SUBSTANCES AFFECTING ADULT TISSUE IN VITRO

II. A GROWTH INHIBITOR IN ADULT TISSUE

BY HENRY S. SIMMS AND NETTIE P. STILLMAN

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York)*

(Accepted for publication, September 15, 1936)

INTRODUCTION

In another paper (1) it was shown that the initial growth of adult chicken aorta tissue was greatly accelerated if the tissue had been digested with trypsin (or papain) previous to being planted in a culture medium. It was suggested that this stimulation resulted from the removal of an inhibitor from the adult tissue by the action of the enzyme.

In the same paper it was shown that dormant tissue cultures (in addition to fresh tissues) could be stimulated to renewed growth by the action of trypsin, and it was suggested that this resulted from the removal of an inhibitor which had been produced by the cells and deposited in the medium.

This paper will show that an inhibitor can be separated from the fluid in which adult aorta tissue has been digested.

Selection of Tissue

In choosing a suitable adult tissue for studying dormancy, as described in this and other papers, it was necessary to select one of simple structure which gives a clearly distinguishable growth in vitro, with a suitable lag period and growth rate, and which gives reproducible results. Adult heart, liver, thyroid, skin, muscle, and brain were all unsatisfactory. Arteries were better but it will be seen in Figs. 1 and 2 that these vary considerably. The thoracic aorta gave the best results. Different layers of the thoracic aorta are seen in Fig. 2 to differ in their growth. The inner layer of the thoracic aorta has been used in most of our work with satisfactory results. This consists of the inner half of the media together with the intima.

*This investigation has been aided by a grant from the Josiah Macy, Jr. Foundation.
ADULT TISSUE. II

EXPERIMENTAL

In the previously described stimulation of growth, the volume of dilute trypsin solution was considerably greater than that of the tissue. In attempts to obtain the inhibitor larger amounts of tissue in proportion to the fluid were used.

In the first experiments the digestion fluid was tested directly without fractionation. It was prepared by digesting strips of chicken aorta with 0.1 per cent trypsin solution at 20°C. for 18 hours under sterile conditions. Part of the fluid thus obtained was tested without heating. Part was heated at 58°C. for 20 minutes and part was heated at 100°C. for 5 minutes.

![Fig. 1](image1.png)

![Fig. 2](image2.png)

**Fig. 1.** Comparison of growth of various artery tissues (freed from adventitia) from the same adult chicken. The same relation was found in other chickens.

**Fig. 2.** Comparison of three layers of the thoracic aorta (also a sample of abdominal aorta).

In later experiments the digestion fluids from dog or sheep aortas were used. A typical preparation (17) was made from 15 gm. of sheep aorta (intima and media). This was minced with scalpels and treated with 20 ml. of 0.2 per cent

---

1 The latter were obtained from experimental sheep by the courtesy of the Physiology Department.
solution of Fairchild's trypsin \(^5\) at 37°C for 3 hours. \(^5\) The pH was kept at about 7.6 by occasional additions of 0.1 ml of 0.5 M NaOH. Near the end of the digestion the pH was allowed to drop to 6.8 (a little HCl was added in other experiments). The fluid was then removed by centrifuging and filtering. It contained 0.15 per cent non-protein nitrogen and 0.12 per cent protein nitrogen (equivalent to 0.76 per cent proteins).

This fluid was treated with an equal volume of absolute alcohol and was allowed to stand at 4°C overnight. The precipitate was dried in a desiccator and half of it was then taken up in 3 ml of "Na-K-G" solution. \(^4\) This fraction (I7A1) was pasteurized at 58°C for 20 minutes and was found to be quite inhibitory (Fig. 4) when mixed with serum (as compared with the serum control and with a portion of the inhibitor which had been heated at 100°C for 5 minutes).

The above precipitate was produced by the addition of alcohol. The remaining fluid (45 ml) was next treated with 1 ml of 5 per cent CaCl\(_2\) plus 1.6 ml of 0.1 NaOH. The resulting precipitate was allowed to settle at 4°C for 3 hours, was centrifuged off, and was taken up in Na-K-G solution. It (I7B) was also found to be active (Fig. 5).

A number of fractions were dialyzed. For example, a CaCl\(_2\) precipitate (I8B; prepared like I7B, above) was taken up in 5 ml of "Na\(_2\)-K\(_2\)-G\(_2\)" solution. \(^6\) This was dialyzed 5 hours in a rocking dialyzer (2) against flowing water and 1 hour against 30 ml of Na-K-G solution in a swirling shaker, with the gradual addition of 1 ml of 0.5 M NaOH to the outside solution, to adjust the pH.

