Fibrinolysis VI. Activity of Aspergillin O (Mold Fibrinolysin) *in vitro*.* (27185)

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The preparation of Aspergillin O has been described(1,2). Some of its physico-chemical properties(2) and activity *in vivo* as a fibrinolytic agent in animals(3) and man(4) are being reported. The present study describes the effects on human plasma *in vitro* and other properties of the agent.

Materials. *Aspergillin O* used in these studies was supplied by the Connaught Laboratories, University of Toronto. Its percentage chemical composition was as follows: ash, 5; nitrogen, 7.68; hexoses, 45.3; hexosamine, 0.5. Its activity was 2,000 units/mg as described from this laboratory(1). The material was dissolved in borate buffer (pH 7.4) to final concentrations of 200, 100, 50, 25 and 12.5 γ/ml, *Blood,* collected from "normal" individuals into Silicone-coated test tubes using 1/10 volume of sodium citrate 3.8% as anticoagulant, was centrifuged at 1,200 rpm/4°C/10′ to obtain *platelet-rich plasma* and at 4,000/4°C/30′ to obtain *platelet*-poor plasma. To obtain *serum,* normal blood was allowed to clot, incubated at 37°C for 3 hours and finally centrifuged at 2,000 rpm/4°C/10′. Commercial bovine *fibrinogen* (Armour & Co.) was used to prepare fibrin plates for studies of the fibrinolytic activity of Aspergillin O. Other materials are described in the text.

Methods. The activity of Aspergillin O on the blood coagulation mechanism was studied with technics previously described, as follows: clotting time of recalcified plasma(5); partial thromboplastin time test(6); thrombin generation test(7); thromboplastin generation test(8); prothrombin time of plasma (5, using 1/10 dilution of plasma with saline); plasma prothrombin concentration(9); labile factor(10), stable factor(9) and Stuart factor(11) activity; plasma fibrinogen level (5); plasma antithrombin (III) activity (12).

Clotting time of recalcified plasma, plasma prothrombin time and concentration, activity of labile, stable and Stuart factors, plasma fibrinogen level were determined immediately and 60 minutes after mixing one volume of diluted Aspergillin O with 9 volumes of platelet-poor plasma at 37°C. In the partial thromboplastin time test, one volume of diluted Aspergillin O was added to 9 volumes of plasma (platelet-poor) in Silicone-coated test tube. This was shaken at room temperature for 5′ and 1/10 ml of the mixture was used as the plasma reagent in the test. In the thrombin generation test, one volume of diluted Aspergillin O was mixed with 9 volumes of platelet-rich plasma (300,000 platelets/cu mm). The test tube was incubated at 37°C for 5′, and 0.6 ml of the mixture was used as the plasma reagent. In the thromboplastin generation test, 0.2 ml of absorbed normal plasma, 0.2 ml of serum, 0.2 ml of cephalin complex (0.03% in saline), 0.1 ml of buffered saline and 0.1 ml of Aspergillin O solution were transferred rapidly to a test tube. 0.2 ml of 0.25 M $CaCl_2$ was added immediately and the test carried out by the original technic. To investigate the effect on native human thromboplastin, Aspergillin O was added 5′ after mixing the other reagents. The test was then carried out as usual. In the antithrombin III test, 75 γ of Aspergillin O in 0.5 ml volume were added to an equal vol-

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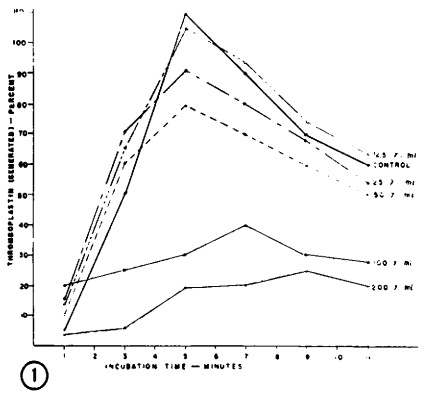

