THE EFFECT OF DILUTION OF PLASMA MEDIUM ON THE GROWTH AND FAT ACCUMULATION OF CELLS IN TISSUE CULTURES.*

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PLATES 43 TO 45.

Since the method of cultivating tissues *in vitro* will no doubt be used in investigating the action of various substances on the growth and function of cells, it is important that the influence on cells, growing under these conditions, of certain easily modified physical and mechanical factors should be understood. The importance of these factors is indicated by the fact that so simple a matter as the thickness of the hanging drop of plasma influences markedly the extent of the outgrowth of cells from the original fragment of tissue.

In a former paper[1] we discussed the influence of temperature, including the effect of variations in the temperature of incubation as well as the effects of exposure of the cultures to high and low temperatures before incubation. In the present paper we shall consider the influence of another physical factor, namely dilution of the plasma medium, on the behavior of cells in cultures. Carrel and Burrows[2] (1911) were the first to describe the effect of dilution with distilled water on cell wandering, and the subject was also discussed in a recent paper by Burrows[3] (1913). Some of the work reported in this paper was begun soon after the appearance of the experiments of Carrel and Burrows, with the object of testing the correctness of the interpretation of their results regarding the

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1 Lambert, R. A., *Anat. Rec.*, 1912, vi, 91.

2 Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 562.

3 Burrows, M. T., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 77.

stimulating action of hypotonic plasma. Later, the use of the method of dilution for the study of the accumulation of fat by cells *in vitro* was suggested to us, and further experiments with this aim in view were carried out. We shall report here the results of two sets of experiments, (1) upon the effect of dilution on the migration and multiplication of cells in cultures, and (2) upon the effect of dilution on the accumulation of fat by cells in cultures.

THE EFFECT OF DILUTION OF PLASMA ON THE MIGRATION AND MULTIPLICATION OF CELLS.

Carrel and Burrows[4] reported that the addition of a limited quantity of water to chick plasma caused a more extensive outwandering of cells in cultures of chick embryo spleen. They also observed that the addition of hypertonic salt solutions was harmful. The stimulating effect in the first instance was attributed to the hypotonic state of the medium. No experiments with isotonic solutions as diluents were made. Burrows has recently published the results of studies upon the effect of the addition of such solutions. A summary of his work is given below in connection with the results that we have obtained.

We have repeated the experiments of Carrel and Burrows with hypotonic plasma, preparing at the same time cultures in plasma diluted with serum and with Ringer's solution. In addition to chick embryo spleen, we have used for cultivation chick embryo heart, bone marrow and intestine, rat embryo skin, and mouse carcinoma.

The Effect of Dilution on the Migration of Cells.—The first experiments consisted in the preparation of cultures of chick embryo spleen in the following media: (1) undiluted plasma, (2) plasma diluted with distilled water in the proportion of 3 to 2 (the optimum medium of Carrel and Burrows), (3) plasma similarly diluted with Ringer's solution, and (4) with serum, and (5) plasma diluted with serum in the proportion of 1 to 2.

Care was taken to have the pieces of tissue of uniform size, and the drops of plasma of as nearly uniform thickness as possible. Ten preparations were made with each medium. At the end of forty-eight hours' incubation, after which time experience had

