ACTION ON FIBROBLASTS OF THE PROTEIN FRACTION OF EMBRYONIC TISSUE EXTRACT.

By LILLIAN E. BAKER, Ph.D., and ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 15, 1926.)

It is known that various tissues will grow indefinitely *in vitro* in a medium consisting of one part of plasma and one part of embryonic tissue juice,[1] and that it is the embryonic tissue juice and not the plasma that furnishes the necessary substances for cell nutrition and multiplication.[2] Whether there is in embryonic extract a specific substance with a function of initiating or producing cell division, or whether the embryonic extract simply contains the essential nutrient substances required by the cells, is not known. Neither is there any knowledge concerning the chemical nature of the substances present in the extract which are utilized by the cells. The work reported in this paper is a preliminary examination of the action of the protein-containing fraction of embryonic extract in relation to its ability to promote the growth of fibroblasts in pure culture, or from heart tissue.

The protein of the embryonic tissue extract was precipitated in a variety of ways and redissolved in a volume of Tyrode solution equal to the volume of tissue juice from which the precipitate was obtained. It was found that the protein precipitate, provided it could be entirely freed from the reagent used for its precipitation and again brought into solution, contained some of the growth-stimulating action of the original extract. Ammonium salts, trichloracetic acid, picric acid, pyridine, etc., were too difficult to remove and were toxic to the tissues. Carbon dioxide passed through a diluted solution, ethyl,

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14; 1914, xx, 1. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755. Carrel, A., and Ebeling A. H., *J. Exp. Med.*, 1923, xxxviii, 487. Ebeling, A. H., *J. Exp. Med.*, 1925, xli, 337.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317. Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255; *Brit. Med. J.*, 1924, ii, 140.

methyl, and isopropyl alcohol, acetone, and acetic acid, all yielded precipitates which showed decided activity. When a comparison was made between these precipitates and the original extract, none was found to be quite as active as the original. However, this was not surprising for, if a sufficient concentration of precipitant such as acetic acid or alcohol was used, some of the protein became denatured as could be seen by a difference in the translucency of its solution. When a sufficiently small concentration of the precipitant was used to prevent denaturation, a part of the protein was not precipitated and the filtrate as well as the precipitate possessed growth-promoting properties. To afford some basis of comparison the method was adopted, therefore, of determining the concentration of nitrogen in the solution of the precipitate or the filtrate, and in the original extract, and then diluting the original extract with Tyrode solution so that it contained the same per cent of nitrogen as the experimental medium.

Tests on a number of precipitates were made in which the cultures were kept for only a few passages. In most cases, the tissue cultivated in the control extract and that in the solution of the protein precipitate grew at the same rate. No case occurred in which the precipitate showed any greater activity than the original extract. In some cases it was less active, because the method of fractionation either denatured the protein or destroyed some activating substance, for the activity of embryo juice is easily destroyed by heat, aging, and many chemical substances. Precipitation by alcohol, even at low temperature, always gave a precipitate less active than the original extract.

A fractionation of the protein by alcohol was carried out by adding a small amount of alcohol, centrifuging the precipitate, and then increasing the concentration of alcohol, etc., until ten fractions were obtained. The first three or four of these precipitates were quite active, the activity decreasing as the concentration of alcohol increased until practically no activity was obtained from the last fractions. The filtrate from precipitation by an equal volume of alcohol contains a considerable amount of active substance, also a considerable quantity of protein. A 50 per cent alcoholic solution will extract from embryo

pulp an appreciable quantity of protein and produce an active extract when the alcohol is removed.

A few adsorption experiments were also tried, with charcoal, kaolin, and alumina as adsorbents. If a sufficient quantity was used to adsorb all the protein, the filtrate remaining was without any noticeable activity. If all the protein was not adsorbed, the filtrate generally exhibited some activity. The experiments tried on redissolving the active substances from the adsorbents were not successful.

These preliminary tests indicate that the protein of the extract is the essential nutritive substance upon which fibroblasts live *in vitro* and if, besides the protein, there is some specific substance essential for cell multiplication, it is united to the protein by a chemical or physical bond or is readily adsorbed on the protein as it precipitates.

