but this was undoubtedly due more to the generally poor condition of the growth than to the hypertonic medium. The same round mitochondria are found on slides of Locke-Lewis solution when the growth is poor, and are probably due to some metabolic disturbance.

In order to observe the direct effect of this solution upon the normal cells I treated heart tissue, which was growing well in normal Locke-Lewis solution, with Locke-Lewis 1.5 per cent sodium chloride. In most cases it killed the cells. In some instances normal growths were used, other pieces of which were growing in the hypertonic 1.5 per cent solution, but the shock of this hypertonic solution to the delicate cells, thinly spread out on the cover-slip, was usually so great that they died. I noted especially the fate of two interesting cells (Text-fig. 28) in a 48 hour growth from a 10 day chick heart, which was treated with Locke-Lewis sodium chloride 1.5 per cent solution at 11.15 a.m. The reaction was immediate (Text-fig. 29). Their clear processes were retracted and formed long, fine processes on cell *A*, cell *B* contracting into an irregular mass. The fine processes on *A* were gradually withdrawn until all had disappeared (Text-figs. 30 to 33). *B* showed two distinct kinds of protoplasm—a clear, outer part, resembling the ectoplasm of an ameba and similarly active; and a denser, granular part in the center of the cell. This central mass was more stationary and the clear protoplasm flowed around it, either evenly, or forming blunt processes, which would be withdrawn and then form anew at another place, as described by Cash for cells treated with ether vapor. Later cell *A*

developed the same kind of movement (Text-fig. 34), but at noon was an inactive mass of cytoplasm. By 2.40 p.m. *B* had recovered completely; *A* was still an irregular mass, though the outer cytoplasm was again active (Text-fig. 35). By 4 p.m. (Text-fig. 36) both were active—*B* normal, *A* abnormal. The following day the cells had migrated and changed positions, so that it was impossible to say

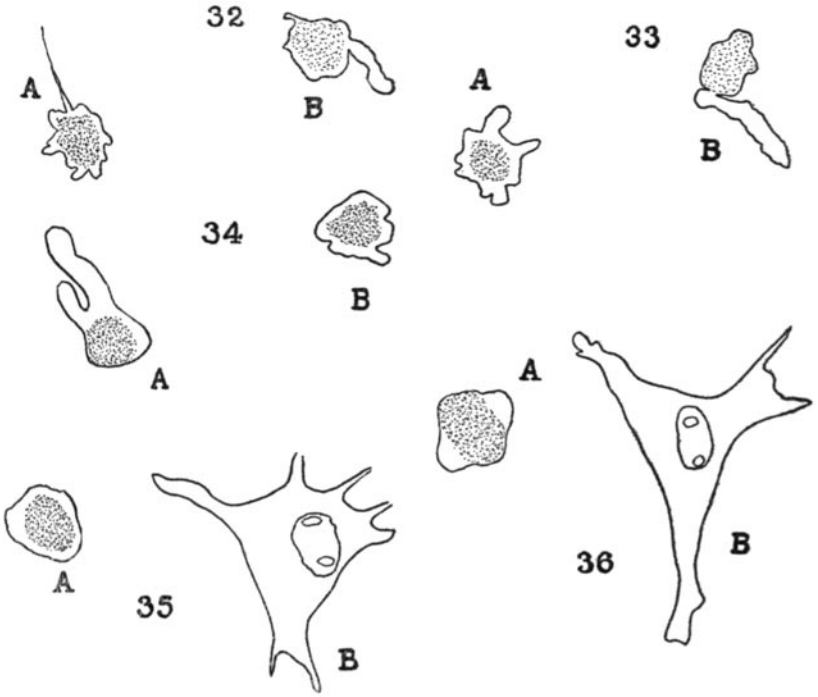


TEXT-FIG. 28. Two normal fibroblasts, *A* and *B*, before treatment with Locke-Lewis solution (1.5 per cent sodium chloride content) at 11.15 a.m.

