STUDIES ON THE GROWTH OF TISSUES
IN VITRO

I. SOME EFFECTS OF THE MECHANICAL PROPERTIES
OF THE MEDIUM ON THE GROWTH OF CHICK
HEART FIBROBLASTS

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(With Two Text-figures.)

INTRODUCTION.

Tissues grown in vitro may behave in one of two ways. The growth may be
organised, and the tissues may differentiate and develop in a manner closely similar
to that in which they would if left in situ; or the growth may be unorganised and
cells emigrate from the central implant and proliferate without showing any
tendency to differentiate. Exactly what are the conditions which determine this
difference in behaviour is not yet certain, and in this paper reference is made
entirely to unorganised growth and the conditions which control the extent to which
it can take place.

Until recently the most practicable method of measuring the rate of growth of
tissues in vitro has been to measure the area occupied by the tissue from day to day
(Ebeling, 1921). This has been done either by projection, or by making outline
drawings by means of the camera lucida of the periphery of the culture and measur-
ing the area enclosed. The area so obtained, however, is affected by at least two
factors. It depends partly on the rate at which the culture is actually increasing by
the production of new cells equal or nearly equal in size to the parent cells, and
partly on the extent to which the cells present in the original implant are capable of
wandering out into the medium. The method of measurement can take no account
of the thickness of the layers of cells, and so it may be difficult to determine how
much of the increase in area is actually due to growth as distinct from outwandering. Mayer (1930) has developed a photometric method for estimating the thickness of the tissues, but this again is not very satisfactory, since the cells themselves are not always of the same optical density owing to the presence of fat and other inclusions, so that a culture may become opaque for other reasons than thickness.

Since so much of the increased area of a culture may be due to migration it becomes important to examine the conditions of cultures to see how these affect the rate of migration, and if possible to devise a method by which the processes of growth and migration can be correctly estimated. Experiments on the effects of various substances on the growth rate of tissues are for the most part performed in “flask cultures” in which the tissue is embedded in a plasma coagulum over whose surface there is placed a layer of fluid medium. Now Fischer (1928) observed in “test-tube cultures” that if the fibroblasts were deeply embedded they often failed to grow, while those near the surface tended to grow freely. This being the case it is probable that slight alterations in the depth of the plasma coagulum in a culture flask have a profound influence on the area attained by the culture. That this is so is illustrated by the following experiments.

EXPERIMENTS.

Six “Carrel” flasks, 3 cm. diameter, were taken and three fragments of ventricular wall from the 10-day chick heart were placed in each. The fragments were selected to be as nearly equal in size as possible. In three flasks they were embedded in a coagulum composed of 0.4 c.c. fowl plasma and 0.4 c.c. of extract of embryo tissue in Tyrode solution (one 10-day chick embryo in 5 c.c. of Tyrode), and in the other three the coagulum consisted of 1.5 c.c. of plasma and 1.5 c.c. of embryo juice. The approximate depths of these clots over the bottom of the flask were therefore about 1 and 3 mm. There is some tendency for the clot to become deeper at the edges. Each clot was covered with a fluid medium of 1 c.c. of embryo tissue extract. The mechanical structure of the clot was therefore the same in both sets of flasks, and the only variables were the total quantity of the medium and the depth of the coagulum. If the area depended on the available nutrient material then it might be expected that the outgrowth would be greater in the flasks containing the deeper coagulum; but actually the reverse is the case, the growth in the deeper coagulum occupies a considerably smaller area than that in the thinner one. It is therefore the distance of the implant from the surface which is responsible for the great difference in apparent growth. An examination of the cultures shows also that the type of growth is quite different in the two series. The tissues in the shallow clot are widely spread out, almost in a single layer, and the cells are relatively clear and free from fat. The central implant may be almost completely dispersed, and to the naked eye the space initially occupied by the thick central implant appears surrounded by a transparent area, which under the microscope is seen to be occupied by several layers of perfectly hyaline cells. It is probable that most of the fibrin from the coagulum has been digested away from this area. In the deep coagulum the
Studies on the Growth of Tissues in vitro 319

growth although smaller in area is more compact, the cells lying closer together and being generally more spindle-shaped and containing noticeably more fat. The cells lie in several layers and it is probable that the volume of new growth is closely similar in both cases, but the areas occupied are widely different.

The areas of growth were measured by means of camera lucida drawings on squared paper; and the results are shown in Fig. 1.

In a second series of experiments the total depth of the clot was kept constant, but its mechanical properties were altered by varying its plasma content. The fibrin clot is composed of a very fine network of fibres, and it is probable that the cells work their way along these fibres, digesting them as they go. Consequently the more dense the network the slower might be expected to be the rate at which the cells move through the medium. This is clearly illustrated in Fig. 2. In each case the total volume of the coagulum was 1.2 c.c., but the amount of plasma varied from 1.0 to 0.25 c.c., its place being taken in the weaker clots by an equivalent quantity of Tyrode solution. The amount of embryo-tissue extract was kept constant at 0.2 c.c. and each culture was covered with 1 c.c. of this fluid, which was renewed on the third day.

