Use of Antitryptic Agents in Tissue Culture. II. Egg-White Antitrypsin.*† (17901)

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An earlier communication(1) from this laboratory reported studies on the use of crude soybean trypsin-inhibitor as a means of preventing the digestion of plasma and fibrin clots that frequently occurs during the growth of cell colonies *in vitro*. This substance, though effective as an antiproteolytic agent, was found to inhibit the growth of the cultures and to prolong the coagulation time of the plasma. These results agreed closely with those obtained by Fischer(2), who employed crystalline soybean antitrypsin. Since the toxicity of the soybean factor appeared to limit its usefulness in tissue culture, an investigation was made of ovomucoid(3), the antitryptic substance in egg white.

Methods. Egg-white antitrypsin was prepared from freshly-laid hens' eggs by the trichloracetic acid and acetone method of Lineweaver and Murray(3). Other preparations were made by a salt-fractionation procedure(3) and by the ammonia and alcohol method of Balls and Swenson(4). In certain experiments, untreated egg yolk, untreated egg white and lyophilized egg white were employed. The activity of each preparation was determined by its ability to prevent the digestion of casein by crystalline trypsin, according to the method of Kunitz(5). These tests were carried out as described for the experiments with soybean antitrypsin(1). All solutions were sterilized by passage through UF fritted glass filters (Corning) and were stored in the refrigerator. Culture strains of fibroblast-like mesenchyme cells were derived from the leg muscle of 11-day chick embryos and were cultivated in D-3.5 flasks through at least 7 to 10 passages (weeks) before use. The culture medium was comprised of chicken plasma, a balanced salt mixture(6) and chick embryo extract. These ingredients, the manner in which they were combined, and the actual culture procedures have already been described(1). In all experiments, an additional layer of the plasma-saline-extract mixture was added to half of the control cultures to minimize digestion, and in certain experiments *all* cultures received the extra layer. This procedure, sometimes referred to as "patching," permitted culture growth without noticeable digestion, so that any growth-inhibiting effects of the antitrypsin could be detected.

Experimental. Egg-white antitrypsin, prepared by the trichloracetic acid and acetone method, was added to several series of sister cultures at final concentrations of 5.0, 2.5, 1.0, 0.5, 0.25 and 0.1 mg per ml. These cultures, together with their controls (without antitrypsin) were incubated at 38° and were observed each day for signs of digestion. It was found that all cultures containing egg-white antitrypsin completely digested the plasma coagulum within 3 days. It was also found that digestion occurred in the presence of antitrypsin even though, in some instances, the control cultures, with and without an additional plasma layer, did not digest the coagulum (Fig. 1-4). The presence of high concentrations of antitrypsin (5.0 to 2.5 mg per ml) caused a cloudiness in the clot, but this effect was not apparent at lower levels.

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2. Fischer, A., *Science*, 1949, v109, 611.

3. Lineweaver, H., and Murray, C. W., *J. Biol. Chem.*, 1947, v171, 565.

4. Balls, A. K., and Swenson, T. L., *J. Biol. Chem.*, 1934, v106, 409.

5. Kunitz, M., *J. Gen. Physiol.*, 1947, v30, 291.

6. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

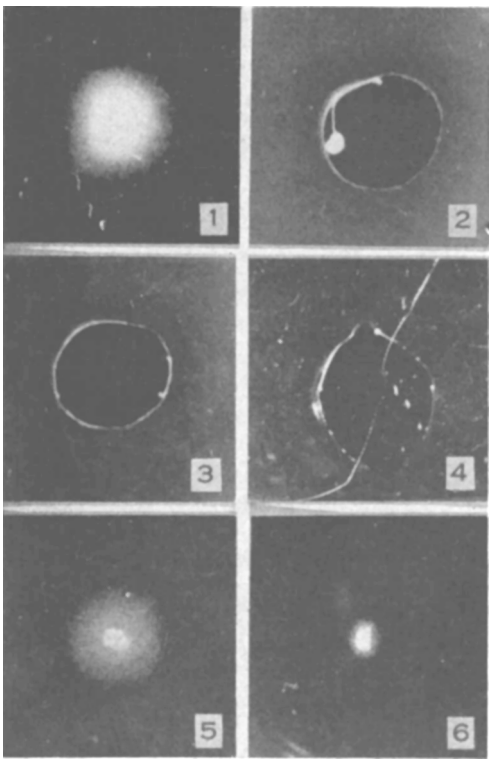

FIG. 1, 2, 3, and 4.
Seven-day sister cultures of 7th passage chick fibroblasts cultivated in a plasma coagulum in the absence of antitrypsin (Fig. 1), in the presence of 5.0 mg egg-white antitrypsin per ml (Fig. 2), 2.5 mg per ml (Fig. 3), and 1.0 mg per ml (Fig. 4). × 2.

FIG. 5 and 6.
Seven-day sister cultures of 16th passage chick fibroblasts cultivated in a plasma coagulum in the absence of antitrypsin (Fig. 5), and in presence of 0.5 mg egg-white antitrypsin per ml (Fig. 6). × 2.

