ARTIFICIAL ACTIVATION OF THE GROWTH IN VITRO OF CONNECTIVE TISSUE.*

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INTRODUCTION.

If the factors that bring about the multiplication of cells and the growth of tissues were discovered, it would perhaps become possible to activate artificially the processes of repair. Then aseptic wounds could probably be caused to cicatrize more rapidly. If the rate of the reparation of tissues were activated ten times only, a cutaneous wound would heal in less than twenty-four hours, and a fracture of the leg would be cured in four or five days. It is permissible to think that this hypothesis is not unreasonable. Jacques Loeb, in his fundamental experiments on artificial parthogenesis, has demonstrated that cell division can be induced by slight changes in the composition of the sea water in which the sea urchin's eggs are placed. It might even be supposed that certain modifications of the *milieu intérieur* of the tissues of mammals would bring about the multiplication of their cells. In 1907 and 1908 I began, therefore, to study the processes of reparation of small cutaneous wounds and the action of a great many substances on the rate of their cicatrization. It was found that the proliferation of epithelium and of connective tissues was activated under certain conditions by dressings made with the pulp of tissues and organs. For instance, thyroid gland pulp deposited on cutaneous wounds of the dog brought about the formation of exuberant granulations. Applied to bones, it produced a marked thickening of the periosteum. The external coat of an artery preserved in cold storage in a mixture of thyroid gland and Locke's solution, and transplanted afterwards into a dog's carotid, underwent an enormous hypertrophy. How-

* Received for publication, June 6, 1912.

ever, it was difficult to study with precision the influence of these substances on the tissues of living dogs. It became evident that the changes brought about by them could be more precisely observed if the tissues were isolated from the organism and made to live in a medium of known composition. Therefore, I undertook to adapt to the cultivation of mammalian tissues the method used by Harrison in his researches on the growth of the central nervous system of frog embryos in a drop of lymph. In some experiments that I made in 1911 with the collaboration of Dr. Burrows, it appeared that the growth of chicken tissues were activated when extracts of the Rous chicken sarcoma and of chick embryo were added to the culture medium.

In 1912, by using a more precise technique, I was able to study quantitatively the influence of tissue juices on the growth *in vitro* of connective tissue and some of the characteristics of their activating power.

METHOD.

The method consists in measuring the extent of the growth of fragments of tissues placed in normal plasma and in plasma containing a known quantity of a tissue juice. The extracts were made of chick embryos from six to twenty days old, of spleen, kidney, muscle, etc. of the adult chicken, of the Rous sarcoma, of thyroid gland, spleen, and muscle of the adult dog, and of spleen of the adult rabbit. The tissues were cut into very small fragments, or cut and ground with sand in a mortar, or cut, ground, and frozen in ice and salt, and then put for a short time in the incubator at 38° C. Afterwards, to one volume of tissue were added from one third of a volume to four or five volumes of Ringer solution. The tubes containing the mixtures were put in cold storage. After a period varying from a few minutes to twenty days, the tubes were centrifugalized. The supernatant fluid was used pure or diluted with Ringer solution.[1] In some experiments the fluid was heated at 56° or 70° C. for ten, twenty, or thirty-five minutes. In other experiments, it was filtered through paper or through a Berkefeld or a Chamberland filter.

The culture medium was composed of one volume of extract and two volumes of hypotonic plasma. The hypotonic plasma was obtained by adding two volumes of distilled water to three volumes of normal plasma.

The majority of the experiments were performed on hearts of chick embryos from seven to fifteen days old. The ventricular wall was divided into small fragments almost identical in size. The fragments, placed in the culture medium, were rapidly surrounded by a dense ring of connective tissue cells. Fragments of skin were also used. In the experiments performed on dog's tissues, fragments of periosteum were employed. With each experiment control cultures in normal hypotonic plasma were made. In order that the results should be comparable, the cultures were prepared with extreme care and precision.

The specimens were examined after twenty-four, forty-eight, and seventy-two hours. The area of the new issue was calculated accurately after the diameter of the original fragment and the width of the ring of new tissue had been measured by the micrometer. But the thickness of the new tissue could not be known exactly. In all the experiments where extracts were added to the medium, the growth was not only more extensive but also denser than in the cultures which contained no extracts. The increase in thickness was generally not considered in the calculation of the value of the acceleration of the growth, this calculation being based only on the increase in area of the tissue. The increase of the tissues was really greater therefore, than appears in the description of the results.

RESULTS.

In every experiment the fragments of heart, skin, and periosteum, cultivated in plasma containing an extract, grew more rapidly than their controls. It is certain, then, that tissue juices have the power to activate *in vitro* the growth of connective tissue.