The method of testing the activity of the inhibitors consisted in incubating adult aorta tissue in the presence of these materials previous to planting the tissue in a plasma medium. The inner layer of fresh adult chicken aorta was cut into pieces 1.5 × 2 mm in size, as previously described (1). Four pieces were placed in each of 10 or 11 sterile 13 × 100 mm tubes. To these tubes 1.5 ml portions of various test fluids, including controls, were added. The inhibitor fractions were mixed with one third volume of serum. The pH was kept about 7.4 with CO\(_2\)-air mixture for 4 days while the tubes were incubated at 37°C. CO\(_2\) is needed

\(^5\) The trypsin was dissolved in an isotonic solution containing 8 gm. of NaCl, 0.2 gm. of KCl, and 50 mg. of phenol red, per liter. The purpose of this solution was to avoid the presence of divalent cations.

\(^6\) In some experiments this 3 hours incubation was divided into two 1.5 hour periods on successive days, the flask being kept in the refrigerator in the interim.

\(^4\) The "Na-K-G" solution contained, per liter: 8 gm of NaCl, 0.2 gm of KCl, 1 gm of glucose, and 50 mg of phenol red. The absence of calcium and magnesium made the inhibitor fractions more soluble.

\(^6\) This is a 7 molar acetate buffer containing 0.6 equivalent of sodium acetate. It is made up with 570 gm of Na\(_2\)H\(_5\)O\(_2\)-3H\(_2\)O, 159 ml of glacial acetic acid plus 54 ml of H\(_2\)O.
by the tissue. The tissue was then cut into 0.6 mm. pieces and planted as already described (1).

All test solutions were made up so that they contained glucose and the inorganic constituents of serum. The osmotic pressure was always about isotonic (0.29 osmolar) it having been found that a 40 per cent decrease or increase (to 0.18 or to 0.40 osmolar) could be tolerated but that greater changes are harmful, growth stopping at 0.5 osmolar (70 per cent increase).

**DISCUSSION**

An inhibitor has been found in the fluid after adult aorta tissue has been digested with trypsin. In Fig. 3 the unheated digestion fluid itself (mixed with serum) is seen to inhibit growth of fresh tissue (as compared with the serum control); although it lost this inhibitory power upon heating, and became somewhat stimulating to fresh tissue (perhaps due to the protein digestion products). The loss of inhibitory power upon heating is attributed to the destruction of an inhibitory material which the tryptic digestion removed from the tissue. That this inhibitor could not be the trypsin itself was shown by experiments in which active trypsin was added in various proportions to serum (to duplicate conditions of testing). It was never inhibitory.

**Inhibitory Precipitates.**—When such a fluid is treated with an equal volume of alcohol it gives a precipitate which is inhibitory (see Fig. 4).

The remaining fluid upon the addition of CaCl₂, plus a little NaOH, gives a second precipitate which is also inhibitory as can be seen in Fig. 5.

Since both these precipitates are active and appear to contain the same material, it is not necessary to separate them. The digestion fluid can be treated with alcohol followed by calcium chloride (and NaOH) and the whole precipitate separated. This is active as shown in Fig. 6.

**Dialysis.**—Several of the preparations were dialyzed. Two of these lost all their activity. Two others seemed to be active but the boiled controls were lost by accident or infection. However, a fraction is seen in Fig. 7, to have been inhibitory after dialysis.

**Sterility and Sensitivity.**—In some experiments, such as the one illustrated in Fig. 3 aseptic technique was followed throughout. The
FIG. 3. Fluids from tryptic digestion of aorta, mixed with serum and used to treat fresh aorta previous to planting. Two portions of the digest were heated as indicated (Exp. 34B).

FIG. 4. An alcohol precipitate is seen to be completely inhibitory while a portion heated at 100°C. permitted growth (Inhibitor 7A1, Exp. 50).

FIG. 5. A CaCl₂ precipitate (from the fluid after alcohol precipitation) is strongly inhibitory as compared with a portion heated at 100°C., and with a serum control (17B, Exp. 50).

FIG. 6. Inhibitory action of a precipitate produced by the addition of both alcohol and CaCl₂ (14B, Exp. 41A).
chicken aorta was sterile at the start. However, in all the experiments using dog or sheep aortas the tissue was not sterile. In these experiments the material was usually pasteurized at 58°C. for 20 minutes at some time during the procedure. The inhibitory fractions in Figs. 4, 5, and 6 were all sterilized in this way.