Similar results were obtained with material prepared by salt fractionation and by the ammonia and alcohol procedure. It was concluded, therefore, that egg-white antitrypsin stimulated proteolytic activity instead of preventing it.

The presence of egg-white antitrypsin, even in low concentrations, was found to inhibit, very appreciably, the growth of the cultures. This inhibition was most apparent with antitrypsin prepared by the ammonia and alcohol method (Fig. 5 and 6) but was found to occur to a lesser degree with all antitryptic preparations tested. Comparative experiments were carried out in which fresh egg yolk, fresh egg white and lyophilized egg white were incorporated in the culture medium. The presence of small amounts of egg yolk (0.01 to 0.001 ml) was somewhat inhibitory to the growth of the cultures, whereas larger amounts suppressed growth almost completely. Both raw and lyophilized egg white were inhibitory to growth and did not protect the plasma clots from digestion.

None of the antitryptic preparations tested was observed to prolong the coagulation time of the plasma. In this respect egg-white antitrypsin differs from soybean antitrypsin (1).

In view of the failure of egg-white antitrypsin to prevent plasma digestion by tissues cultivated *in vitro*, test-tube experiments were made to study its effect upon the hydrolysis of casein and fibrin by crystalline trypsin. The activity of the various egg-white preparations was found to correspond closely with that reported by Lineweaver and Murray (3), and to represent roughly an 8-fold increase in potency over that of lyophilized egg white. The antitryptic activity was exerted equally well regardless of whether fibrin or casein was employed as the substrate. From these results, it was concluded that the preparations found to be ineffective in tissue cultures were fully potent in their ability to prevent the proteolytic action of crystalline trypsin. It was also observed that the digestion of casein and of fibrin was increased by the presence of very small amounts of egg-white antitrypsin. This effect was exerted by concentrations considerably below the levels found to be effective in preventing the action of the standard amount of trypsin (10 μg).

Discussion. Early tissue culture workers attempted to solve the troublesome problem of plasma digestion by the addition of various protective substances. Carrel and Ebeling (7) incorporated 0.25% egg-yolk suspension in their media; and Carrel (8) reported the use of serum, sodium linoleate and egg-yolk suspension to prevent clot lysis. These attempts were not particularly successful,

7. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, v37, 759.

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however, and complete digestion of the coagulum is now commonly avoided by the successive addition of fresh layers of plasma, as they become necessary. Since this procedure is laborious and requires large amounts of plasma, it was hoped that the problem might be solved by the use of antitryptic agents. The present experiments, involving more than 150 cultures, would seem to eliminate egg-white antitrypsin as a possible antiproteolytic agent for use in tissue cultures.

The failure of egg-white antitrypsin to prevent plasma digestion in tissue cultures is in marked contrast to its ability to prevent the proteolytic action of trypsin upon either casein or fibrin substrates. However, soybean trypsin-inhibitor has been found effective both in tissue culture and in test-tube experiments(1). These divergent results may possibly be attributed to a difference in the degree of specificity of the two antiproteolytic agents. Egg-white antitrypsin has been shown(9,10) to be almost entirely specific for trypsin, whereas soybean antitrypsin has been found to be a general inhibitor of proteolytic enzymes(10). The general antiproteolytic activity of the soybean trypsin-inhibitor might account for its interference with normal plasma coagulation, whereas egg-white antitrypsin, which is active only against trypsin, does not interfere.

Digestion of the plasma coagulum by cells cultivated *in vitro* has been attributed to the secretion of proteolytic enzymes(8,11) or to the elaboration of a substance that activates profibrinolysin to fibrinolysin(12,13). The present findings do not furnish evidence in support of either of these hypotheses, but they do render it unlikely that plasma digestion is caused by *trypsin.*

Summary. Egg-white antitrypsin not only fails to prevent but actually stimulates the digestion of plasma by cells cultivated *in vitro.* The presence of even low concentrations of antitrypsin inhibits tissue-culture growth but does not interfere with normal plasma coagulation. The antitrypsin preparations shown to be ineffective in tissue cultures strongly inhibit the proteolytic activity of crystalline trypsin in test-tube experiments.

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Antibacterial Activity of Hydrolyzed Red Blood Cells *in Vitro.** (17902)

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In our recent studies(1) the presence of an antibacterial factor in ticks was interpreted mainly as a biological phenomenon which may have bearing on the natural transmission of arthropod-borne infections. The higher antibacterial potency of blood engorged ticks as compared with unfed specimens was a significant indication of a possible growth inhibitory role of blood *per se.* Preliminary tests gave evidence that the tryptic digest of guinea pig blood exhibits a marked growth inhibition of *Bacillus subtilis*(1). This observation stimulated the present inquiry as to whether the blood of various animals may serve as a source of antibacterial substances. The possible relationship of the following antibacterial substances of animal origin have been considered in the present study: Flem-

* Aided by a grant of Lilly Research Laboratories.

1. Anigstein, L., Whitney, D. M. and Micks, D. W., *Tex. Rep. Biol. and Med.*, 1950, v8, 86.