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11. Part of this work was performed while I was a guest at the Institut für Mikrobiologie und Infektionskrankheiten der Tiere, Munich, Germany. Dr. A. Koestner provided the electron photomicrograph. Supported in part by grants FR05463 and 1F 10 NB 1802 from NIH.

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Antithrombin III: Protection against Death after Injection of Thromboplastin

Abstract. *Intravenous injection of autologous lipoprotein (thromboplastin) or thrombin produced a lethal, hemorrhagic syndrome in chicken embryos. The embryos could be protected from this fatal result by injection of antithrombin III, an alpha$_2$-globulin (molecular weight 60,000 to 80,000) purified from human, bovine, and guinea pig blood. Heparin also protected the embryos, but other inhibitors were less protective.*

It is well known among researchers in several branches of experimental biology that the intravenous administration of autologous cell homogenates or even of improperly washed cell suspensions may be fatal to the recipient animal. This fact may be viewed as an experimental model for the study of that part of homeostasis that deals with something as basic as the defense against the breakdown products of the individual's own cells.

Because thromboplastic material (of poorly defined lipoprotein nature) may be extracted from almost any kind of tissue and because the pathological findings in animals injected with toxic cell preparations are usually indicative of hemorrhagic disorders, it is natural to turn to experimental hematology for further elucidation. As early as 1947 Schneider (*1*) used the lethality that results from intravenous injection of mice as a bioassay of the thromboplastin content of homogenates of placenta and other tissues. Schneider (*1*) and Thomas (*2*) demonstrated clearly that the same toxic syndrome in mice could be circumvented by preincubating the thromboplastic material in vitro with normal mouse serum and concluded that the latter must contain a natural antithromboplastin; however, they did not succeed in defining this substance much further. Although later workers have investigated the changes in the blood level of various coagulation factors after injection of thromboplastic material, there is surprisingly little known about the nature of the inhibitor, or inhibitors, that hold in check the presumably never-ceasing flow of thromboplastins from decay of natural or pathological tissues (*3*). Our own work shows that a progressive antithrombin with the mobility of an alpha$_2$-globulin (in agarose) is one of these inhibitors. (The word "progressive" refers to the observed time-dependence of the inactivation of thrombin.)

We used chicken embryos as recipients of lipoproteins (LP), the thromboplastic material being prepared from allantoic fluid or the homogenized lungs from 17-day chicken embryos. The allantoic fluid was collected carefully in order to avoid contamination with blood or tissue fluid. Lipoproteins, partly purified by dialysis against phosphate-buffered saline followed by differential centrifugations (1,500 and 150,000*g*), contained 35 to 40 percent protein, 50 to 60 percent lipid, and 2 to 5 percent RNA.

The intravenous injection of LP from either of these sources produced a fatal hemorrhagic syndrome in chicken embryos (Fig. 1). The amount of LP required to produce the reaction was roughly proportional to the body weight of the 13-day embryos that were used in the present experiments; 12 μg of LP produced a fatal reaction in nearly all embryos. Death was ascertained by transillumination of the eggs and usually occurred within 1 to 4 hours after the injection. The following findings strongly suggest that the syndrome is caused by the thromboplastic effect of the injected LP. (i) Heparin could prevent death when administered before or simultaneously with the LP; (ii) there was a close correlation between different preparations with respect to their in vivo toxicity and their thromboplastic effect on chicken plasma in vitro; (iii) the injection of chicken and bovine thrombin produced similar lesions; and (iv) a marked thrombocytopenia developed before death.

The hemorrhagic syndrome could be prevented by preinjections of plasma or serum from chickens as well as by preincubation of LP with serum and plasma from various mammals. Chromatography of guinea pig serum or human plasma on diethylaminoethyl-cellulose (*4*) separated two fractions that had a protective effect in vivo. One of these had no anticoagulant effect in vitro,

Table 1. Comparison of in vivo protective effect of antithrombin III. Each dose was tested on eight to ten eggs. Controls (not shown) ensured that a lack of protection was not due to toxicity of the test substance. Lethality in 24 hours is recorded as follows: −, no significant difference from controls treated with lipoprotein; +, 25 to 50 percent of group protected; and ++, 50 to 100 percent protected; I.U., international unit.

Test substance	Dose (micrograms per egg)	Protection
Guinea pig antithrombin III	100	++
Guinea pig antithrombin III	25	+
Human antithrombin III		
From Copenhagen	35	++
From Oslo	22	++
Bovine antithrombin III	70	++
Human alpha$_2$-macroglobulin	660	−
Heparin* (1.0 I.U.)	7.1	++
Heparin (0.2 I.U.)	1.5	+
Lima bean trypsin inhibitor†	2000	++
	500	+
Ovomucoid trypsin inhibitor‡	2000	−
Pancreatic trypsin inhibitor‡	200	−
ϵ-Amino caproic acid (EACA)§	20,000	−

* Leo Pharmaceutical, Copenhagen. † Worthington Chemical. ‡ Sigma Chemical. § Kabi, Stockholm.