Experiments on cultures in which the tissue is grown for only one passage of 2 or 3 days are valuable for preliminary information but are not conclusive, since it is quite probable that the tissue may carry along with it from its old medium, either mechanically or already adsorbed, some substance essential for its growth. The only convincing experiments, therefore, are those in which the tissues live for several passages in the experimental medium and its control. This is a time-consuming process, so as yet only results with the protein precipitated by carbon dioxide and its filtrate have been procured in this way. Tests have been made on the precipitate, using the method of small cultures in which the tissue is cut at every passage,[3] and also that of cultivation in flasks[4] where the tissue is not removed but the total accumulated growth over the entire period may be observed (Figs. 1 to 3). It is evident from the curves that the rate of growth of the tissue in the protein precipitated by CO_2 and redissolved in Tyrode is approximately the same as that in the original extract diluted to contain the same per cent of protein. The condition of the cells in the experimental medium was as good throughout as in the control. Since the experiment (Fig. 1) was carried on for 28 days, it seems conclusive that the protein precipitate contains all the necessary nutritive substances. Only a fraction of the protein, however, is precipi-

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

[4] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

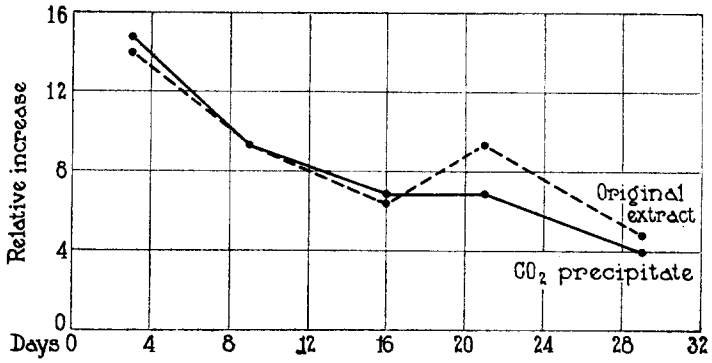

FIG. 1. Comparison of the rate of growth of fibroblasts in the protein precipitated by CO_2 and in the original extract diluted to the same nitrogen content.

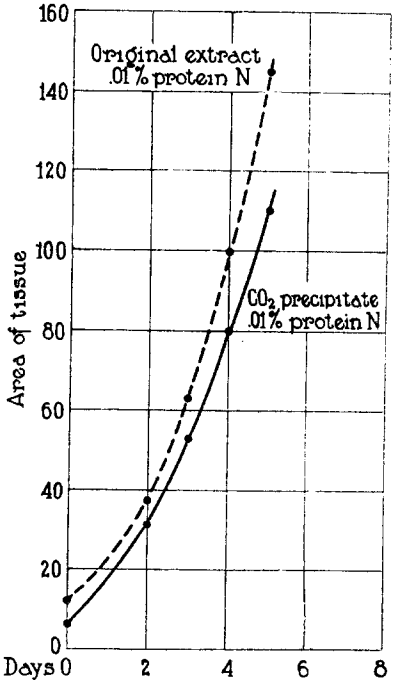

FIG. 2.

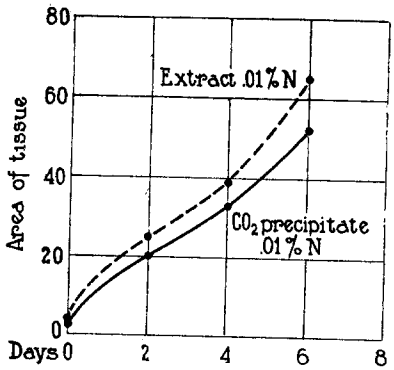

FIG. 3.

FIG. 2. Comparison of the rate of growth of fibroblasts from embryo heart in embryo extract and in the protein precipitated from it by CO_2.

FIG. 3. Comparison of the rate of growth of a 13 year old strain of fibroblasts in embryo extract and in the protein precipitated by CO_2.

tated by CO_2, approximately one-half of that originally present. The filtrate is also quite active (Fig. 4), and relatively in proportion to its protein content. A careful examination of the curves for the CO_2 precipitate and the CO_2 filtrate shows that there is a little activity due to the non-protein nitrogen present. This causes a slightly increased