TEXT-FIGS. 29 to 31. The same cells after treatment. In *A* is shown the formation of long fine processes, which were gradually withdrawn. *B* is an irregular mass showing two kinds of protoplasm, granular and clear, the latter moving like the pseudopodia of *Amœba proteus*.

whether these particular cells had entirely recovered. Half the number of cells on the slide were normal, with stellate forms; hence some of them had been able to withstand the shock. In another case which was followed one cell was apparently killed at once, while a larger

cell with a long process remained active, going through the stage of fine process formation and finally contracting, though the long posterior process remained extended like the posterior end of a *Vorticella*, acting as an anchor.



TEXT-FIGS. 32 to 36. Later stages of the cells shown in Text-figs. 28 to 31. The fine processes of *A* were completely withdrawn and after passing through an ameboid stage it became an irregular, rather inactive mass of protoplasm. *B* after the ameboid stage became normal.

Hypertonic Solution, 1.2 Per Cent Sodium Chloride.—Growth in this solution was very good; 32 per cent of the transplants grew, and one lived 15 days without being opened or treated in any way. Growth, however, was much slower than in the controls or in the hypotonic solutions. Often the cells did not begin to migrate until the 2nd or 3rd day, and then slowly, though in growths in which they were going to form a reticulum, they had done so by the 4th

day. Migration was not greater than in the hypotonic solutions but was often abundant, and the appearance of both migratory and reticular formations was that of healthy, normal cells.

When these cells were stained with neutral red and Janus black No. 2 the neutral red granules were found to be present in varying amounts and were arranged around the centriole, as in the controls. Usually there were some long mitochondria, but these varied in size.

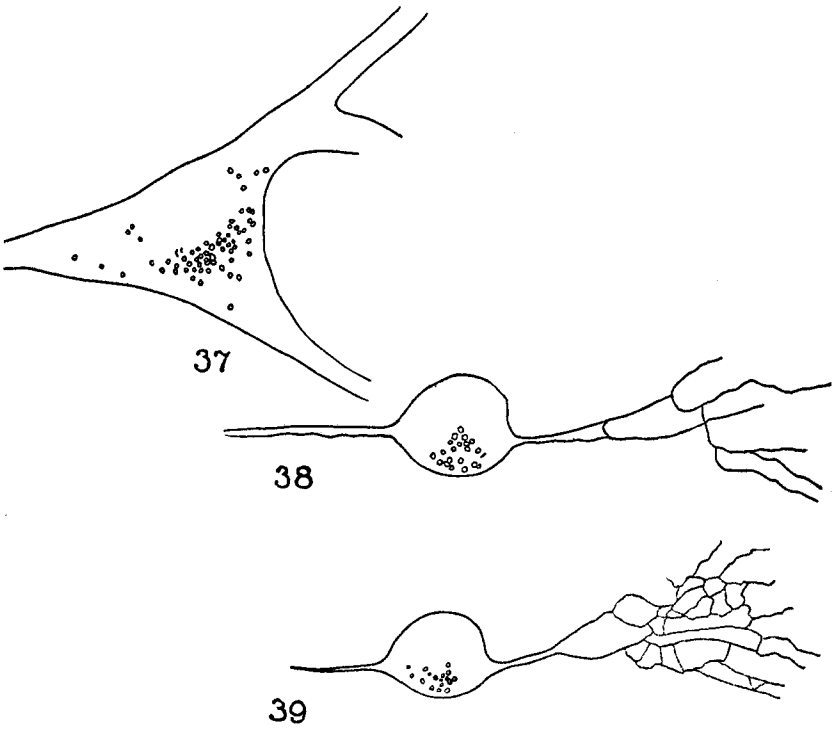
Occasionally the number of migrating cells was very small and these sometimes showed abnormalities. On one slide the cells extended out from the explant like a string of beads, three or four to a string. On another slide, which was later fixed in Zenker's fluid and stained with Ehrlich's hematoxylin, most of the cells were not flat but slightly rotund. The mitochondria were all round, a few neutral red granules were present, and the nucleus in many instances was apparently budding or dividing amitotically, in a manner similar to that shown in some of Macklin's figures. Some cells showed as many as three nuclei of different sizes, though all contained chromatin material. The metabolism of this culture had been seriously deranged, resulting in this change in the nuclear and mitochrondrial conditions.