The value of this power varied according to the method used in the preparation of the extract. An extract obtained by the centrifugalization of embryonic tissue, a few minutes after it had been mixed with Ringer solution, increased the rate of growth two and

a half and three times. The acceleration of the growth was much more marked when the mixture of tissues and Ringer solution was allowed to stand in the refrigerator for twenty-four hours or several days before being centrifugalized. For instance, in experiment 1,734, Ringer solution containing embryonic pulp had been preserved for twenty days in cold storage before being centrifugalized. In twenty-four hours, the area of new connective tissue was thirty times larger in the cultures containing the extract than in the controls. The extracts of tissues cut in small fragments, mashed, and frozen, were generally very active. The experiments made with these different extracts showed that they were able to increase the growth of connective tissue from about three to forty times.

1. *Effect of Concentration of an Extract on Its Activating Power.*—An extract diluted with Ringer solution was less active than one that was not diluted. For instance, in experiment 1,577, fragments of heart were cultivated in plasma containing ⅓, ⅙, 1/12, 1/24, and 1/48 of embryonal juice dissolved in Ringer solution. The control cultures were made with plasma containing a like quantity of Ringer solution. The areas of connective tissue produced in forty-eight hours were respectively 60, 21, 12, 8.25, and 5, in the experiments, and 5 in the controls. In other experiments the quantity of new connective tissue varied also in direct ratio to the concentration of the extract.

2. *Effect of the Nature of the Tissues on the Activating Power.*—The experiments were performed on extracts of chick embryo from six to twenty days old, of spleen, liver, connective tissue, kidney, heart, and blood corpuscles of the adult chicken, of the Rous sarcoma, and of thyroid gland and muscle of the adult dog.

All the extracts activated the growth of the connective tissue. But the degree of acceleration varied in large measure. Embryonal tissue extract was the most active. Extracts of adult spleen and the Rous sarcoma were almost as active as the extract of chick embryos. Kidney and heart extracts were much less active, while the extracts of connective tissue and of blood corpuscles brought about a slight acceleration only of the growth.

The influence of the extracts of thyroid gland and muscle of the dog on the growth of periosteum was very marked, but thyroid extract was more active than muscle extract.

3. *Effect of the Origin of the Tissues.*—The power of an extract of animal tissues seemed to be specific and was confined to the tissues of another animal of the same species. For instance, the extract of chicken spleen activated greatly the growth of connective tissue of a fragment of the heart of chick embryo, while the activating influence of extracts of dog and rabbit spleen on chick tissue was very slight.

4. *Effect of Heat on the Activating Influence of the Extracts.*—The embryonic extracts began to lose their activating power when they were heated at 56° C. for ten minutes. The diminution was more marked when the extracts were heated for thirty minutes. For instance, in experiment 1,565 an embryonic extract heated at 56° C. for ten minutes lost one third of its power. The same extract heated at 56° C. for thirty-five minutes lost two thirds of its power. An extract of the Rous sarcoma heated at 56° C. for ten minutes lost also one third of its activity. The extracts of adult spleen were slightly modified by the heating at 56° C. for ten minutes. Their activating power remained generally unaltered.

The extracts of adult spleen, of the Rous sarcoma, and of chick embryo, heated for ten minutes at 70° C. lost completely their activating power.

5. *Effect of Filtration on the Activating Power of the Extracts.*—The experiments were made on extracts of chick embryo and of the Rous sarcoma, diluted with Ringer solution.

The power of the extracts was not modified by filtering them through filter paper. However, if the extract contained much cellular debris, the filtration increased its power slightly.

Extracts which were filtered through a Berkefeld filter always lost a great part of their activating power. The area of new connective tissue in cultures to which such a filtered extract had been added was about one third or one fourth smaller than in the cultures containing an unfiltered extract.

Filtration through a Chamberland filter suppressed completely the activating power of an extract. The heart fragments did not

produce more connective tissue in plasma containing such a filtered extract than in plasma containing an equal quantity of Ringer solution. For instance, in experiment 1,538 the areas covered by the new connective tissue were respectively 77, 32, 21, and 21, in media containing (1) unfiltered extract of the Rous sarcoma, (2) extract filtered through a Berkefeld filter, (3) extract filtered through a Chamberland filter, and (4) Ringer solution.

SUMMARY AND CONCLUSIONS.

The experiments have shown that extracts of tissues and tissue juices, under certain conditions, accelerate the growth *in vitro* of the connective tissue from about three to forty times. This activating power was found in many tissues. It was much more marked, however, with the extracts of embryos, of adult spleen, and of the Rous sarcoma. The power diminished directly with the dilution of the extracts, and appeared not to apply to the tissues of a heterologous animal. The power was reduced when heated at 56° C., and removed when heated at 70° C. It was diminished markedly by filtration through a Berkefield filter and was completely suppressed by filtration through a Chamberland filter.

Possibly the finding of the activating power of tissue extracts will have no immediate practical application. Nevertheless, it may be indirectly useful by leading to the discovery of some of the factors determining the growth of tissues and of the unknown laws of cell dynamics, and may ultimately throw light on the mechanism of the cicatrization of wounds.