[4] Carrel, A., and Burrows, M. T., *loc. cit.*

shown that little further migration of spleen cells took place, the cover-glasses with the clots attached were placed in formalin and later mounted on slides. With a projection drawing apparatus rough drawings were made in which the pieces of tissue and the halos of migrated cells were outlined. By taking the average of the radii of the tissue fragments and the average width of the cell halos, a composite drawing for each series of preparations was constructed. While the cultures in a single series differed little from one another, the composite drawings of the several series showed certain differences (figures 1, 2, 3, 4, and 5). The most extensive migration is seen to have taken place in the plasma diluted with two volumes of serum. There is little difference in the extent of migration about the pieces of tissue in plasma diluted with the smaller quantities of serum, Ringer's solution, and water, although in each the outwandering was more extensive than in the controls in undiluted plasma. In other words, 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 result is in agreement with the recent work of Burrows in which he found that the extent of migration of spleen cells in plasma diluted with varying quantities of serum increases with the amount of serum added. The effect is attributed to the diminution in the amount of fibrin in the clots. Since this reduction in fibrin results, in the beginning at least, in a coarser meshwork in the clot, the explanation of the increased migration of cells would seem to lie in the diminished resistance to the progressive movements of the cells. In order to test this explanation several other tissues were used, including chick embryo heart, intestine and bone marrow, rat embryo skin, and mouse carcinoma, the cells of which vary considerably in their migratory ability.

Briefly stated, it was found that cells which are relatively small and actively motile, such as those of the bone marrow and spleen, show increased migration in diluted plasma, whereas cells whose power of locomotion is limited, such as those of intestinal epithelium, rat embryo skin, and mouse carcinoma, which tend to spread out slowly in cultures in groups or large sheets, are not influenced by dilution of the medium. Cells which occupy a midway position

in the matter of motility, such as connective tissue cells, are only slightly influenced in this way. The mechanical explanation of diminished resistance to active cell locomotion offered by the coarser fibrin meshwork seems to us to account for these results.

The Effect of Dilution on Cell Multiplication.—An analysis of the results suggests other criteria for determining a true stimulating effect on tissue cultures than the extent of the outwandering of cells through the clot. It occurred to us that the extent of cell multiplication, as determined by the number of cells that undergo division in the preparations in a given time, would afford a much more satisfactory test. As we have pointed out, all stages of mitotic division can be observed with comparative ease in the living cell. Since division at body temperature is completed, as a rule, in from twenty to thirty minutes, the number of mitoses in a preparation may be hourly recorded without danger of counting the same cell twice. By making observations on small groups of preparations from the several series over a period of twenty-four hours or longer it is possible to arrive at a definite conclusion as to which series shows the most active multiplication of cells. We have studied cultures of chick embryo heart in pure plasma and in plasma diluted with water and with Ringer's solution in the proportions given above. There were five preparations in each set. Each preparation was examined six times during a period of forty-eight hours. The total number of mitoses for each of the three sets in the order stated was, 88, 96, and 81. In another experiment in which four observations were made the total numbers were, 76, 62, and 70. We concluded from these two experiments that dilution of plasma within these limits is without appreciable effect on cell multiplication.

THE EFFECT OF DILUTION ON THE ACCUMULATION OF FAT BY THE CELLS.

In previous communications we have referred to the appearance in the cells of tissue cultures of small droplets of fat which tend to increase in size and number with the age of the culture. After five to seven days the cells may be actually distended with the droplets.

We[5] reported observations which seemed to show that the accumulation was in all probability the result of a disturbance in cell metabolism. There was evidence that the vitality of the cells was not seriously impaired, since they were seen to move about actively and to undergo normal mitotic division. We also observed that cells growing in a thin drop of plasma accumulated less fat than those in a thick coagulum. This phenomenon can be easily demonstrated in a single preparation by shaking the drop of plasma before coagulation takes place, causing the drop to touch the slide cavity, and then by manipulating the tissue fragment so that it lies along the inner edge of the drop. The cells that grow in one direction into the thick clot accumulate a large quantity of fat in their cytoplasm, while those growing in the other direction in the thin film of medium on the cover-glass accumulate very little fat. It is difficult to say whether this difference in fat accumulation is due to the difference in the quantity of fat at the disposal of the cells, or to a difference in oxygen supply to the cells under the two conditions, as has been suggested by Burrows.