The results following the addition of Locke-Lewis 1.2 per cent sodium chloride solution to normal growths of 1, 2, and 3 days were varied. The reaction was much slower than with the other hypertonic solutions. On three slides of 3 days growth few of the cells showed any contraction after 6 hours of treatment. Many were abnormal and contained a great number of deeply staining neutral red vacuoles. These cells lived and the vacuoles retained the neutral red stain for 1 to 3 days after treatment, or until some of the cells were 8 days old.

On other slides of 2 and 3 days growth interesting changes in the cells were observed. The long, fine, straight processes were also present here. Text-fig. 37 shows a normal cell which at 10.45 a.m. was treated with Locke-Lewis 1.2 per cent sodium chloride solution. At the end of half an hour little change was noted. Within the next hour the cell contracted and a fine network of protoplasmic processes formed at one side, quite a distance from the cell, yet connected with it by a long strand of clear protoplasm (Text-fig. 38).

By 2.10 p.m. (Text-fig. 39) this network had become more intricate, the neutral red granules had almost disappeared, and the cell was practically dead. Later, large clear vacuoles formed in the central mass.

In another cell which was followed the central mass contracted, leaving the clear cytoplasm in a large, thin sheet, stretching out at



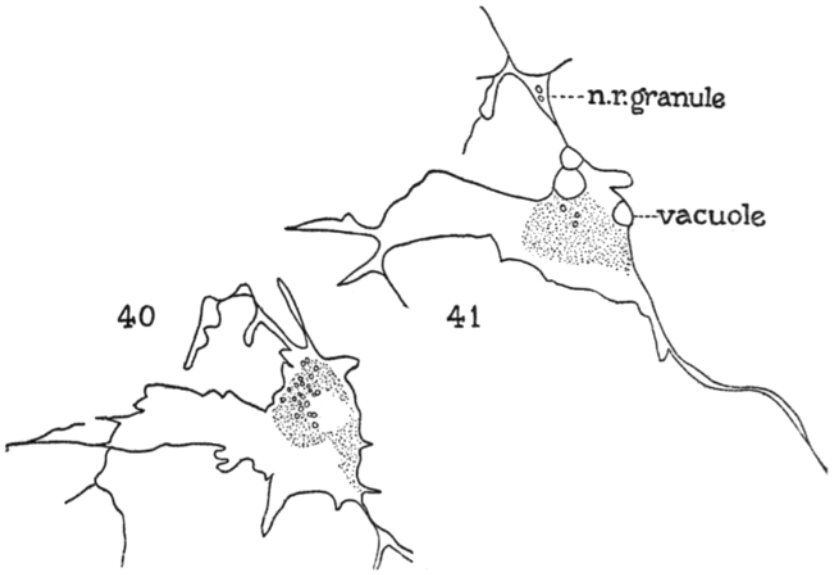
TEXT-FIG. 37. Normal fibroblast of a 7 day chick embryo heart; 3 days growth.

