SUBSTANCES AFFECTING ADULT TISSUE IN VITRO

I. The Stimulating Action of Trypsin on Fresh Adult Tissue

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PLATE 1

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INTRODUCTION

Previous workers (1–5) have grown various normal adult tissues *in vitro* and have found that these cultures resemble those from embryo tissue, but are harder to maintain.

In this and subsequent papers a study of dormant adult tissue will be described. The dormancy is characterized by the *lag period* preceding the onset of growth *in vitro*. Adult chicken aorta has a lag period of 3 to 5 days before the first new fibroblasts can be seen, while embryo tissue starts to grow in a few hours (6). In this paper it will be shown that digestion of the tissue with trypsin not only shortens this lag period (*i.e.*, overcomes the dormancy) but also increases the growth rate.

EXPERIMENTAL

Our methods differed from the standard tissue culture technique (7–12) in the following details: In each experiment a chicken was bled aseptically from the carotid artery (aided by suction) and was then killed either by clamping the trachea, or with ether. Using sterile technique the thoracic aorta was removed,

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and sometimes other tissues. These tissues were placed temporarily in "glucosal" solution (containing neither $NaHCO_3$ nor phenol red) until the aorta had been stripped of its adventitia and examined for fatty areas. The tissue could be stored 3 or 4 days, if desired, in the ice box in pH 7.4 Tyrode solution (containing 0.7 gm. of $NaHCO_3$ and 0.05 gm. of phenol red per liter) providing the aorta had not been split (see below) but it gave best results when fresh.

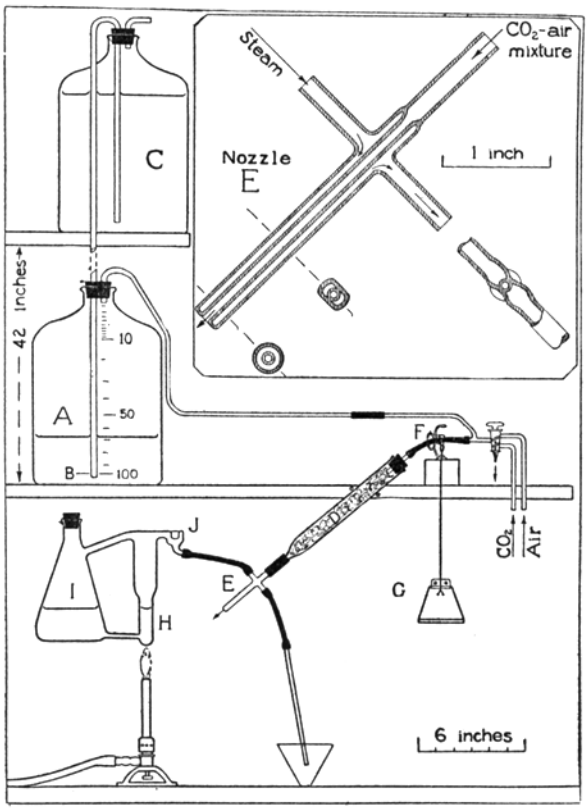
Treatment of Tissues with Trypsin.—The portion of the thoracic aorta which was to be used on a given day was split through the media. The outer media was discarded. The piece containing the inner media and intima was used. It was cut into strips about 1.5 × 2.0 mm. Four of these strips were used for each part of an experiment and were placed in a 13 × 100 mm. stoppered tube with 1.5 ml. (or 2.0 ml.) of sterile trypsin solution. The pH was adjusted to 7.6 as indicated by phenol red in the solution. This adjustment was made with sterile CO_2-air mixtures (about 3 per cent) with the addition, if necessary, of a little 0.15 M NaOH (or $NaHCO_3$) during the digestion. Usually 0.1 per cent Fairchild's trypsin was allowed to act 3 hours at 37°C. or, better, 24 hours at 22°C. The experiments were controlled by tissue treated with Tyrode solution under the same conditions. As a further control fresh tissue was planted without treatment

Planting the Tissue.—After this treatment the four strips of tissue from each tube were cut into pieces 0.6 mm. in diameter and were planted in two special 32 mm. Carrel flasks, 16 pieces in a flask. Before the tissue was introduced about 0.08 ml. of chicken plasma (which had been diluted with two parts of Tyrode solution, containing phenol red) was spread over the bottom of each flask. The sixteen pieces of tissue were then planted in even rows with the aid of a metal guide. The flasks were corked and allowed to stand ½ to 1 hour until the plasma clotted. The surface was then rinsed with Tyrode solution which was drawn off by gentle suction. 0.7 ml. of diluted plasma was added, spread over the surface, and allowed to clot. The pH was adjusted to about 7.3 with 5 per cent CO_2-air mixture introduced with a sterile gas injector (see Text-fig. 1). The flasks were placed at 37°C. Fresh CO_2-air was added each day.