Several methods suggested themselves for testing the effect of a reduction in the quantity of fat in the medium on the amount of fat accumulated by the cells: (1) the removal of the fat by extraction with ether or chloroform; (2) the use of an artificial fat-free medium, such as agar or salt solutions, in which Lewis[6] states that embryonic chick cells will grow for a time; and (3) the use of plasma in which the amount of fat has been reduced by dilution with Ringer's or physiological salt solution. The first method was found impracticable because prolonged shaking of serum with pure ether or chloroform seemed to produce some change, probably in the serum proteins, as the result of which the serum even when combined with sufficient plasma to cause clotting did not afford a satisfactory culture medium. The second method could not be successfully used, because, after washing the tissue fragments in salt solution to remove the tissue lymph before preparing the cultures, it was found that the cells in the outgrowths lived only eighteen to twenty-

[5] Lambert, R. A., and Hanes, F. M., *Virchows Arch. f. path. Anat.*, 1913, ccxi, 100.

[6] Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

four hours; that is, not long enough to make comparative observations. Very little fat is present in the pure plasma preparations at the end of twenty-four hours' incubation. We therefore resorted to the use of the third, or dilution method. Dilutions of chick plasma in Ringer's solution were made in the following proportions: 1 part of plasma to 2, 5, 10, 12, 15, and 20 parts of Ringer's solution. Clotting was not interfered with by the high dilution of the plasma, although the coagula formed by drops of the 1 to 15 and 1 to 20 dilutions were often not firm.

It was found that cells from pieces of chick embryo heart, previously washed in salt solution, showed active migration and multiplication in each of the diluted media. However, those growing in the 1 to 20 medium underwent early disintegration, frequently in less than twenty hours. The lower dilutions only were therefore used in the majority of the experiments. A single protocol will be sufficient to show the effect of dilution on the accumulation of fat by the cells; for it was found that when proper care was exercised in the preparation of the cultures, including the thorough washing of the pieces of tissue to prevent a relatively great modification of the dilution from the addition of tissue lymph, and in the care of the instruments and glassware used, the results in a number of experiments were practically uniform.

Experiment iii–5.—Plasma was obtained by bleeding a fowl from the wing vein. The heart of a ten day chick embryo was cut into small pieces of the size suitable for culture preparations. Dilutions of plasma were made by adding 1 part to 2, 5, 10, 12, and 15 parts of Ringer's solution. Ten pieces of tissue were put up in each medium, including undiluted plasma.

All preparations upon examination after twenty-four hours showed the usual radial extension of spindle and irregularly shaped cells. Small droplets of fat were visible in the cells in pure plasma and in those in the 1:2 and 1:5 dilutions; only occasional granules were seen in the preparations of the remaining series. After forty-eight hours the differences were more striking. There was a marked increase in the number and size of the granules in the cells in pure plasma and in the 1:2 dilution. In the 1:5 dilution the cells showed a moderate amount of fat, but decidedly less than those in undiluted plasma. Those growing in the higher dilutions were practically free from fat. Three of the preparations from each series were fixed in formalin at this time in order that the observations on the living cells could be verified by staining with Sudan III (figures 6 and 7). Some of the preparations in diluted media became disintegrated on the third and fourth days. Those remaining alive as long as four days presented striking pictures when compared with the controls, the former

showing only occasional granules of fat, while in the latter the cells appeared distended with numerous large droplets.

In order to exclude the possibility that in the diluted plasma the diminution in the amount of fibrin might have influenced in some way the fat metabolism of the cells, plasma diluted with serum instead of salt solution was used in another set of experiments. It was found that the accumulation of fat under these circumstances was practically the same as in undiluted plasma. Repetitions of these experiments, including several in which pigeon spleen and mouse carcinoma were used, gave results that varied little from those just stated. Two further observations, however, should be mentioned. First, it was occasionally observed that some of the cells that wandered farthest into the clot of highly diluted plasma showed a small number of rather large fat droplets, while the cells in the denser outgrowth immediately about the tissue fragment were practically fat-free; secondly, in a few of the preparations in diluted plasma in which the outgrowth consisted of only fifteen to twenty cells, these cells contained more fat than those in preparations showing a luxuriant outgrowth. These observations seem to be in harmony with the findings stated, for it may be assumed that if the number of cells in a culture or in a particular area of the clot is small the fat supply for these cells is relatively greater.