TEXT-FIGS. 38 and 39. The same cell after treatment with Locke-Lewis solution (1.2 per cent sodium chloride content), showing fine protoplasmic network developed from the processes of the cell.

one side of the cell with many slender processes extending in various directions. In Text-fig. 40 can be seen the concentration of neutral red granules in one part of the cell, while in Text-fig. 41 two of the granules have been caught in one of the processes when the central mass began to contract. Here blebs are forming and the large

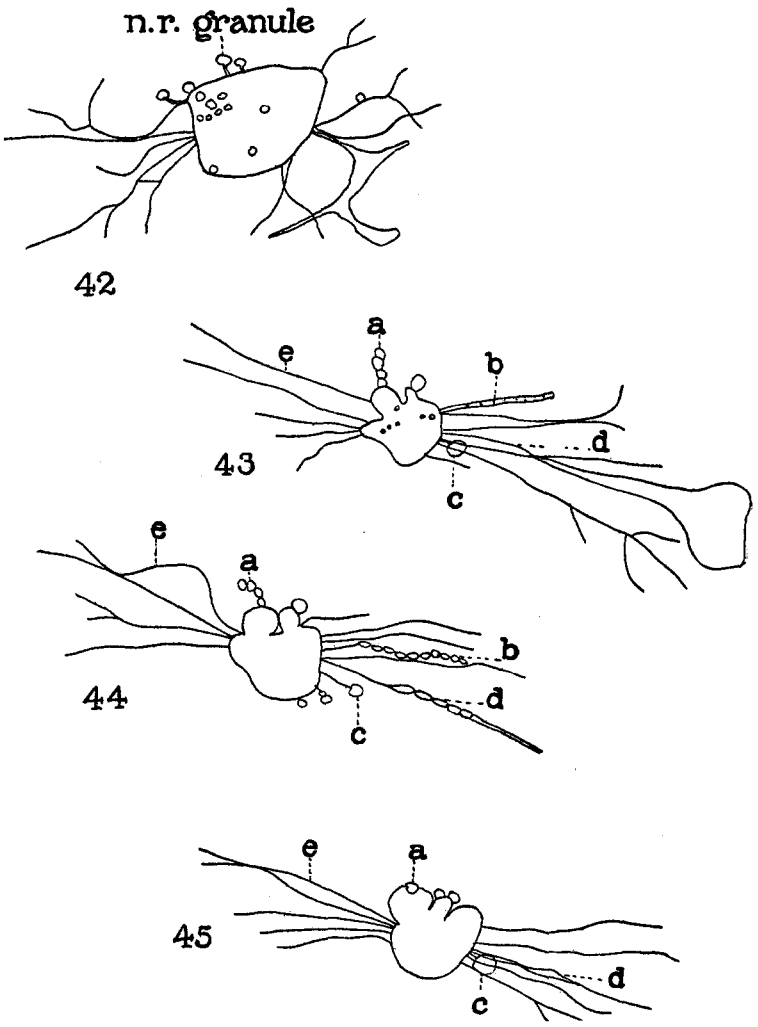
vacuoles indicate that the cell is nearly dead. In Text-fig. 42, from another cell, small neutral red granules are shown alongside of the processes and evidently held to them by fine protoplasmic threads, for they remain stationary though vibrating all the time with Brownian movement.

Still other interesting formations are seen in Text-figs. 43 to 45, from a 2 day growth of a 7 day chick heart. This cell was one of a



TEXT-FIGS. 40 and 41. Fibroblast after treatment with Locke-Lewis solution (1.2 per cent sodium chloride content), showing a contracted clear flat area of cytoplasm where normally there were processes. In Text-fig. 41 two neutral red granules have been caught in one of the processes during contraction of the cytoplasm.

group of three which had been followed after its treatment with Locke-Lewis 1.2 per cent sodium chloride solution at 11 a.m. In Text-fig. 43 at *a* there is a chain of protoplasmic beads which waved back and forth (Text-fig. 44) in the medium. Later, three of these beads broke away and disappeared. At *b* (Text-fig. 43) a slightly thicker process is seen which at 2.40 p.m. showed light and dark areas like a striated muscle. These quickly became constricted into a chain of proto-



TEXT-FIG. 42. Fibroblast treated like that in Text-fig. 40, showing neutral red granules fastened to the cell by slender threads of cytoplasm.

