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Differentiation of Plasma Antithrombin Activities.* (20829)

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It has been suggested that the antithrombin activity of plasma can be divided into 4 effects, for which the names antithrombin I, II, III and IV were proposed(1). Antithrombin I refers to the adsorption of thrombin on fibrin. Antithrombin II is the plasma co-factor of heparin, which acts with heparin to interfere with the action of thrombin on fibrinogen. The term antithrombin III applies to the capacity of plasma to inactivate thrombin. This occurs independently of heparin. Antithrombin IV is the action of plasma which inactivates thrombin as it is being formed from prothrombin. This action does not destroy preformed, purified thrombin. This last antithrombin activity has been demonstrated only in ether treated plasma which is free of antithrombin III.

Although it has become possible to categorize plasma antithrombin activity into 4 distinct actions, it has not been established whether or not these effects represent four distinct chemical entities. In this paper information is presented which may offer some insight into this problem. It has also been of interest to consider the plasma factor which acts as a co-factor of heparin to inhibit the activation of prothrombin(2), and which is referred to as the '39 factor. This effect was discovered in 1939, and has not

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received further attention since that time.

Methods. In assaying for antithrombin II, 0.3 ml of bovine plasma or plasma fraction, which had been heated at 56°C for 3 minutes to remove fibrinogen, was added to 0.2 ml thrombin reaction mixture(3) and 0.1 ml of either saline or heparin (liquaemin-Roche-Organon, Inc., diluted 1/100 with saline). Then 0.3 ml of this mixture was added to 0.1 ml purified fibrinogen(4), and 0.1 ml purified thrombin(5) was added, and clotting times were noted. The concentration of the thrombin solution was 100 units/ml. A measure of the activity of antithrombin II could thus be obtained on the basis of the difference between the clotting times with and without heparin. Antithrombin III was assayed quantitatively by the method of Seegers, *et al.*(6). Antithrombin IV was determined on bovine plasma by the thrombin titer method described by Seegers, Johnson and Fell (1). In order to test fractions they were added to plasma so that the antithrombin IV activity could be compared with the plasma to which there was no addition. Before adding such fractions they were treated with $BaCO_3$ to remove prothrombin and with ether to remove antithrombin III. In assaying '39 factor, a prothrombin product(7) containing Ac-globulin was mixed with thromboplastin, calcium, and plasma or plasma fraction. Heparin, diluted 1/30 with saline, was also added. Thrombin concentrations were plotted against prothrombin activation time, and the '39 factor was estimated on the basis of the inhibition of thrombin formation with heparin, as compared with the formation of thrombin in an identical reaction mixture to which no heparin was added. Ether treatment for the removal of antithrombin III was carried out as previously described(1).

Results. The effects of various procedures on the antithrombin reactions are summarized in the table. Although an attempt was made to do exact quantitative studies, we have limited ourselves to qualitative statements, since the experimental error in some of our procedures may have been fairly large, and because quantitative standards for antithrombins II and IV, and the '39 factor have not as yet been established.

The three antithrombins and the '39 factor all resisted heating at 60°C for 3 minutes, but were largely or entirely inactivated by heating at 70°C. When plasma was dialysed against cold water to a specific resistance of 2500 ohms, all 4 factors were found in the water soluble fraction, but not with the water insoluble globulins. The factors are not adsorbed on $BaCO_3$. Crystalized bovine plasma albumin, a commercial product of Armour and Co., was assayed and found to contain none of the 4 factors.

It was reported previously that antithrombin III is removed from plasma or serum by treatment with ether, while antithrombin IV activity remains(1). This observation has been confirmed, and antithrombin II and '39 factor assays showed that these were also present in ether-treated plasma. It is of interest that antithrombin III activity could not be detected in the ether extract of plasma, or in a combination of such an extract with ether treated plasma. Therefore, while ether treatment is a valuable distinguishing feature, it does not necessarily establish a complete separation of antithrombin III from the other factors, since it is not thus far possible to decide whether antithrombin III is removed by ether, or simply modified and left in the plasma.

In the ammonium sulfate fractionations fresh bovine plasma was diluted with an equal volume of distilled water, fractionated at 50% and 70% of saturation, dialysed against water to a specific resistance of 1000 ohms, and lyophilized. The fractionation work was done at low temperatures. Samples from the fractions were then reconstituted to their original concentrations in physiologic saline.