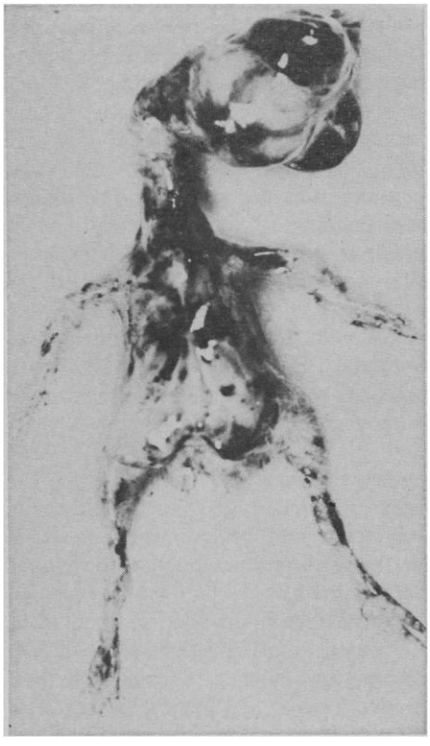

Fig. 1 (left). Hemorrhagic syndrome in 13-day chick embryo killed by intravenous injection of 0.1 ml of allantoic fluid.

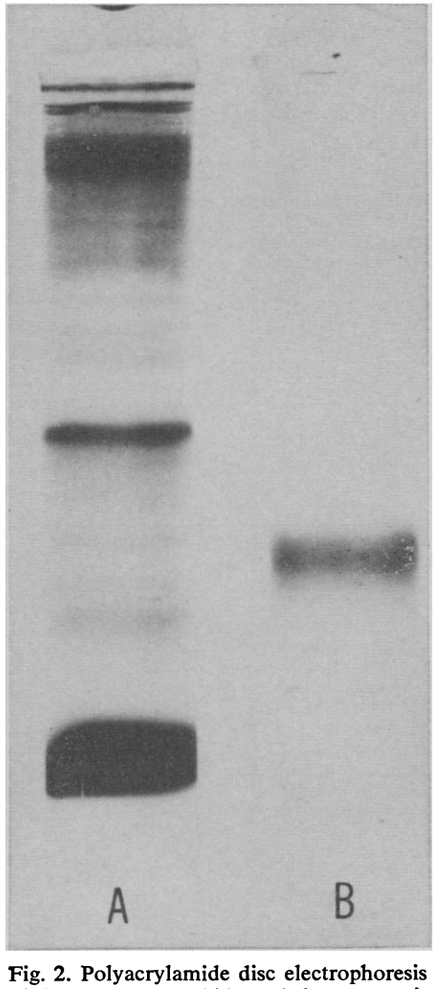

Fig. 2. Polyacrylamide disc electrophoresis of human serum (A) and human antithrombin III (B).

and attempts at further purification were unsuccessful. The other fraction inactivated thrombin progressively in vitro. These antithrombin fractions (guinea pig and human) were further purified by chromatography on carboxymethyl cellulose, preparative disc electrophoresis, and gel filtration. The final preparations were alpha$_2$-globulins with molecular weights around 70,000, as shown by gel filtration. The final human preparation was homogeneous (see Fig. 2), whereas the guinea pig preparation showed traces of two impurities in immuno- and disc electrophoresis. The molecular weight of the guinea pig protein, as judged by its behavior on Sephadex G-200, was in the range of 40,000 to 80,000. Analytical ultracentrifugation at a concentration of 6 mg/ml gave an $s_{20,w}$ of 3.4. Immunochemical analysis indicated the absence of the well-known protease inhibitors, alpha$_1$-antitrypsin and alpha$_2$-macroglobulin, in the human preparation.

The studies described up to this point were carried out by the group in Copenhagen. Recently, however, Abildgaard, in Oslo, reported the isolation of antithrombin III from bovine and human blood (*5*). Since this protein apparently is the main inhibitor of thrombin in plasma (*6*), we undertook joint studies on the antithrombins. Double diffusion in agar against rabbit antiserums demonstrated identity of the human preparations. Further, Abildgaard (*5*) showed that guinea pig antithrombin possessed heparin cofactor activity and was a weak inhibitor of trypsin, as were human and bovine antithrombin III.

The in vivo protection provided by our preparations and some other agents is summarized in Table 1; antithrombin III was protective in an amount present in 0.1 ml of human plasma. In comparison, 0.05 ml of human plasma or guinea pig serum offered a similar protective effect. Since the volume of plasma of the embryo was about 1 ml, it is reasonable to assume that the embryo contained very little endogenous inhibitors. Purified alpha$_2$-macroglobulin (*7*), another in vitro inhibitor of thrombin (*8, 9*), was ineffective in vivo in an amount representing 0.3 ml of human plasma. Some other protease inhibitors were less effective or not effective at all (Table 1).

Thromboplastin shows considerable species specificity and a human preparation (750 μg, Ortho Pharmaceuticals) was well tolerated by chicken embryos in vivo and was ineffective in vitro. In contrast, both chicken thrombin and bovine thrombin (1 NIH unit) produced lethal reactions that could be prevented by either heparin or antithrombin III. Since antithrombin III prepared from several mammalian species was protective, this inhibitor shows low species specificity.

Our in vivo test entails an overloading of the blood with thromboplastic material. In this sense, it may have features in common with the severe hemorrhagic disorders seen after major surgery, pathological deliveries, and in malignancy. If this is correct, treatment of the disorders with antithrombin III might be possible. Related to this question is the role of antithrombin III for homeostasis. A reduction of the antithrombin III level to about 50 percent of normal, as observed in a study of individuals in a family with a hereditary deficiency of antithrombin activity (*10*), is associated with a marked tendency to thrombosis. From this it may be inferred that antithrombin III plays an important role in man and is normally present in concentrations with a relatively small margin of safety.

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