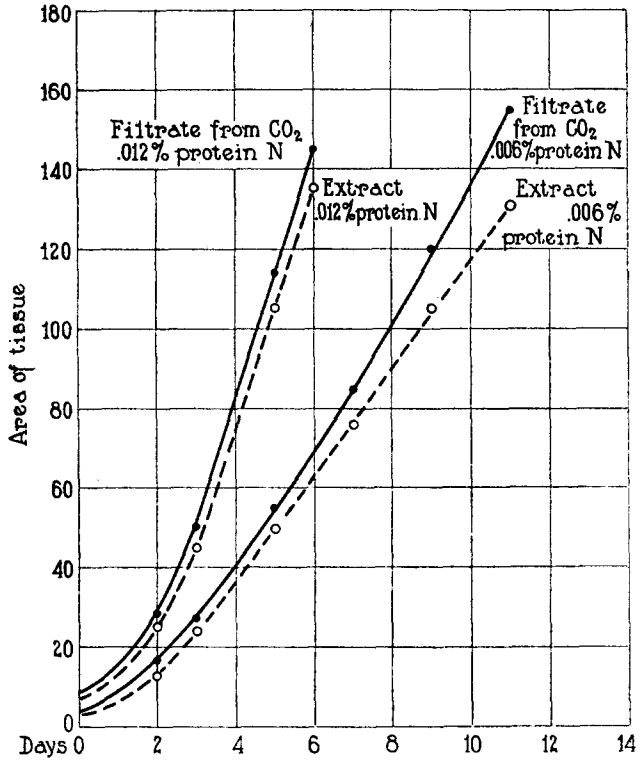

FIG. 4. Comparison of the rate of growth of fibroblasts from embryo heart in embryo extract and in the CO_2 filtrate containing equal concentrations of protein nitrogen.

area in the growth of tissue in the extract over that in the CO_2 precipitate, which contains only protein nitrogen and a slightly greater area in the CO_2 filtrate than in the diluted extract. The basis of comparison here was an equal content of protein nitrogen. As has been shown previously[5] amino acids are capable of producing a slight

[5] Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

stimulation of growth, causing greater migration of the cells, but they do not produce an increase in the mass of the tissue, as is done by the protein fraction of embryo juice. Further confirmation of these findings is reported in the following paper.

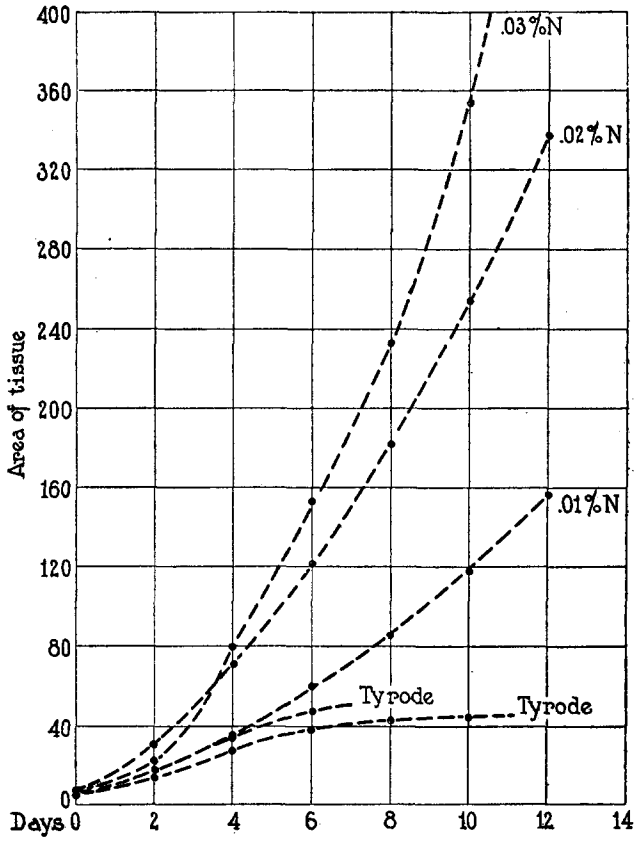

FIG. 5. Rate of growth of fibroblasts in embryo extract diluted to contain 0.01, 0.02, and 0.03 per cent of nitrogen. Note that the shape of the curve for media containing nutrient materials is quite different from that for Tyrode solution.

The rate of growth of fibroblasts is proportional to the concentration of active substances in the medium, a fact well illustrated by their growth in embryonic tissue extract at different dilutions. In the experiments recorded in Fig. 5, one sample of tissue juice was diluted so as to contain 0.01, 0.02, and 0.03 per cent nitrogen. Tissues do not,

however, grow at the same rate in different preparations of extracts, even when they contain the same per cent of protein. Curves such as those figured are, therefore, truly indicative of the concentration of growth-activating substances in the experimental medium when compared with a sample of the original extract from which the experimental medium was prepared.