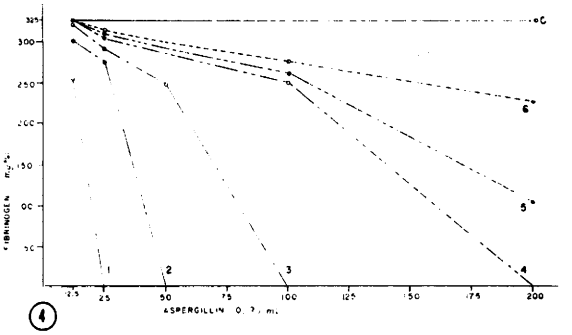

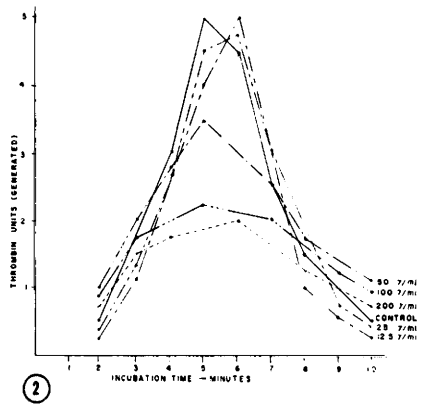

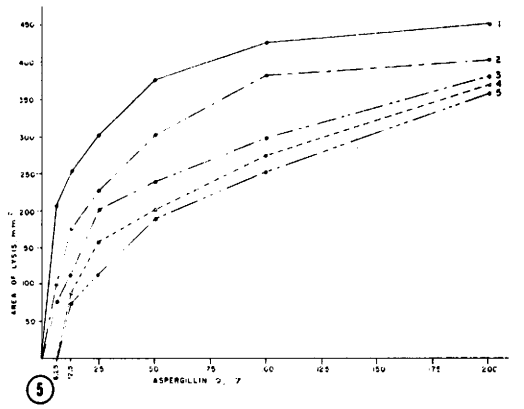

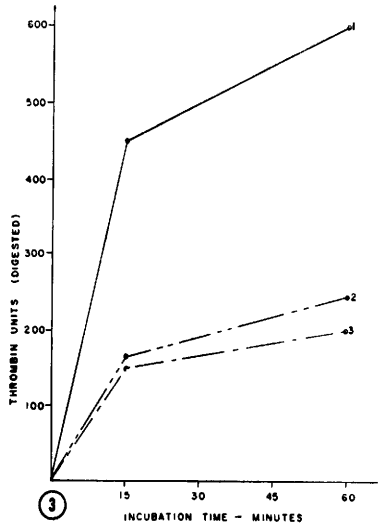

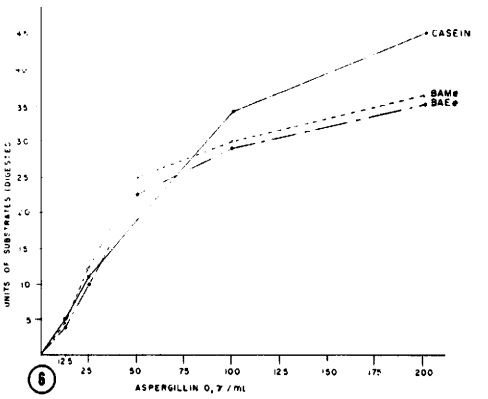

FIG. 1. Effect of Aspergillin O on generation of thromboplastin *in vitro* (see text). Avg results of 5 experiments for each study.

FIG. 2. Effect of Aspergillin O on generation of thrombin *in vitro* (see text). Avg results of 5 experiments for each study.

FIG. 3. Antithrombin activity of Aspergillin O and its inhibition by human serum (see text). Avg results of 5 experiments for each study. Curve 1: Mixture of thrombin and Asper-

ume of thrombin solution containing 2,000 NIH units/ml. The ability of the mixture to clot a standard solution of fibrinogen (300 mg%) was then tested immediately and 15′, 30′, 60′ later. To test the inhibitory activity of normal serum on the antithrombin activity of Aspergillin O, the same volume and concentration of Aspergillin O was incubated with an equal volume of serum at 37°C for 60′. The reagent was then added to equal volume of thrombin solution and the clotting ability of the mixture evaluated after 15′ and 60′ of additional incubation. A mixture of serum and of thrombin represented the control.

Fibrinolytic activity of Aspergillin O was investigated using heated and non-heated plates, with a modification of the technic of Astrup and Muellertz(13). 0.05 ml of each dilution of Aspergillin O was transferred onto the fibrin plate, which was then incubated at 37°C for 6 hours. Details of the method as well as calculations of results have been described(13). Caseinolytic activity was tested by the method of Norman(14) using a 2% solution of casein and esterase activity by the method of Brown(15) using benzoylarginine methyl ester (BAMe) and benzoylarginine ethyl ester (BAEe) as substrates. To investigate the inhibitory effect on the fibrinogenolytic activity of Aspergillin O, normal human serum was added to a 1% solution of bovine fibrinogen in veronal buffered saline (pH 7.3), to final volumes of 2.5, 5, 10 and 20%. Nine volumes of the fibrinogen-serum mixture were then incubated with one volume of Aspergillin O solution at 37°C for 60′. The fibrinogen level was then determined with the tyrosine method. To study the inhibition of fibrinolysis, 2.5, 5 and 10% volumes of serum were added to a solution of fibrinogen containing 350 mg%. The serum-fibrinogen mixture was then clotted by addition of thrombin (as for the preparation of standard fibrin plates). 0.5 ml of Aspergillin O at the desired solution was transferred onto the serum-fibrin plates. These were incubated at 37°C for 6 hours, when the area of lysis was calculated. Borate buffer instead of serum was used in the control plates.