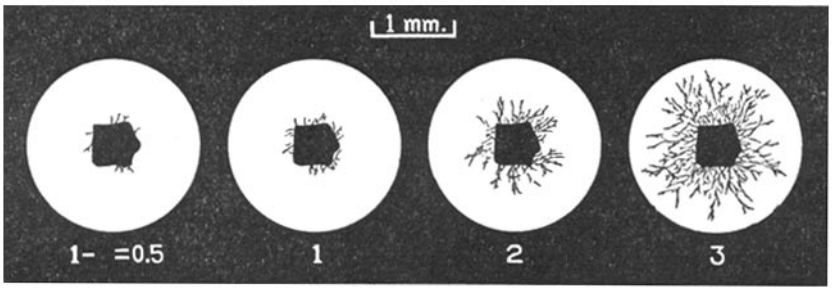
Evaluation of Growth.[1] Each colony was examined daily under the microscope. The nature of the growth was such that the following system of evaluation was found most satisfactory (see Text-fig. 2).

0 = no growth.
1– – (= 0.25) = 1 to 6 new cells.
1– (= 0.5) = about 10 new cells.
1 = about 30 new cells.
2 = growth extended into medium (about 90 new cells).
3 = abundant growth but not visible to naked eye.
4 = new growth clearly visible to naked eye.
5 = colony 1 cm. or more in diameter.

[1] The word "growth" is used to indicate the production of new cells visible under the microscope.



TEXT-FIG. 1. A sterile gas injector for introducing CO_2-air mixtures into flasks or tubes. Steam from boiler tube, H, keeps nozzle, E, sterile. The gas mixture from bottle, A, is sterilized by passing through sterile cotton, D, and is injected into the culture flask through E. The stopper of the flask can be placed in the sterile cup, J.



TEXT-FIG. 2. Amount of growth corresponding to the indicated ratings

Intermediate ratings of 1+, 2+, 3+, and 4+ (equalling 1.5, 2.5, 3.5, and 4.5 respectively) were also used.

The sum of the ratings of the sixteen colonies in a flask gave the "growth value" of that flask. Each value in this paper is the mean of two duplicate flasks (thus representing 32 pieces of tissue).

Enzyme Solutions

The trypsin solutions were prepared as described below and were then sterilized by Berkefeld filtration and stored in the refrigerator.

Fairchild's trypsin was made up as a 1 per cent suspension, filtered through paper, then through a Berkefeld filter, and kept as a stock solution. This "1 per cent" solution was diluted with 9 volumes of Tyrode solution before using.

A "purified trypsin" was prepared from Fairchild's trypsin by fractionation with ammonium sulfate. It was dialyzed against water, then against Tyrode, and used without further dilution.

Northrop's *crystalline trypsin* and *chymotrypsin*[2] were made up to 1 per cent solutions in 0.003 M HCl and dialyzed against water; against saturated sodium chloride; then against water; and finally against 0.003 M HCl. They were diluted with Tyrode before use.

Papain solution was prepared as a 1.5 per cent solution. This was activated by adding one-fifth volume of a neutralized 1 per cent solution of cysteine hydrochloride and heating 1 hour at 36°C. In using this on a tissue a sufficiently low pH was obtained by introducing a 40 per cent CO_2-air mixture. This did not materially injure the tissue.

Activity of Trypsin.—The proteolytic activities of the trypsin solutions were determined by digestion of casein solution using the procedure of Northrop and Kunitz (13).

5.0 ml. of 5 per cent casein solution was warmed to 35.5°C. 1.0 ml. of enzyme solution (or 0.5 ml. diluted to 1.0 ml.) was added. The mixture was maintained at 35.5°C. for 20 minutes. Trichloracetic acid was then added and the non-protein nitrogen was determined. The weight of non-protein nitrogen liberated per milliliter of the enzyme solution was taken as the measure of activity.

Treatment of Cultures with Trypsin.—To each flask culture 1 ml., or less, of 0.1 per cent Fairchild's trypsin solution (in Tyrode) was added. The flasks were placed at 37°C. and observed at frequent intervals. As soon as the digestion in a given flask had reached the cells the fluid was quickly drawn off and fresh plasma was added. Sometimes when the digestion was slow, the fluid was replaced with serum at the end of 4 hours, and the treatment repeated the next day. Caution was then necessary to prevent complete liquefaction of the clot.

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[2] These crystalline enzymes were obtained through the kindness of Dr. John H. Northrop.