However, this amount of heating can destroy the inhibitory activity. It will be seen that the fractions in Figs. 3 and 7 which were heated at 58°C. lost their activity, and the activity is displayed only by the unheated fractions. In the latter experiment the original tissue was contaminated, but by using aseptic technique we ended with sterile material owing to the bactericidal action of the alcohol and the acetate buffer.

*Similarity to Lactenin.*—The procedure described above was adopted because of the apparent similarity between this tissue inhibi-
H. S. SIMMS AND N. P. STILLMAN

Both these inhibitors withstand moderate heating at 58°C but are destroyed at 100°C. They are both precipitated by the addition of one volume of alcohol, and by addition of the calcium ion. Both are dissolved again under conditions favorable for the solution of calcium phosphate. They both withstand a moderate digestion with trypsin and are thereby liberated from much of the accompanying proteins. Both survive dialysis (i.e., they do not pass through the collodion bag) for a limited time providing the action of the trypsin has not gone too far. Furthermore, both inhibitors often lose their activity for some unaccountable reason when conditions seem to be carefully controlled.

It was therefore decided to test for their identity by seeing if each would perform the function of the other.

First it was found that whey (containing lactenin) would inhibit adult tissue growth. In Fig. 8 it will be seen that pasteurized whey incorporated in the medium in which aorta tissue was planted, caused a reduction of growth as compared with the boiled whey control.

However, the reverse test gave a negative result. A preparation of tissue inhibitor failed to inhibit the growth of scarlet fever streptococcus. This has not yet been repeated.

The Inhibitor in Tissue Cultures.—Observations on cultures from adult tissues (1) led us to suppose that the cells produce an inhibitor in vitro, which is deposited in the surrounding medium, and which can be removed by digestion with trypsin. This inhibitor, if it exists, may be identical with the inhibitor in dormant adult tissue.

Tumor Inhibitors.—Sittenfeld, Johnson, and Jobling (4) found that Rous tumor extract contained an inhibitory protein fraction which could be separated from other proteins, and from the causative agent, by precipitation. It mitigated the potency of the tumor agents—but not of tumor cells.

Murphy and Sturm (5) have worked with an extract from desiccated chicken

This was the same strain of streptococcus which the late Dr. Frederic S. Jones had used in our previous studies. It was found to be still susceptible to lactenin. Dr. Charles A. Slanetz was kind enough to test the action of the tissue inhibitor on this organism.
sarcoma tissue which restrained sarcomas in chickens and mice—but not a carcinoma. Furthermore, extracts from desiccated embryo skin, placenta, and mammary glands inhibited some mouse carcinomas, but not sarcomas. These extracts withstand 55°C but not 65°C., and in this respect resemble both lactenin and our adult tissue inhibitor. Whether there is further similarity remains to be determined.

Morton and Beers (6) found that an extract of human rectus sheath sometimes restrained rat carcinoma transplants. Unsaturated fatty acids and pancreas extracts have been claimed to retard the growth of chicken sarcomas and tumors (7).

**Significance.**—We believe that the tissue inhibitor described in this paper plays a large role in restraining growth in the adult animal body, thereby keeping the cells in their normal dormant state. It is suggested that the cells elaborate the inhibitor and deposit it in the surrounding intercellular space where it remains because of its insolubility.

**SUMMARY**

Digestion of adult tissue with trypsin has been shown to stimulate its initial growth *in vitro*. This stimulation appeared to result from the removal of an inhibitory material from the tissue due to the proteolytic action of the trypsin.

This paper shows that the fluid after the digestion contains material which inhibits the initial growth of adult chicken aorta tissue.

This tissue inhibitor has been obtained from chicken, dog, and sheep aortas. It is partly precipitated by an equal volume of alcohol, and is more completely precipitated by the further addition of CaCl₂ (plus a little NaOH). The inhibitor is destroyed at 100°C, but usually withstands 58°C for 20 minutes. Moderate tryptic digestion renders it soluble without destroying it. It sometimes withstands dialysis, but at other times is lost.

Its physical and chemical properties, as far as we know them, coincide with those of lactenin (a bacteriostatic substance in milk). Lactenin was found to inhibit adult tissue growth; but the tissue inhibitor failed to restrain bacterial growth.

This tissue inhibitor is believed to play a role in limiting the growth of tissue in the adult animal.
REFERENCES