Results. The effects of Aspergillin O on the coagulation mechanism *in vitro* seemed related to the concentration used. At concentrations higher than 50 γ/ml the material accelerated the clotting time of recalcified plasma (especially with short periods of incubation) and the partial thromboplastin time, while it inhibited the generation of thromboplastin (Fig. 1) and of thrombin (Fig. 2). The activity of prothrombin and of labile factor and concentration of prothrombin were depleted slightly. Activity of stable and Stuart factor was unaffected (Table I). The activity of thrombin was inhibited drastically, an effect reversed in part by addition of human serum (Fig. 3). At concentrations lower than 50 γ/ml Aspergillin O did not affect appreciably any of the clotting constants.

The fibrinogenolytic activity of Aspergillin O varied with its concentration, and with the medium used as source of fibrinogen. Purified fibrinogen preparations were digested actively by Aspergillin O even at minimum concentrations, an effect inhibited by human serum (Fig. 4). Platelet-poor plasma was also used

gillin O. Curve 2: Mixture of thrombin, Aspergillin O and human serum. Curve 3: Mixture of thrombin and serum.

FIG. 4. Fibrinogenolytic activity of Aspergillin O and its inhibition by human serum. *Line C* is a control study (see text). *Curves 1, 2, 3, 4* and *5* represent results with mixtures containing diluted solution of fibrinogen, Aspergillin O at standard concentration and increasing volumes of human serum (0, 2.5, 5, 10, 20% volume respectively). When platelet-poor plasma represented the course of an optimum amount of fibrinogen as well as of inhibitory agents, fibrinogenolytic activity of Aspergillin O was minimum *(curve 6)*. Avg results of 3 experiments for each study. Figure shows the increasing inhibition of fibrinogenolytic activity of Aspergillin O by added serum and maximum inhibition by human plasma.

FIG. 5. Fibrinolytic activity of Aspergillin O and its inhibition by human serum. Fibrin plates were heated *(curve 1)* or non-heated *(curve 2)*. Other heated plates contained human serum in 2.5% *(curve 3)*, 5% *(curve 4)* and 10% volume *(curve 5)*. Addition of increasing volumes of serum resulted in progressively greater inhibition of fibrinolytic activity of Aspergillin O. Avg results of 3 experiments for each study.

FIG. 6. Caseinolytic and esterase activity of Aspergillin O. Casein, benzoylarginine methyl ester (BAMe) and benzoylarginine ethyl ester (BAEe) were used as substrates (see text). Avg results of 3 experiments with each substrate.

TABLE I. Effect of Aspergillin O at Various Concentrations on Plasma Coagulation Factors *In Vitro*.*

Conc. of Aspergillin O, γ/ml	Clotting time of recalcified plasma, sec.† 0′	60′	Prothrombin time of plasma (1/10 dil.), sec.	Prothrombin conc., %	Activity— Labile factor	Stable factor	Stuart factor (%)	Partial thromboplastin time, sec.
0‡	180	190	26	100	90	100	75	400
12.5	180	200	30	90	80	110	80	210
25	180	205	30	85	75	110	80	160
50	144	210	30	85	70	110	80	135
100	140	215	30	85	65	100	75	95
200	110	185	40	80	60	80	70	95

* Avg results of 5 experiments, using normal human platelet-poor plasma (unless indicated in the text). Values given are those at the end of 1 hr on incubation of plasma with Aspergillin O.

† Under column 0′ are values taken immediately after mixing of plasma and Aspergillin O.

‡ This tube contained borate buffer instead of Aspergillin O and represented the control.

to supply an optimum concentration of fibrinogen and of inhibitors of Aspergillin O. The activity of the material was minimum under this experimental condition.

The fibrinolytic activity of Aspergillin O increased in proportion to its concentration. The agent does not activate the fibrinolytic system and is inhibited by components of plasma and serum. Accordingly, its fibrinolytic activity was higher on the heated than on the non-heated fibrin plates and was inhibited again by addition of serum to the fibrin plates (Fig. 5). Finally, Aspergillin O also exhibited strong caseinolytic and esterase activity (Fig. 6), in direct relation to its concentration.

Discussion and conclusions. At adequate concentrations, Aspergillin O has significant fibrinolytic, caseinolytic and esterase activity with only negligible effects on the mechanism of blood coagulation. The fibrinolytic activity is detectable clearly even in the presence of plasma or serum, which inhibit strongly the activity of Aspergillin O. These conclusions correlate well with findings *in vivo*(4), where, with maximum concentrations of 7 to 10 γ/ml of blood, Aspergillin O induces evidence of clot lysis without untoward effects on the coagulation mechanism.

Summary. 1. Aspergillin O is a material obtained from cultures of Aspergillus oryzae B-1273 which, at high concentrations and *in vitro,* depletes slightly the activity of prothrombin and labile factor, accelerates slightly the clotting time of recalcified plasma and the partial thromboplastin time. At lower concentrations, it does not affect appreciably the activity or concentration of various factors or constants of the blood clotting process, while still exhibiting marked proteolytic, esterase and fibrinolytic activity. 2. Its activity is inhibited by addition of plasma or serum.

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