duction of free-moving particles or by a possible increase of osmotically-active particles, is to be considered as another example of stress with which the adrenalectomized organism fails to cope.

Summary. All frogs employed in this study may be conveniently divided into 4 groups (18-22 animals/group): the adrenalectomized frogs whose postoperative body weights were either controlled, to within ± 1.5 g of their individual pre-operative values, or uncontrolled; and the controls, both renal damaged and unoperated frogs. The individuals of each group were subjected to a constant dehydrating force and the resulting data were statistically analyzed with respect to the following points: *a*, the rate of loss and *b*, the total loss of body weight (water) and *c*, the duration of survival on exposure to a constant force of dehydration.

1. A comparison of the difference between respective means of the 2 groups of adrenalectomized frogs shows no significance as regards the foregoing items *a*, *b*, and *c*. 2. Comparison between the 2 groups of controls (renal damaged and unoperated frogs) gives no significance with respect to items *b* and *c*, though it does for *a*. 3. A comparison between the respective means for either group of adrenalectomized frogs and their controls (renal damaged) produces a significance for *a* and *b*, and for *c* as it affects the death point of the controlled but not of the uncontrolled adrenalectomized frogs. 4. On the basis of the known cardiovascular embarrassment subsequent to adrenalectomy, it is suggested that the increased osmotic pressure resulting from the forced water loss and the attendant decrease in peripheral circulation brings an increased osmotic stress to bear on an already weakened heart action. It is suggested that this stress is the deleterious factor in affecting the physiological points raised.

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17292. Use of Antitryptic Agents in Tissue Culture. I. Crude Soybean Trypsin-Inhibitor.*†

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Substances that inhibit the proteolytic activity of trypsin have been found in serum and plasma,[1] in egg white,[2] in navy beans and soybeans,[3] and in extracts of pancreas.[4,5] Because of the great activity of these antitryptic agents, it seemed of interest to investigate the possibility of using them in tissue culture as a means of preventing the digestion of the plasma coagulum that frequently occurs during the growth of cells *in vitro*.[6-8] The soybean antitrypsin,‡ which has been

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2 Balls, A. K., and Swenson, T. L., *J. Biol. Chem.*, 1934, **106**, 409.

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6 Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **13**, 495.

7 Losee, J. R., and Ebeling, A. H., *J. Exp. Med.*, 1914, **19**, 593.

8 Santesson, L., *Acta path. et microbiol. Scand.*, 1935, Suppl. **24**

‡ It is interesting to note that Fischer has just reported (Fischer, A., *Science*, 1949, **109**, 611) a series of experiments with crystalline soybean trypsin inhibitor supplied by Kunitz.[9] His results are in complete accord with those reported here.

9 Kunitz, M., *J. Gen. Physiol.*, 1947, **30**, 291.

shown by Kunitz[9] to be a protein of globulin nature, was selected as the first agent to be studied in the present investigation.

Methods. Crude trypsin-inhibitor was prepared from solvent-extracted unheated soybean flakes§ by the method of Kunitz.[10] This material was dried from the frozen state, and the resulting powder was kept in the refrigerator. The activity of each preparation was determined by its ability to prevent the digestion of casein by trypsin, according to the procedure of Kunitz.[9] In these tests, graded amounts of antitrypsin were added to a standard solution of trypsin and the degree of proteolysis was then measured colorimetrically as tyrosine liberated by Folin's reagent. For use in tissue cultures, the dried powder was dissolved in 0.01 N HCl, and the material was sterilized by passage through a UF fritted glass filter. Stock solutions were diluted further in sterile glass-distilled water to the desired concentrations. All sterile solutions were kept in the refrigerator, and were found to retain their antitryptic potency for a period of at least 6 to 8 months.

Culture strains of fibroblast-like cells were derived from the leg muscle of 11-day chick embryos, and were cultivated in D-3.5 flasks through at least 10 to 12 passages (weeks) before use. In some experiments, use was made of fresh tissue explants.

The coagulum added to each flask consisted of 0.3 ml chicken plasma, 0.3 ml chick embryo extract, 0.3 ml Earle's modification of Tyrode's solution,[11] and 0.1 ml phenol red (0.025% in Earle's solution). In addition, a feeding mixture was added to each culture flask on the second day and was renewed on the fourth and sixth days. This feeding mixture was comprised of 0.3 ml embryo extract, 0.6 ml Earle's solution, and 0.1 ml phenol red solution. At no time was the original coagulum reinforced by the addition of another layer of plasma.

Embryo extract was prepared by grinding 11-day chick embryos that were suspended in a roughly equivalent volume of Earle's solution in a graduated all-glass homogenizer. After the cell debris had been separated by centrifuging, the supernatant was frozen and thawed twice. This concentrated extract was diluted in Earle's solution to 1 part in 4 and was kept as a stock solution in the refrigerator. The stock solution was further diluted in Earle's solution to a final concentration of 1 part in 20 immediately before use.