Stimulation of Fresh Tissues by Trypsin

There is a lag period of 3 to 5 days before the first growth of fibroblasts can be seen from adult chicken *thoracic aorta* which has been planted in a medium of diluted plasma and kept at 37°C. But if the

TABLE I

Stimulation of Abdominal Aorta with Trypsin

The digestion was followed by 2 days at 4°C. in Tyrode solution (Exp. 61C).

Treatment before planting	Growth values after planting					Relative growth on 5th day
	1 day	2 days	3 days	4 days	5 days	
Tyrode control	0	0	0.5	2.0	3.0	1
Trypsin 3 hrs. 37°C	0	0.7	2.0	5.7	8.5	3

TABLE II

Stimulation of Adult Chicken Liver. (Exp. 30A and B)

Tissue treatment before planting	Growth values after planting				
	1 day	2 days	3 days	4 days	5 days
Untreated	0	1.2	5	8	11
Trypsin 3 hrs. 24°C	0	3.0	8	10	19

TABLE III

Dog Granulation Tissue, Stimulated by Trypsin

(2 hours at 37°C. and 22 hours at 0°), planted in chicken plasma (Exp. 49).

Tissue	Treatment before planting	Growth values after planting		
		1 day	3 days	5 days
Vascular area (newer growth)	Tyrode control	0	0	0
	Trypsin treated	2.5	6	9
Fibrous area (older growth)	Tyrode control	0	0	0
	Trypsin treated	7	12	19

tissue is first treated with trypsin its lag period is definitely reduced. Furthermore (see Text-figs. 5, 7, 8, 9, and 10, discussed below) the growth rate is greatly accelerated.

Other adult tissues are similarly stimulated. These include the

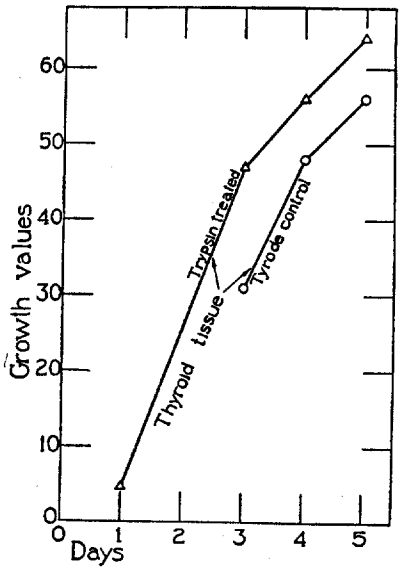

FIG. 3

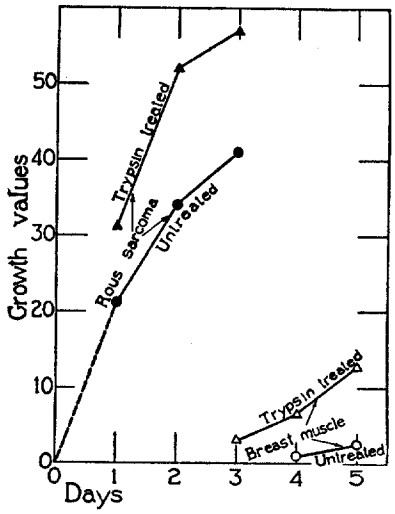

FIG. 4

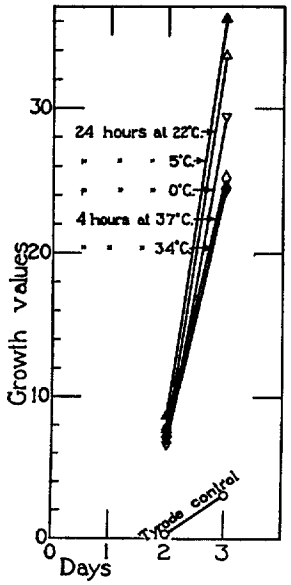

FIG. 5

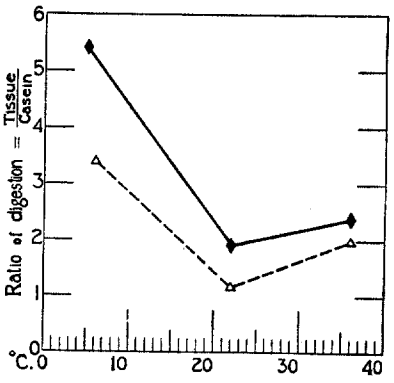

FIG. 6

abdominal aorta (Table I), adult *liver* fibroblasts and epithelial cells (Table II), adult *thyroid* (Text-fig. 3), muscle fibroblasts (Text-fig. 4), and dog *granulation tissue*[3] (Table III).

Rous sarcoma tissue was stimulated by trypsin treatment (Text-fig. 4) even though the untreated tissue had no lag period. Of a number of human brain tumors[4] which were studied one *meningioma* grew 15 per cent faster after 2.5 hours in trypsin at 37°C., and one glioma was slightly stimulated (not evaluated).