It has therefore been concluded that the effect of dilution of the plasma with salt solutions reduces the quantity of fat accumulated by the cells by reducing the quantity of fat in the medium.

SUMMARY.

1. Dilution of plasma with isotonic solutions causes a more extensive migration in cultures of cells of the actively migratory type, such as those of spleen and bone marrow. Dilution with a limited quantity of distilled water produces the same effect. Less actively motile cells are influenced little or not at all by dilution. The effect on cells of the first type is probably due to the reduction in the quantity of fibrin in the clot producing lessened resistance to cell locomotion.

2. Dilution of plasma with either isotonic solutions or distilled water is without effect on cell multiplication, as is shown by records of the number of mitoses in living culture preparations.

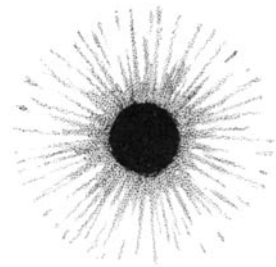

FIG. 1.

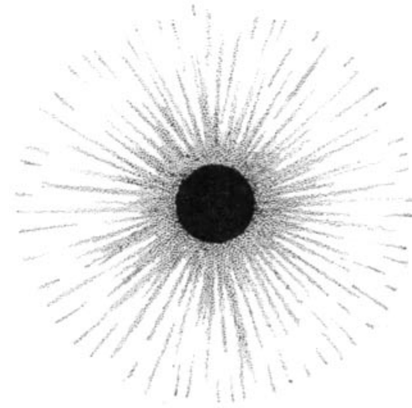

FIG. 2.

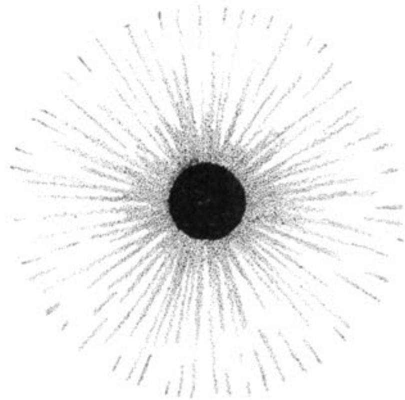

FIG. 3.

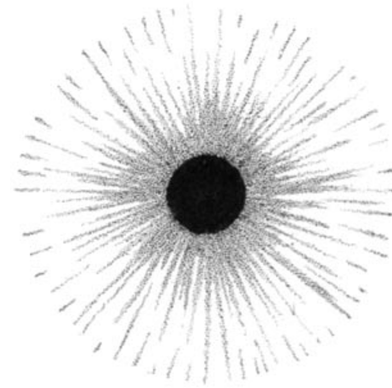

FIG. 4.

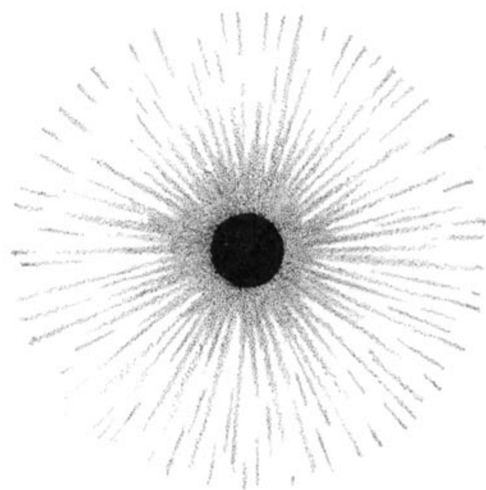

FIG. 5.

(Lambert: Growth of Cells in Tissue Cultures.)

FIG. 6.

(Lambert: Growth of Cells in Tissue Cultures.)