TEXT-FIGS. 43 to 45. Fibroblast treated with Locke-Lewis solution (1.2 per cent sodium chloride content). Many of the fine processes showed movement. The beaded formations at *a* and *b* moved back and forth; *c* was continually oscillating; *d* was moving and *e* waved until it became attached to another fibril (Text-fig. 44); then it moved like an undulating membrane until the cell was dead.

plasmic beads which were free moving and waved about in the surrounding medium (Text-fig. 44). At 3.00 the process broke loose, became entangled with the other thin processes, and disappeared. *c* was a round process of protoplasm attached by a fine thread, which also oscillated back and forth. *d* was apparently attached to a process from a neighboring cell, and in Text-fig. 43 was only a thick process. In Text-fig. 44 it had become beaded, the beads being of different sizes, and was undulating gently. Later (Text-fig. 45) it had become practically straight again, and as the cell was dying the movement gradually lessened. *e* always remained a thin, thread-like process. At first free moving (Text-fig. 43), it later became attached to another process (Text-fig. 44) which was stationary. However, it continued to wave like an undulating membrane until nearly 4 p.m., when motion in the processes ceased and the cell was dead, the color in the neutral red granules having also disappeared.

This long, waving fiber answers the description of those grown by Baitsell in plasma. He believes that they are not outgrowths of the embedded tissue but come from fibrin in the plasma clot. His evidence for this is not complete, as his stains give two different results, one confirmatory of his theory, the other pointing toward the cellular origin of the fibers. In the present experiments there was no plasma and consequently no fibrin. Furthermore, this fibril is directly connected with the cell and remains in active motion only as long as the cell is alive. Its place of origin, the clear, protoplasmic area which contracts to form long, fine processes, is the same as that given by Lewis (1917). She describes the formation of the fibrils of connective tissue from the ectoplasm of the cell.

Summary of Results with Hypertonic Solutions.

1. Hypertonic solutions were made by boiling down Locke-Lewis solution until the sodium chloride content was 1.2, 1.5, and 1.8 per cent respectively.

2. Tissues grew in the first two of these solutions.

3. Tissues did not live so long in these solutions as in normal Locke-Lewis solution and growth was slower.

4. The cells of normal growth were killed by treatment with hypertonic solutions with a sodium chloride content of 1.8 and 1.5 per cent.

5. When treated with hypertonic solutions the cells usually contracted, their thin processes became long and thread-like and were later drawn into the body of the cell.

6. Connective tissue fibrils formed from these thread-like processes. They moved and anastomosed with other fibrils.

7. Neutral red channels formed in many cells.

8. The cytoplasm frequently became alveolar when the death process set in.

9. These three hypertonic solutions showed a definite gradation in their effects on the processes of the fibroblast. In Locke-Lewis solution containing 1.8 per cent sodium chloride the processes contracted rapidly, leaving many thread-like structures in their places. These were quickly withdrawn and the cell soon died. In Locke-Lewis solution containing 1.5 per cent sodium chloride the thread-like processes were frequently formed, but the cells did not all die; some recovered. In Locke-Lewis solution containing 1.2 per cent sodium chloride the processes still formed but more slowly. They also showed motion, which lasted as long as the cell was alive.

Hypertonic versus Hypotonic Solutions.