The stimulation of initial growth of dormant tissue seemed to depend upon obtaining just sufficient digestion of the tissue. Too much digestion killed the tissue. At the optimum point the tissue was perceptibly softer (but we do not believe this softness caused the stimulation) and was more translucent as seen under the microscope. It sometimes contained visible round cells which later developed into fibroblasts.[5] The digested aorta tissue, when stained with hematoxylin, was seen to have lost its mucoid material. The digestion removed 10 to 20 per cent of the nitrogen from the tissue with a corresponding increase in the nitrogen of the digestion fluid. There was considerable variability between chickens in the susceptibility of their tissues to digestion and this did not seem to be related to age, sex, or breed.

TEXT-FIG. 3. Stimulation of thyroid tissue by trypsin (Exp. 65A).

TEXT-FIG. 4. Stimulation of Rous sarcoma, and of breast muscle fibroblasts, by trypsin digestion (2 hours at 37°C.) (Exp. 48).

TEXT-FIG. 5. Relative growth after trypsin treatment under different conditions (Exp. 63B).

TEXT-FIG. 6. Relative digestion of aorta tissue and of casein by trypsin at different temperatures. The solid line represents an experiment (65D) in which the digestion fluids were maintained at pH 7.6. The duration of the digestions were: 24 hours at 5°C. and at 22°C., and 4 hours at 36°C. The broken line represents a similar experiment (64A) in which the pH of the tissue digestion fluids dropped to lower values.

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[3] Kindly furnished us by Dr. Margaret Murray. It was prepared by planting small tubes in dog muscle for 12 days.

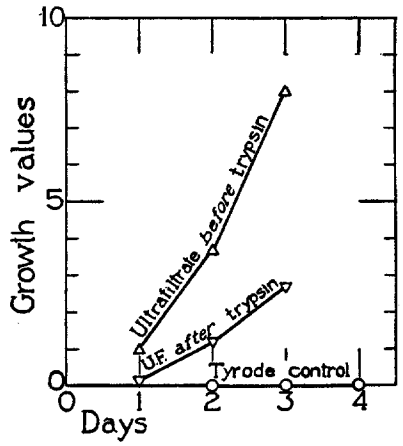
[4] Surgical material obtained with cooperation of Dr. Abner Wolf.

[5] Rous and Jones (14) obtained similar round cells by drastic digestion of embryo cultures. We likewise observe a retraction of fibroblast processes during partial digestion of our cultures (as in Fig. 1, Plate 1).

The control tissues in Text-figs. 3, 5, 7, 8, 9, 10, and 11 were treated with Tyrode solution under the same conditions as the trypsin treatment. Most of the experiments were also controlled with fresh untreated tissue (as in Text-fig. 4). There was no significant difference between the Tyrode controls and the untreated controls.

Temperature of Treatment.—Usually a slow action at a low temperature stimulated better than the faster digestion at higher temperature. In Text-fig. 5 the relative stimulations under different conditions are compared.

The degree of stimulation at 0°C. was somewhat surprising. It seemed strange that sufficient digestion could occur at that temperature. This is explained by the results in Text-fig. 6 in which the amounts of nitrogen liberated from aorta tissue and from casein are compared under different conditions. If the relative susceptibility



TEXT-FIG. 7. Stimulation produced by 3 days incubation of the tissue in serum ultrafiltrate *before* digestion with trypsin (Exp. 55C).

(to tryptic digestion) of aorta and of casein were independent of temperature then the three points of each experiment would lie in a horizontal line. (The height of the line is not important.) If we take 22°C. as a standard then the experimental results show that the susceptibility to digestion was relatively greater for aorta than for casein at 36°C. and considerably greater at 5°C. In other words,

aorta was more readily digested at refrigerator temperatures than would be expected from observations on casein.

Incubation of tissue in serum ultrafiltrate (or serum) *before* trypsin digestion produced a much better stimulation than the same treatment after the digestion (Text-fig. 7). The serum ultrafiltrate contains a stimulant (the "A factor"; see later paper (17)), needed for the initial growth of adult tissue. The A factor may render the cells more susceptible to the stimulating action of trypsin. The presence of this agent *during* the digestion increased the stimulation only 10 per cent. However, the presence of serum in the trypsin solution prevented digestion and gave a stimulation only equal to that of serum alone.