The effects of the increased density of the clot on the character of the cells were similar to the effects produced by increased depth. The cells tended to migrate less and consequently remained in several layers, and fatty globules became more

![Fig. 1. 1 plasma, 1.5 c.c.; extract, 1.5 c.c. 2 plasma, 0.4 c.c.; extract, 0.4 c.c.](image-url)
abundant in the cytoplasm. An area of clear cells developed in the thinner coagulum, and the cells became more scattered and in the periphery tended to assume a triangular shape rather than remaining spindle-shaped.

DISCUSSION.

By far the most convenient method of measuring the activity of cultures in different media is by measuring the area occupied by the culture on successive days, but the two experiments described in this paper show that if the measurements obtained are to be reliable they must be made with every attention to the composition of the medium and the purely mechanical factors which may affect the area attained by the tissues. It may therefore be useful to discuss some of the factors which are known to affect the growth of cultures in flasks (using the term growth in its widest sense).

In the first place the tissue itself is a variable quantity and is apt to behave somewhat capriciously. On the whole, tissue which has been grown in vitro for some time tends to behave more regularly than tissue fresh from the animal, but this difference in behaviour is often greatly exaggerated, and unless a particular region of the culture is selected each time for sub-cultivation it is probable that the culture takes a very long time to become pure, and as Olivo and Delorenzi (1932) have shown, tissue even after being subcultured over two thousand times still shows cells with very
Studies on the Growth of Tissues in vitro

widely different intermitotic periods, so that it is very doubtful whether in measurements of growth rate the use of subcultured tissue is really as helpful as it is sometimes made out to be. Frequently experiments are performed on the two halves of a tissue which has been previously cultured in vitro, and although, as Mayer (1930) has pointed out, this method is not without its disadvantages, it is probably the most satisfactory means of obtaining comparable tissue, but even then the experiments should be performed on several fragments, for the variations in the behaviour of the two fragments are often great.

Although in hanging drop cultures the initial size of the fragment of tissue may be of great importance, in flasks, especially if the medium provides most of the essentials for growth, the initial size is of less significance. It is noticeable that when cultures have been growing freely under the same conditions for a few days in flasks the areas attained by the tissue fragments become very uniform. This only applies, however, to cases where they are supplied by nutrient media: where they are merely surviving on their own resources then the initial size will be important in modifying the amount of the available food supply and so limiting the final dimensions of the outgrowth (Ephrussi and Teissier, 1932). Consequently where measurements of area are relied upon to give information with regard to the growth of tissues in vitro it is preferable to obtain data from a large number of fragments, even though they may differ slightly at the start, rather than to trust to the behaviour of one or two selected fragments. Even so, the greater the uniformity in the tissue chosen the greater will be the chance of obtaining satisfactory results.

The measurement of the area of cultures, although the most convenient method of estimating growth, is far from satisfactory, since the increase in area is so much dependent on the facility with which the cells can wander out into the medium, and this outwandering is something which is quite independent of the actual growth, although it may often happen that actively growing tissues may also show active cell migration. The migration depends far more on the mechanical properties of the plasma coagulum, which are themselves variable, than does the actual growth of the tissues, by cell division. The two experiments described in this paper show clearly the extent to which the area of growth can be modified by the mechanical constitution of the clot, and there is no evidence that the actual growth by cell division is materially different in the various media, so that unless precautions are taken to ensure uniformity in the plasma coagulum, area measurements may be misleading. Incidentally the experiments emphasise the desirability of obtaining a coagulum with rather more standard properties than plasma. Attempts were made to use gelatin after fixation with formalin, which has a similar mechanical constitution to plasma, consisting of a network of fibres, but even after prolonged washing it remains toxic to tissues planted on it. Moreover, it has the disadvantage that the tissues cannot be embedded in the gelatin in the same way as they can in plasma.

Measurements of area of cultures are only satisfactory as a method of measuring growth if they are combined with a study of the occurrence of cell division within the culture, or if the tissue is repeatedly divided and subcultured. In the latter case, if the tissue can be frequently subdivided without loss of size, actual growth, i.e.
increase in mass, of the tissue must be taking place. A satisfactory method for the study of the occurrence of cell division in tissue cultures is one of the main requirements of the tissue culture technique, for only by observations on the rate of cell division is it possible to study the actual process of growth.

SUMMARY.

Experiments are described which demonstrate the importance of standardising the coagulum for the growth of tissues in vitro in respect to (a) depth and (b) plasma content, in order to obtain, by daily measurements of the area of the cultures, a true indication of the actual rate of growth of the tissues.

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REFERENCES.