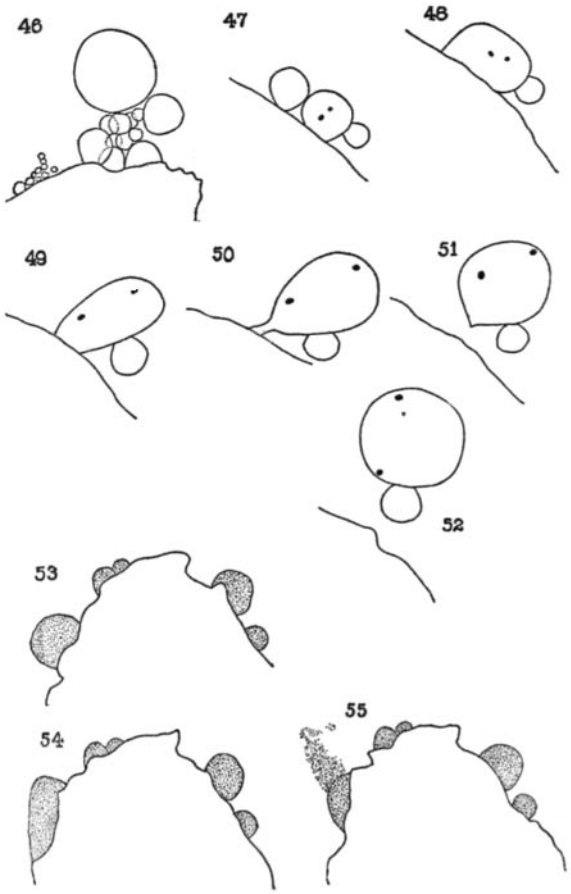
In comparing the growth in hypertonic with that in hypotonic media the following points may be emphasized. In the former the migration is slower than in the latter. In hypertonic solutions the optimum growth is reached on the 3rd day or later, in hypotonic solutions on the 2nd day. The growths live longer in hypertonic solutions but are, as a rule, smaller than in hypotonic solutions. Table I shows this difference of growth in the various media.

TABLE I.

Amount of NaCl in medium.	Total No. of explants.	No. that grew.	No. that died.	Growth.	Greatest No. of days any culture lived in the medium.
per cent				*per cent*	
1.8	38	0	38	0	0
1.5	130	12	118	9	7
1.2	100	32	68	32	15
0.9	282	188	94	66.6	19
0.54	23	20	3	86.9	7
0.45	202	90	106	44.5	12
0.225	12	0	12	0	0

There seemed to be a possibility of proving whether or not the neutral red granules and vacuoles are the product of metabolism, as Lewis (1919) believes, by an experiment in which parts of the same heart were grown respectively in Locke-Lewis normal solution, Locke-Lewis solution with sodium chloride content of 0.45 per cent, and with 1.2 per cent sodium chloride content, the rate of growth being different in each of these media. Accordingly, pieces of heart from a 7 day chick embryo were planted in the three solutions and incubated for 48 hours. At the end of 24 hours nine of the normal Locke-Lewis cultures, eight of the 0.45 per cent sodium chloride, and two of the 1.2 per cent sodium chloride showed growth. After another 24 hours of incubation eight of the Locke-Lewis, four of the 0.45 per cent sodium chloride, and five of the 1.2 per cent sodium chloride cultures were alive. Here is seen again the slow growth in hypertonic solutions. These cultures were stained with neutral red and Janus black No. 2. Growth in both the Locke-Lewis solution and 1.2 per cent sodium chloride was good, and each showed a normal arrangement of the neutral red granules. In the hypotonic solution growth had been poor. Few cells had migrated, and in these the large neutral red vacuoles were full of deeply stained material. The cells, in their poor condition, were evidently having difficulty in getting rid of the waste products which were accumulating from metabolism.

In working with hypertonic solutions, occasionally with normal Locke-Lewis solution, and in a few instances with hypotonic solutions, I observed an interesting attempt on the part of the tissue to adapt itself to the new medium. Tissues were planted in Locke-Lewis solution containing 1.8 and 1.5 per cent sodium chloride. On the following day transparent, balloon-like structures were noted, varying in size from quite small to very large, which formed along the edges of the culture. Some explants had many of these balloons around the sides, others only a few large ones. Sometimes balloons of all sizes piled up in masses (Text-fig. 46), those at the top being free from the tissue. Upon close observation these structures were seen to begin as small hemispheres rising out of the explant. In time they became almost spherical and increased in size as though something from the tissue was being poured into them. Occa-



TEXT-FIG. 46. Masses of balloons of all sizes piled up at the edge of the heart tissue which had been planted in Locke-Lewis solution of 1.5 per cent sodium chloride content 48 hours before.