Stimulation of Tissue Cultures by Trypsin

Flask cultures of adult chicken aorta fibroblasts which have reached a state of partial degeneration and retarded growth have been treated with trypsin until the plasma clot has been digested sufficiently to expose some of the cells. Fresh plasma was added and allowed to clot. There was an immediate vigorous stimulation to renewed growth (while control cultures failed to grow appreciably whether they were washed with heparin plasma, serum, serum ultrafiltrate, Tyrode solution, or embryo extract; or if untreated). Plate 1 illustrates one of many such cultures. This untreated 11 day culture had not grown for 3 days and was partly degenerated when treated with trypsin. Fig. 1 was taken immediately after digestion; Fig. 2 after 20 hours, and Fig. 3 after 3 days.

Similar stimulation has been obtained with cultures of Rous sarcoma, Walker rat sarcoma 319 cultures,[6] and a human meningioma culture.

Weekly digestion with trypsin (and intermediate washings with serum or serum ultrafiltrate) has made it possible to maintain adult cultures in the same flasks for long periods; but this is not recommended owing to the difficulty of controlling the digestion.

The gentle digestion, by leaving the colonies more or less intact, has made it possible to study the stimulating mechanism (see below).

[6] These pure sarcoma cultures had been grown *in vitro* over a year and a half by Dr. Warren H. Lewis, who furnished them to Dr. Joseph Victor, of this department.

It has no similarity to the drastic digestion of embryo cultures by Rous and Jones (14) and by Rous, McMaster, and Hudack (15).

Mechanism of the Trypsin Stimulation of Fresh Tissues

Considering first the action of trypsin on fresh tissues (rather than on cultures) three explanations suggest themselves:[7] First, the stimulation might be due to protein split products which Baker and Carrel (16) showed would stimulate active cultures of embryo fibroblasts. Second, the stimulation might be due to lipase or some other impurity in the Fairchild's trypsin, and not due to its proteolytic action. Third, the trypsin might stimulate solely because of its proteolytic action on the tissue.

Protein Digestion Products.—Our use of trypsin was originally intended to stimulate growth by virtue of the protein split products produced by the digestion (16). However, we obtained results which could not be explained on this basis. In several experiments tissues were thoroughly washed *after* trypsin treatment, previous to planting.

TEXT-FIG. 8. Comparison between the growth stimulation of tissue washed before, and of tissue washed after trypsin treatment (Exp. 51A). The tissues were washed 1 day in Tyrode solution at 0°C.

TEXT-FIG. 9. Stimulation produced by three trypsin solutions of different purity but equal activity toward casein (Exp. 27A).

TEXT-FIG. 10. Stimulation produced by equally active solutions of Northrop's crystalline trypsin and chymo-trypsin compared with commercial trypsin (Exp. 59B).

TEXT-FIG. 11. Growth stimulation produced by papain compared with trypsin (Exp. 60C).

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[7] It might be thought that the softening of the tissue is mechanically responsible for the stimulation of growth. We do not believe this plays an important rôle. Hardness cannot account for the dormancy of adult tissues, hence softness cannot account for the onset of growth. Soft tissues such as liver or brain have as long a lag period as the firm aorta tissue. It may be seen in Table III that none of the dog granulation tissue grew without the action of trypsin, in which case the firmer tissue grew faster.

Furthermore, the dormant cells in an old culture flask are surrounded, before digestion, with a medium much softer than the aorta tissue after digestion. What is more, growth is generally better in firm plasma clots than in soft ones.