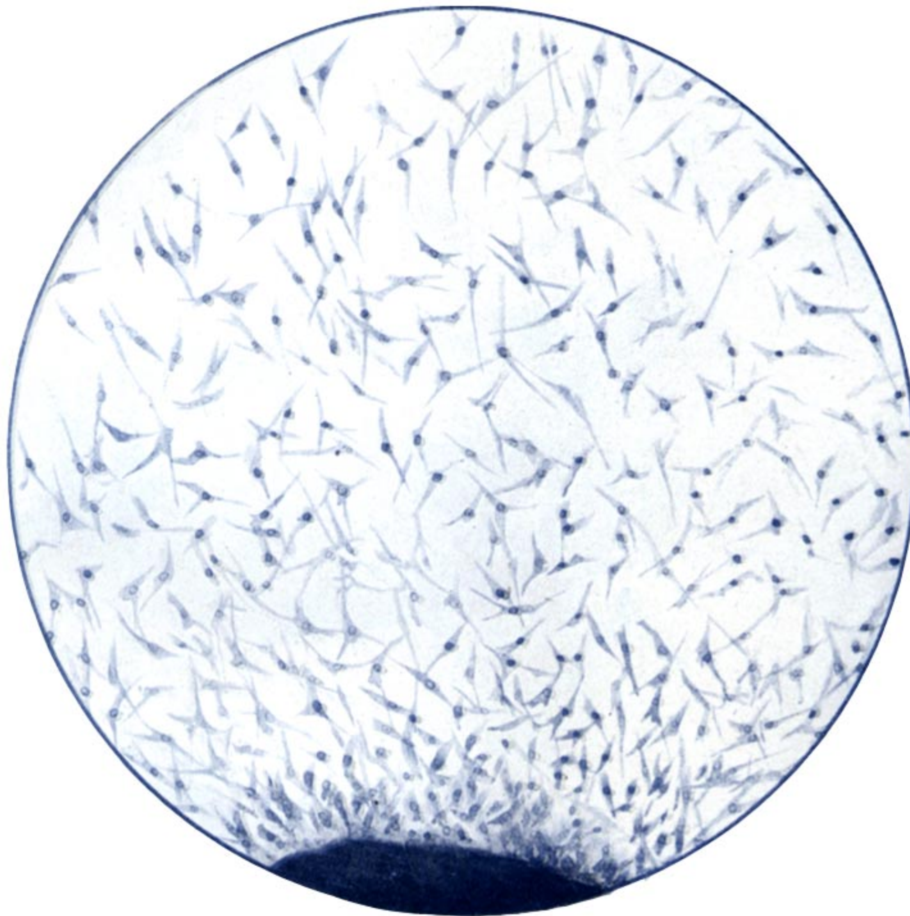

FIG. 7.

(Lambert: Growth of Cells in Tissue Cultures.)

3. Dilution of plasma with suitable quantities of Ringer's solution causes a marked diminution in the quantity of fat accumulated by the cells. This reduction is to be attributed to the decrease in the quantity of fat in the medium. The accumulation of fat by cells in cultures is therefore not to be regarded as the result of a cell degeneration, but as an accumulation, the source of the fat being the medium in which the cells are growing.

EXPLANATION OF PLATES.

Plate 43.

Drawings showing the extent of migration of spleen cells in undiluted chick plasma and in plasma diluted with serum and with salt solutions.

Fig. 1. In undiluted chick plasma (control).

Fig. 2. In plasma diluted with serum in the proportion of 3:2.

Fig. 3. In plasma diluted with distilled water in the same proportion.

Fig. 4. In plasma diluted with Ringer's solution in the same proportion.

Fig. 5. In plasma diluted with serum in the proportion of 1:2.

Plate 44.

Fig. 6. Three day culture of chick embryo heart in undiluted plasma. Stained with hematoxylin and Sudan III to show the accumulation of fat by the cells.

Plate 45.

Fig. 7. Three day culture of chick embryo heart in plasma diluted with Ringer's solution in the proportion of 1:15. The staining shows that fat granules are practically absent. It is also to be observed that the cells are smaller and stain somewhat more deeply with hematoxylin than in the control preparation.