TEXT-FIGS. 47 to 52. The formation of a large balloon by the fusion of two small ones (Text-figs. 47 and 48). This large one elongated (Text-fig. 49), formed a stalk (Text-fig. 50), was pinched off (Text-fig. 51), and became free floating (Text-fig. 52).

TEXT-FIG. 53. Granular hills along the edge of a piece of heart planted in Locke-Lewis solution of 1.5 per cent sodium chloride content.

TEXT-FIGS. 54 and 55. One of these granular hills enlarged and discharged its contents into the surrounding medium.

sionally two fused together, as shown in Text-figs. 47 and 48. This particular one continued to increase in size until the pressure became so great that it elongated (Text-fig. 49). A small stalk was formed (Text-fig. 50) which quickly snapped, thus freeing the balloon (Text-fig. 51). It rounded up into a ball within a few seconds (Text-fig. 52) and rolled around over the tissue when the slide was tilted back and forth. It appeared to be taking in culture medium or else flattening out, as it continued to increase in size until one side grew faint, then disappeared, and the balloon went to pieces.

Some cultures were full of these balloons floating around singly or in masses. They were frequently found being given off on the 2nd day, but were not seen to form after that, though those already formed persisted for several days. One slide 11 days old still showed masses of them, but these disappeared on the 12th day. The balloons were clear and transparent, and occasionally contained small particles like cells. There was a distinct surface tension which quickly rounded them up after they were given off. They were also very delicate structures, as evidenced by the fact that when attempts were made to transfer the cover-slip, with the culture in its hanging drop, from the depression slide with the vaseline ring to a straight slide with a large drop of the same hypertonic solution in which the tissue was planted, the balloons nearly always went to pieces, no matter how carefully the cover-slip was lowered onto the new drop of medium. Occasionally this transfer was successful, and on placing the slide under the microscope with dark-field illumination the balloons showed as small, milky white structures not only at the edges, as had appeared with the ordinary illumination, but all over the explant.

There was another type of structure, which I have termed granular hills, that also appeared over the explants. These were more stable and lasted as long as the cultures were kept. They grew in size, sometimes becoming quite large, though they were most frequently seen as small balls or hills along the edges or between the angles of the tissue (Text-fig. 53). They were very finely granular. Sometimes the surface tension would be taxed too much and the granular hill would break open at one place, pouring the fine granules into the surrounding medium (Text-figs. 53 to 55).

Both balloons and granular hills would appear on the same explant. These explants never showed growth or migration of cells, though they would often continue to beat for several days. The structures described above were evidently a means of adjusting the tissue to its new medium. Some days every tissue planted in Locke-Lewis 1.8 and 1.5 per cent sodium chloride solutions would show the balloons or the granular hills, sometimes both. Again, a new medium, made in the same proportions of Locke solution, bouillon, and dextrose, boiled down to the same volume as that used in the preceding experiments, would give few if any of these structures, and also practically no growth. The balloons and granular hills were not the large precipitations, with irregular, angular outlines, which are frequently observed in tissue cultures and which are much more coarsely granular. They may be the same material which Burrows and Neymann state is liberated from the cells when they are removed from their normal habitat to an oxygen-containing plasma or salt solution. These authors state that the substances are "almost transparent, their refraction not very different from that of the original medium, and they accumulate at the surface of the medium to form a membrane." Perhaps the growth in plasma with a different osmotic pressure from Locke-Lewis solution may account for the membrane formation instead of the balloons and granular hills found in my experiments. On the other hand, they may be quite different substances, as Burrows and Neymann state that theirs "are liberated in large amounts from a tissue fragment rich in cells." While the structures described above rarely appeared when there was cell migration, a few times I have found near the explant, after the cells had degenerated, granular hills which had been completely covered over by the cells.