The results of these experiments are summarized in the table. Antithrombin II was found in both the 0-50% and the 50-70% fractions, and in higher concentration in the latter fraction. Antithrombin III was found only in the 0-50% fraction, and in a concentration considerably lower than its concentration in unfractionated plasma. Antithrombin IV activity was not detected to any significant degree in any of the fractions, although there appeared to be a trace in the

TABLE I. Effects of Various Procedures on Antithrombins II, III and IV, and on the '39 Factor.

Procedures	Antithrombin II	Antithrombin III	Antithrombin IV	'39 factor
A. Heating plasma				
1. 60° for 3 min.	P*	P	P	P
2. 70° " 5 "	A	A	A	A
B. Dialysis of plasma against H_2O				
1. H_2O insol.	A	A	A	A
2. H_2O sol.	P	P	P	P
C. $BaCO_3$ treatment	N. ad.	N. ad.	N. ad.	N. ad.
D. Ether "	R	Re	R	R
E. $(NH_4)_2SO_4$ fractionation				
1. 0-50%	T	P	A	A
2. 50-70%	P	A	T	P
3. Supernatant from 70%	A	A	A	A

* P = Present; A = Absent; N. ad. = Not adsorbed; T = Trace; R = Remains; Re = Removed.

50-70% fraction. The '39 factor was found only in the 50-70% fraction. While our experiments did not demonstrate this factor in other fractions, this does not conclusively rule out the possibility that it was not present in these fractions, since other experiments indicated that a high concentration of accelerators of prothrombin conversion could overcome the inhibiting effect of heparin and its co-factor. We made a number of variations in our assays to take these variables into consideration.

clusive, it seems quite possible that the '39 factor is identical with antithrombin II. This would then mean that heparin acts with the one co-factor at two distinct points in the blood coagulation mechanisms; namely at the prothrombin activation stage and at the fibrinogen activation stage.

It is interesting to note the report of Gorter and Nanninga(9) that heparin can act as an antithrombin in the absence of co-factor. They even consider the possibility that the co-factor does not exist. Perhaps their results

that heparin requires a co-factor for full activity.

We are not yet able to relate antithrombin IV to the other antithrombins. It is possible that this activity represents another separate entity. It is also possible that more than one substance is involved, and that these substances are known by other manifestations in the complex interactions of the blood clotting mechanisms.

Summary. The antithrombin capacity of plasma can be differentiated into 4 main effects. Antithrombin I is the adsorption of thrombin on fibrin. Antithrombin II is the co-factor of heparin. Antithrombin III neutralizes thrombin activity even in the absence of heparin, while antithrombin IV destroys thrombin only while it is being formed from prothrombin. Some of the properties of antithrombins II, III and IV have been investigated, together with those of the factor which acts with heparin to inhibit the activation of prothrombin, and which is referred to as the '39 factor. The 4 activities have the common property of resistance to heating at 60°C for three minutes, but are destroyed by heating at 70°C. They are not adsorbed on $BaCO_3$. They are water soluble. None of these activities is found in a commercial crystalized bovine plasma albumin. Antithrombin III activity is the only one removed by ether treatment of plasma. In ammonium sulfate fractionation antithrombin II is found predominantly in the 50-70% fraction, while antithrombin III is found only in the 0-50% fraction. It is believed that these 2 factors are two distinct entities. The relation of antithrombin IV to these 2 is not yet clear. It is possible that the '39 factor and antithrombin II are identical.

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Monolayer Tissue Cultures I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells.*† (20830)

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Recent reports by Dulbecco(1,2) on the use of monolayer tissue cultures for the study of certain animal viruses has made possible the application, in studies of animal viruses, of quantitative technics similar to those used with bacterial viruses. Dulbecco has demonstrated production of plaques of degenerated cells by Western equine encephalitis virus in monolayer chicken-fibroblast cultures(1) and similarly in monolayer monkey kidney cultures, plaques have been produced by the Brunhilde strain of Type 1 poliomyelitis virus(2).‡

Methods for treatment of kidney tissue fragments with trypsin to reduce the time required for cell outgrowth in plasma clot cul-

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