The antitryptic factor (0.1 ml of an appropriate dilution) was added to both the original coagulum and the feeding mixture in place of 0.1 ml of Earle's solution. An equal volume of glass-distilled water was added to all control cultures.

In some experiments, the effect of antitrypsin was tested on cell colonies cultivated in fibrinogen-thrombin clots prepared by the method of Porter and Hawn.[12] To these cultures, 0.3 ml of serum was added to provide the nutrient materials ordinarily supplied by the plasma.

When the tissue transplants were prepared from strain material, each of the cultures chosen for an experiment was cut into 4 fragments of approximately equal size, and 3 of the fragments were embedded in separate flasks containing graded amounts of antitrypsin. The fourth fragment in each set served as a control and was cultivated in the absence of antitrypsin. Each experiment consisted of at least 3 sets of cultures containing the same ingredients.

To test for possible inhibition of migration and growth by the antitryptic agent, outline drawings showing the increase in surface area were made daily by means of a projectoscope, and the increments were measured with a planimeter.

Results. Prevention of clot digestion. In preliminary experiments, crude antitrypsin, prepared as described, was added to a series of 12th passage sister cultures at final concentrations of 5.0, 3.0, 2.0, 1.0, 0.5, 0.25, 0.1, and 0.05 mg per ml. These cultures together with

§ Obtained through the courtesy of Dr. W. D. McFarlane, Canadian Breweries, Ltd., Toronto, Ontario.

10 Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

11 Earle, W. R., *J. Nat. Cancer Inst.*, 1943, **4**, 165.

12 Porter, K. R., and Hawn, C. v. Z., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 309.

their controls were incubated for 7 days and were observed each day for signs of digestion of the coagulum. It was found that antitrypsin levels between 5.0 mg per ml and 0.25 mg per ml completely prevented digestion of the plasma coagulum, while lower concentrations were not effective. Fig. 1 and 2 show two representative cultures selected from this series and photographed at 11 days.

Comparable results were also obtained in other experiments in which fibrinogen-thrombin clots were used. In these instances, the effective concentrations of antitrypsin were found to be between 5.0 and 0.25 mg per ml.

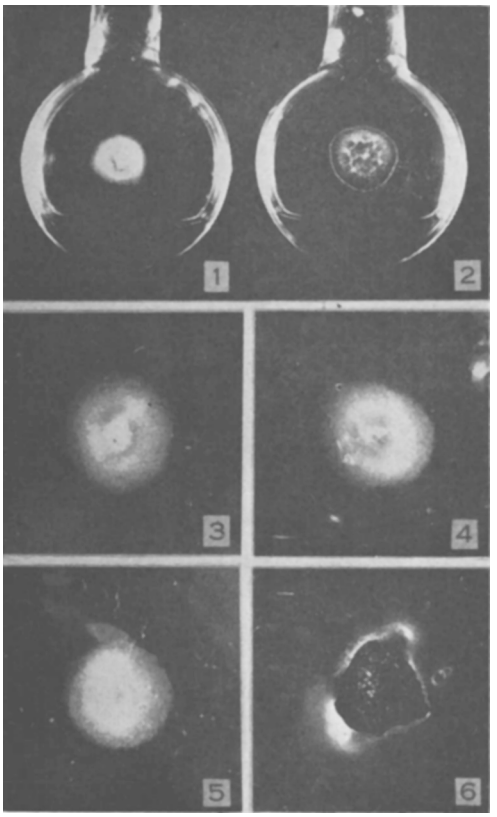

FIG. 1 and 2.
Eleven-day sister cultures of 12th passage chick fibroblasts cultivated in plasma medium in the presence of 0.5 mg antitrypsin per ml (Fig. 1), and in the absence of antitrypsin (Fig. 2). × 1.

FIG. 3, 4, 5 and 6.
Seven-day sister cultures of 15th passage chick fibroblasts cultivated in plasma medium in the presence of 1.0 mg antitrypsin per ml (Fig. 3), 0.5 mg per ml (Fig. 4), 0.25 mg per ml (Fig. 5), and in the absence of antitrypsin (Fig. 6). × 3.

In both types of coagulum, control cultures usually gave evidence of initial digestion within 24 to 48 hours. This appeared first as a thin ring in the medium a short distance from the tissue fragment, and progressed rapidly to form a crater containing liquefied plasma. The tissue fragment contracted very considerably, and in most cases was found floating freely in the liquid plasma. Cell growth frequently occurred on the surface of the glass beneath the digested area.