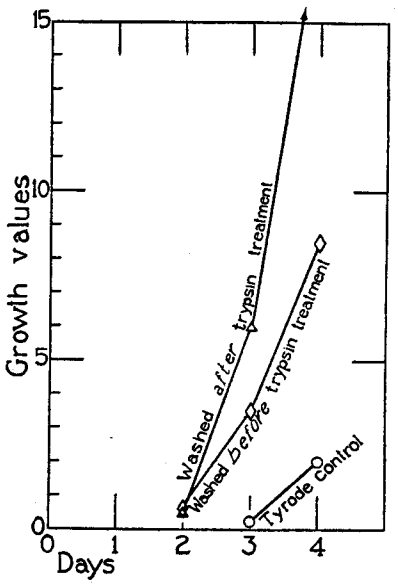

FIG. 8

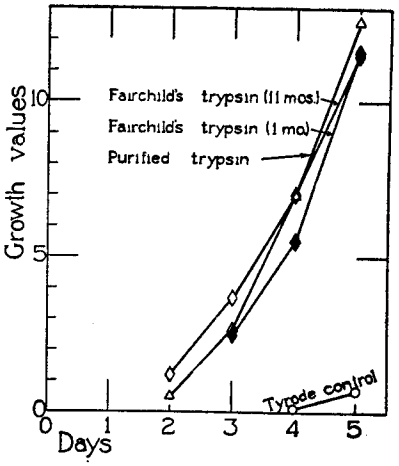

FIG. 9

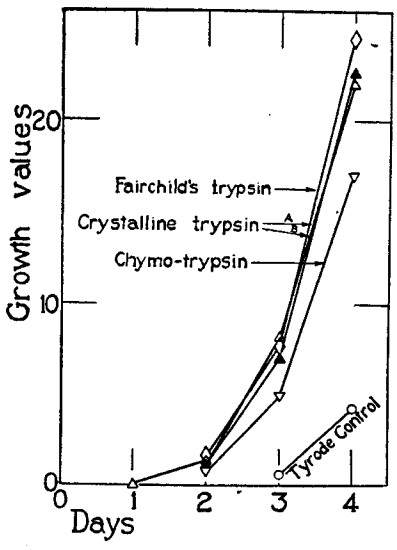

FIG. 10

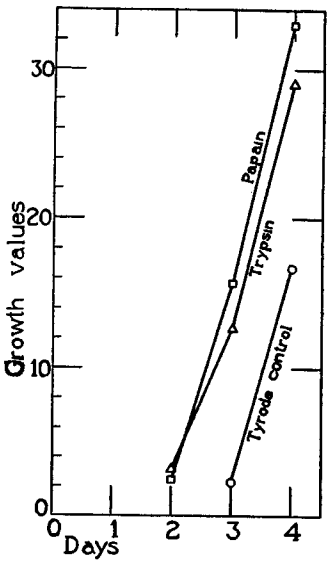

FIG. 11

These tissues grew as well as, and sometimes better than, the unwashed ones. Fig. 8 gives an example.[8] Note the growth on the 4th day. It is seen that both of the trypsin treated tissues were markedly stimulated (as compared with the control) but the tissue which was washed *after* the digestion grew much faster in spite of the

TABLE IV

Determination of Trypsin Activities

5.0 ml. of 5 per cent casein digested with enzyme solution for 20 minutes at 35.5°C. (Exp. 27A).

Trypsin solution	Volume of enzyme solution	Concentration of enzyme	Non-protein nitrogen liberated	Relative activity	Calculated concentration for equal activity
	ml.	*per cent*	*mg. per ml. of enzyme*		*per cent*
Fairchild's (11 mos.)	1.0	0.1	0.49	100	0.100
Fairchild's (1 mo.)	0.50	0.1	2.2	453	0.022
Purified	0.50	X*	0.87	174	0.58X

* X equals the unknown concentration of trypsin in the purified solution.

TABLE V

Determination of Trypsin Activities

5.0 ml. of 5 per cent casein digested with 1 ml. of 0.2 per cent enzyme solution for 20 minutes at 35.5°C. (Exp. 59B).

Trypsin solution	Non-protein nitrogen liberated	Relative activity	Calculated concentration for equal activity
	mg. per ml. of enzyme		*per cent*
Fairchild's	3.0	100	0.100
Crystalline trypsin A	6.0	200	0.050
Crystalline trypsin B	5.4	180	0.055
Crystalline chymo-trypsin	6.6	220	0.045

removal of digestion products. Had the stimulation been due to the digestion products the reverse effect would have been observed.

It might be supposed that some of the digested proteins remained

[8] Text-fig. 8 should not be confused with Text-fig. 7. In one case the tissue was washed in Tyrode solution at 0°C. In the other case the tissue was incubated at 37°C. in the presence of the stimulating "A factor."

in the tissue despite the washing. However, we have found that the digestion fluid does not stimulate, but on the contrary is slightly inhibitory (see the following paper). The possibility of stimulation due to trypsin retained by the tissue is remote, since trypsin incorporated in the clot of a culture does not stimulate, and furthermore tissue which has been insufficiently digested has a good opportunity to adsorb enzyme—but is not stimulated. Hence we may discard the idea that the trypsin stimulated the initial growth of adult tissue because of protein split products.[9]

Impurity Versus Proteolytic Action.—The suggestion that the crude trypsin, which we first used, contained an impurity (such as lipase[10] or another enzyme, or some non-enzymatic substance) which produced the stimulation, is untenable in view of the results obtained by comparing enzymes of different degrees of purity. In Table IV are the relative activities of three trypsin solutions, as tested on casein. The values in the last column indicate what concentrations of the three enzymes should have equal proteolytic activity.[11]

The three enzyme solutions were then diluted to these (equally active) concentrations and tested on adult aorta tissue. The subsequent growth of these tissues, as indicated in Fig. 9, shows that the three enzyme solutions produced about equal stimulation. The first trypsin solution had been kept 11 months and contained 4.5 times as much solid matter per unit of activity as did the second solution. The third was a purified trypsin. Hence the amounts of impurities were quite different in the three solutions, but since the activities were about equal, and the stimulations were practically equal, it would appear that the stimulation depends upon proteolytic activity.