Since the protein of the embryonic tissue extract appears to contain the essential nutritive and activating substances, experiments were undertaken to purify it by the process of repeated precipitation. This

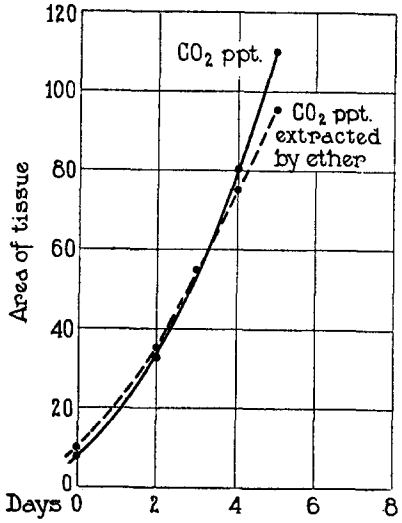

FIG. 6. Comparison of the rate of growth of tissue in the CO_2 precipitate and the CO_2 precipitate extracted by ether.

was tried with the precipitates obtained by carbon dioxide, acetic acid, and alcohol. The precipitation was carried out at low temperature and, if only a part of the total protein was precipitated, it was dissolved in a correspondingly smaller volume of Tyrode solution before being reprecipitated. The activity of the first and third precipitates was tested. The third precipitate was found to be considerably less active in each case; in fact, when it was compared with Tyrode solution, it was found to be almost inactive. Obviously, the protein has either lost some substance attached to it which was essential to the growth of the tissues or it has itself been altered in the process of

purification so as not to be biologically the same, even if it appears to have the same chemical properties.

The protein of embryonic tissue juice has been tested chemically and found to be a mixture of nucleoprotein and a glycoprotein with mucin-like properties. However, when these proteins were isolated in a comparatively pure state from embryo pulp and tested on growing tissues, they did not seem to possess any growth-promoting action.

Pure sodium nucleate from embryonic pulp was prepared and found to be inactive, as were many other substances also, among which were egg albumin, egg globulin, crystalline egg albumin, nucleoalbumin, nucleoglobulin, lecitho-albumin, thymus nucleic acid, and acid metaprotein from crystalline egg albumin. Crystalline egg albumin prepared with ammonium sulfate proved to be toxic even after prolonged dialysis, but that from which the ammonium sulfate was removed by recrystallizing three times with Na and K sulfates, and washing with sodium chloride and acetic acid, was not toxic. A few of the above preparations appeared to be slightly stimulating in the first passage but proved inhibiting after three passages, and in no case was there any growth as large as that obtained with even a very small quantity of embryo tissue extract.

Since the protein precipitated by almost any method carries along with it by adsorption some substances of lipoid nature, these precipitates and the original extract were purified by extraction with ether. As will be reported in another paper of this series, the growth-promoting action was not lost (Fig. 6). Therefore, it is the protein, and not the lipoid associated with it, that carries the activity of the embryo tissue extract.

DISCUSSION AND SUMMARY.

The above experiments indicate that the growth-stimulating substance found in embryonic tissue extract, which has been responsible for the continuous growth of fibroblasts *in vitro* for 14 years, is either protein in nature or closely associated with the protein of the extract and adsorbed by it. If any specific hormone responsible for cell division is present, it is united to the protein or carried along with it in its first precipitation. It seems probable that the tissues utilize this protein for the nitrogen which they build into protoplasm. Whether it

is first hydrolyzed before adsorption by the tissues has not been ascertained as yet. It has been shown in other experiments reported in the following paper that the amino acids of the tissue juice do not suffice for the growth of fibroblasts and that hydrolyzed tissue juice is toxic in the same way that a too concentrated mixture of amino acids is toxic.

The results of the foregoing experiments may be summarized as follows:

1. Fractionation of embryo tissue juice has shown that it is the protein fraction that contains the activating substance.

2. Tissues continue to grow for a long time in the protein of the extract precipitated by CO_2 and at a rate approximately equal to that in the original extract diluted to the same nitrogen concentration.

3. The non-protein nitrogen gives slight stimulation to growth.

4. Purification of the protein by repeated precipitation destroys its growth-promoting properties, but whether this is due to a denaturing of the protein,—which occurs very readily,—or to loss of some substance possibly an enzyme attached to it, has not been ascertained.

5. Preparations of purified proteins from embryonic tissue and egg white have shown no marked nutritive or stimulating action. A number of other pure substances have been tried without effect.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.