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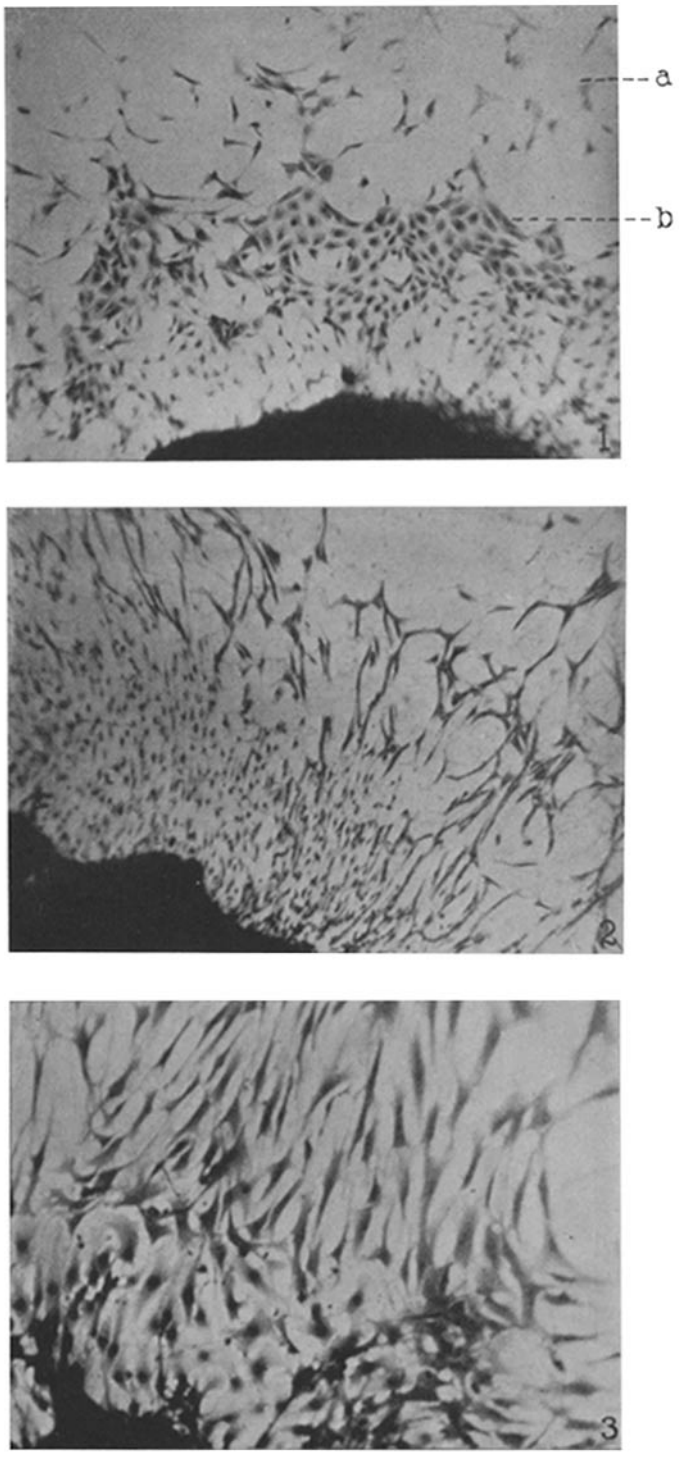
EXPLANATION OF PLATES.

PLATE 56.

FIG. 1. Photomicrograph of normal growth of chick heart tissue, showing fibroblasts at *a* in migration formation, and mesothelial cell in membrane formation at *b*.

FIG. 2. Photomicrograph of 3 day growth of chick heart tissue in hypotonic Locke-Lewis solution (0.45 per cent sodium chloride), showing the early death of cells next to the explant.

FIG. 3. Photomicrograph of a normal 48 hour growth which was treated with hypotonic Locke-Lewis solution (0.45 per cent sodium chloride) and incubated for 24 hours. Some of the cells next to the explant have been killed, but others are migrating out over them.



(Hogue: Fibroblasts of embryo chick heart.)