[9] We have found (in unpublished experiments) that protein digestion products prepared by the methods of Baker and Carrel (16) do produce a mild stimulation of the initial growth of adult aorta tissue under favorable conditions. However, under the conditions of the experiments reported in this paper, these products appear to play a negligible rôle in the stimulation of digested *tissue*. On the other hand there is evidence that digestion products do play a small, but minor rôle in the stimulation of digested *cultures* (see text).

[10] Evidence is also given in a later paper (17) that the stimulating effect of serum is not due to the lipase contained in it.

[11] This calculation assumes that the activity was proportional to the concentration of enzyme. This was roughly true in this range of concentrations.

Similar results were obtained in another experiment in which Northrop's crystalline trypsin and some of his crystalline chymotrypsin were compared with commercial trypsin. In Table V the activities of these solutions toward casein were compared. The concentrations for equal proteolytic activity are given in the last column.[11] These concentrations of the enzymes when used on aorta tissue produced about equal stimulation as seen in Fig. 10. The two solutions of Northrop's crystalline trypsin were prepared separately from the same solid material.

Papain.—In Fig. 11 it will be seen that papain stimulates the growth of adult tissue in the same manner that trypsin does. Hence the stimulation seems to be purely one of proteolysis. Owing to the necessity of treating the tissue at a lower pH with papain than with trypsin it was not attempted to compare their activities quantitatively.

Inhibitor.—If we accept the above data as indicating that these enzymes stimulate because of their proteolytic activity we are confronted with a new question, namely, why the digestion of tissue protein should stimulate growth. We suggest that the enzymes digest away an inhibitory protein material, and thus remove it from the environment of the cells. The following paper will show that the digestion fluid contains an inhibitor.

Mechanism of Trypsin Stimulation of Tissue Cultures

Returning now to the stimulation of tissue *cultures* (see Plate 1) several explanations suggest themselves. It might be supposed that the stimulation resulted from the products of protein digestion (16). However, washing away the digestion fluid before adding the new clot resulted in only a slight decrease in the stimulation. This indicated that the digested proteins did aid the growth slightly but that they played only a minor rôle.[9] Furthermore, the addition of digested plasma[12] to cultures in an advanced state of growth failed

[12] This digested plasma was obtained by digesting plasma clots in Carrel flasks under conditions identical with digestion of the plasma medium of cultures (except that no tissue was present). The enzyme in this digest was not heat killed but control experiments showed that the enzyme could not have been toxic to these cells.

to produce any conspicuous stimulation (although similar cultures were definitely stimulated by digestion to immediate and prolific growth).

These conclusions are further substantiated by many observations on partly digested cultures. Within a given culture marked stimulation was obtained only in those portions where the digestion had removed the clot in close proximity to the cells. Had the digestion products been the sole cause of this stimulation then these products should have also diffused through the slightly thicker clot in other parts of the same culture—but in reality these other portions were only slightly stimulated, if at all.

It might also be supposed that renewal of the plasma clot added some needed material, but washing the cultures with serum (or heparin plasma) failed to stimulate in the way that trypsin did. Hence if serum (or plasma) contains such a needed material it must be too slowly diffusible to pass through the medium. The only poorly diffusible stimulant in plasma that we know of is globulin (17), and this is only mildly active, especially in the presence of albumin. Renewal of the fibrin cannot be essential since the new growth can extend into unused portions of the old clot to some extent.

We have found only one explanation which fits our observations, namely that the old clot immediately surrounding each cell contained inhibitory material which was too poorly diffusible to be washed away. The trypsin in digesting the clot dissolved the inhibitor with it.

It seems unlikely that such an inhibitor could have been produced by the plasma. Aging of plasma (in the ice box) for a year does not materially affect its ability to support growth (unpublished data) and plasma that had been incubated several days before use was still stimulating. Hence this inhibitor, if it exists, is a product of the cells.

SUMMARY

Adult tissue is characterized by a lag period of several days preceding the onset of growth *in vitro*.

Treatment of fresh adult tissues with trypsin before planting them in culture flasks stimulated the tissues to grow sooner and more rapidly.

Best stimulation was obtained by slow digestion at low temperature. The tissues lost nitrogen during the digestion. Lowering the temperature from 22°C. to 5°C. reduced the digestion of aorta tissue much less than it reduced the digestion of casein.

Washing the tissue after trypsin treatment resulted in better stimulation.

Trypsin solutions of different degrees of purity, when diluted to equal activity toward casein, gave equal stimulation to the tissue growth. These included solutions of Northrop's crystalline trypsin and chymo-trypsin.

Papain also stimulated growth in a similar manner.

The results indicate that this stimulation of tissue growth is due entirely to proteolytic action.