Inhibition of plasma coagulation by antitrypsin. It was found that the presence of high concentrations of the crude trypsin-inhibitor (5.0 to 1.0 mg per ml) prevented normal plasma coagulation in the presence of embryo extract. The addition of a few drops of dilute thrombin overcame this inhibition and permitted normal clot formation. This finding is in agreement with the results of Tagnon and Soulier,[13] who reported that both crude and crystalline antitrypsin from soybeans had a marked anticoagulant effect upon whole blood and recalcified plasma, but had no antithrombic activity. In the present investigation, it was also noted that the anticlotting activity of trypsin-inhibitor was more pronounced when heparin or citrate was present in the plasma.

Inhibition of tissue growth by antitrypsin. The presence of crude soybean antitrypsin was found to retard the growth of the culture strains that were used as test material. The effect was most pronounced at high concentrations, 3.0 to 1.0 mg per ml. Cultures maintained in such high antitrypsin concentrations were observed to have a total area that was 20 to 30% less than the area of sister cultures grown in the absence of antitrypsin. The toxicity was less evident at lower levels, 0.5 to 0.25 mg per ml. Inhibition of growth was not uniform, however, in all cultures tested; and in some experiments the inhibition was negligible even at concentrations of 3.0 mg per ml. The effect of graded levels of crude antitrypsin on 15th passage strain cultures is shown in Fig. 3, 4, 5 and 6.

Repeated passage of cultures through media

[13] Tagnon, H. J., and Soulier, J. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 440.

containing inhibitory levels of antitrypsin resulted in a gradual disappearance of the growth-depressing effect. The cultures acquired a tolerance to antitrypsin after 3 to 5 passages, and thereafter appeared to grow normally in the presence of concentrations that had previously been found to be inhibitory.

Effect of trypsin-inhibitor on fresh tissue explants. When a study was made of the effect of several concentrations of crude soybean antitrypsin on fresh tissue explants in their first culture passage, it was found that levels of 1.0, 0.5, and 0.25 mg per ml completely prevented digestion of the plasma coagulum. It was also noted that fresh tissues were much less sensitive to the toxic action of antitrypsin than were strain cultures, and concentrations as high as 3.0 mg per ml had no appreciable inhibitory effect. This absence of growth inhibition was also observed in cultures of fresh tissues carried in fibrinogen-thrombin clots.

Discussion. Digestion of the plasma coagulum by growing cells has presented a considerable problem since the earliest days of tissue culture. Thus, Losee and Ebeling,[7] working with human culture material, attempted to prevent liquefaction of the plasma by the addition of serum, agar and egg albumin. Although these experiments were unsuccessful, it was found that dilution of the plasma with an equal volume of Ringer's solution slowed down the rate of digestion sufficiently to allow short term experiments to be carried out. Carrel[14] reported that he was able to protect fibrin clots from digestion by the addition of a little serum, a small amount of sodium linoleate, or a suspension of egg yolk. Of the various sera that have been employed, horse serum has proved to be most suitable, although it has the undesirable property of retarding the rate of cell multiplication. In many laboratories, the proteolytic action of tissues cultivated in plasma is controlled by the addition of a second layer of plasma, on the day following preparation of the cultures. Subsequent layers of plasma are then added as they become necessary. This procedure takes considerable time and requires large amounts of plasma.

Some investigators[6,14] have attributed digestion of the plasma coagulum to proteolytic enzymes secreted by the cells during active growth. Others[15-17] have advanced the hypothesis that cells in tissue culture elaborate an activating substance that transforms an inactive precursor, profibrinolysin, present in the medium, into an active proteolytic enzyme, fibrinolysin, which then digests the clot. The present observations can be interpreted on the basis of either of these hypotheses.

The results reported in the present paper indicate that the incorporation of antitryptic agents in the culture medium offers promise of a solution to the troublesome problem of clot digestion. It is realized, of course, that the slight inhibition of growth caused by crude soybean antitrypsin presents a serious obstacle to its routine use. But further studies are now in progress with more highly purified preparations of the trypsin-inhibitor in an effort to eliminate the growth-retarding factor. It is also planned to extend the present investigation to a study of antitryptic agents derived from other sources.

Summary. Crude soybean trypsin-inhibitor has been found to prevent digestion of plasma and fibrinogen-thrombin clots by tissues growing *in vitro*. The presence of antitrypsin retards the normal coagulation of plasma but this inhibition can be overcome by the addition of thrombin. The growth of strain cultures of fibroblast-like cells is somewhat depressed by high concentrations of antitrypsin, but the inhibitory effect can be eliminated by repeated cultivation in antitrypsin. Fresh tissue explants are not as sensitive as strain cultures to this inhibitory action.

[14] Carrel, A., *J. Exp. Med.*, 1923, **38**, 407.

[15] Demuth, F., and von Riesen, I., *Biochem. Z.*, 1928, **203**, 22.

[16] Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.

[17] Goldhaber, P., Cornman, I., and Ormsbee, R. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 590.

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