Cultures of adult fibroblasts (and some tumor cultures) having reached a state of retarded growth have been treated with trypsin to digest away most of the plasma clot (used as a medium). Fresh plasma has been added to renew the clot. This treatment has resulted in an immediate renewal of growth.

Reasons are given for supposing that the cells produce an inhibitor *in vitro* which they deposit in the surrounding clot, and which is removed by the action of trypsin.

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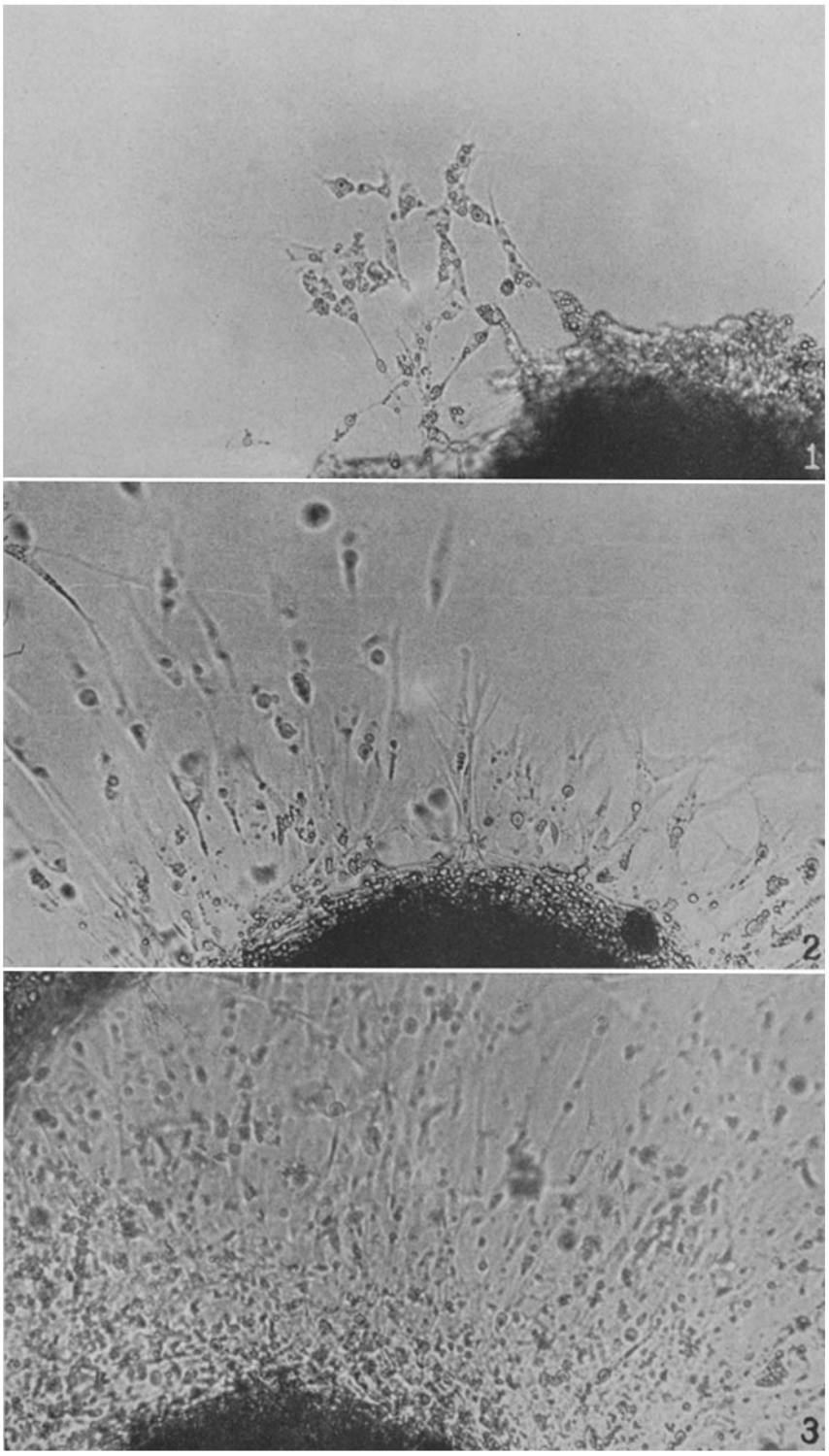
EXPLANATION OF PLATE 1

FIG. 1. An 11 day culture of adult chicken aorta fibroblasts after digestion with trypsin. The dark area is part of the original tissue (culture colony 2779-14, F7-11), ×180.

FIG. 2. The same field after 20 hours (F7-18), ×180.

FIG. 3. The same field after 3 days (F9-8), ×180.



(Simms and Stillman: Adult tissue. I)