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The Influence of Calcium on the Binding of Antithrombin by Antithrombin Inhibitor.* (31101)

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In our initial studies(1) it was shown that plasma antithrombin is normally held inactive by combination with an inhibitor. During clotting, a platelet factor is made available which binds the inhibitor, thereby releasing the antithrombin.

In the present studies, it was noted that the inhibitor requires calcium to bind antithrombin.

Materials and methods. 1. All materials and methods, other than those noted below, were previously described(1).

2. Calcium free plasma was prepared from intact, platelet free, nonanticoagulated plasma. The plasma was treated with 1 mg/ml disodium ethylenedinitrilotetraacetate, dihydrate (EDTA) and dialyzed at 5C against 3 changes of 0.9% NaCl. It was centrifuged clear and stored in plastic vials at —20C. These samples were stable for at least 3 weeks.

3. Calcium free human thrombin was prepared from thrombin(1) which was similarly treated with EDTA. It was dialyzed against 3 changes of veronal buffer and concentrated against polyvinylpyrrolidone in veronal buffer. The thrombin activity of the preparations was unchanged by the processing. Two ml volumes were stored in plastic vials at —20C. Preparations were exhausted in 2-3 weeks without change in potency.

4. All preparations were verified free of calcium by the method of Copp(2).

5. A standard thrombin activity curve was prepared using a sample of thrombin supplied by the Division of Biologics Standards, N.I.H., Bethesda, Md.

6. The method of study was described(1). In brief, it consisted of an incubation mixture composed of the reactants mentioned in the text. At stated intervals, an aliquot of the incubation mixture was added to a fibrinogen solution and the thrombin clotting time measured.

Results. Normal plasma, not deprived of its calcium, had minimal antithrombic activity (Fig. 1, curve 1A)(1). Similarly, calcium free plasma to which physiologic (M/360) calcium chloride was added, had minimal activity (Fig. 1, curve 1). The process of removal and readdition of the calcium chloride did not affect the activity.

In contrast, calcium free plasma had maximal antithrombic activity (Fig. 1, curve 2). With the addition of calcium to this plasma, suppression of antithrombic activity occurred, which varied inversely as the calcium concentration (Fig. 1, Fig. 3, curve 1). It is evi-

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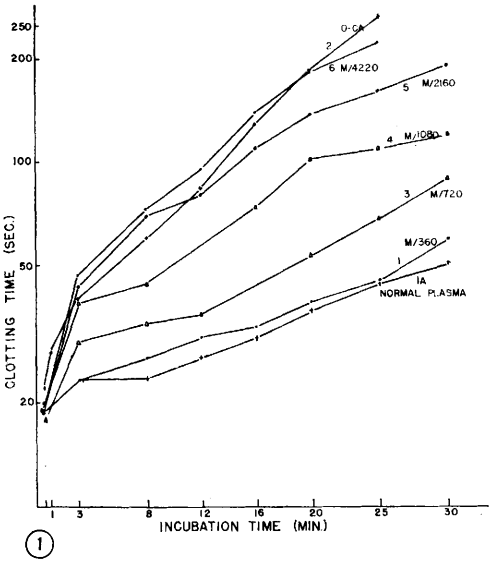

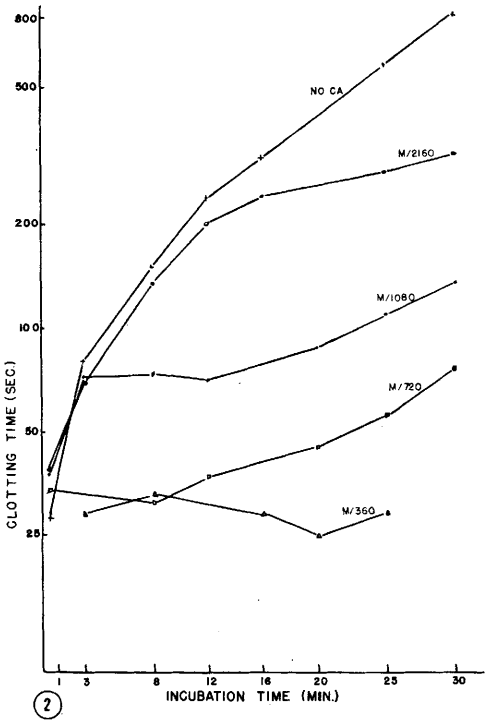

FIG. 1. Influence of calcium on antithrombic activity of plasma. Incubation mixtures consisted of: Plasma, calcium free, .05 ml; $CaCl_2$ to give molarity indicated on curve, various ml; Thrombin, calcium free, .1 ml; Veronal buffer to .9 ml. Curve 1A; normal plasma, non anticoagulated, without added calcium chloride. The reactions were started with addition of the thrombin.

FIG. 2. Influence of calcium on isolated antithrombin and inhibitor. Incubation mixtures consisted of: Isolated antithrombin, .15 ml; Isolated inhibitor, .4 ml; $CaCl_2$ to give molarity indicated on curve, various ml; Thrombin, calcium free, .1 ml; Veronal buffer, to .9 ml. The reactions were started with addition of the thrombin.

dent, therefore, that calcium quantitatively suppressed antithrombic activity. These calcium concentrations in the incubation mixture did not affect substrate clotting.

Concerning the modus operandi of calcium it has been shown that plasma contains both an antithrombin and an inhibitor of antithrombin(1). It was also shown that calcium-containing plasma is capable of suppressing antithrombin because of its content of inhibitor. In the absence of inhibitor, calcium had no influence upon antithrombin as indicated in the following experiment. Plasma was rendered deficient in inhibitor activity by heating for 15 minutes at 56-57C(1). This plasma, containing its full complement of calcium, exhibited maximal antithrombic activity. Similarly, the antithrombic activity of heat treated, calcium free plasma was unaffected by the addition of calcium.

Identical results to those with plasma were obtained with mixtures of isolated antithrombin and inhibitor. With a constant mixture of antithrombin and inhibitor, the antithrombic activity varied inversely with the calcium concentration (Fig. 2).

The effect of calcium upon the antithrombic activity of isolated inhibitor plus antithrombin was quantitatively identical with that of calcium upon plasma antithrombic activity. In Fig. 3, it is noted that the curves of antithrombic activity obtained by varying the concentration of calcium in plasma (curve 1) and by varying the concentration of isolated inhibitor in the presence of constant antithrombin and calcium (curve 3) were identical with that of isolated antithrombin (curve 2). The identity of these curves suggests that the effect of the calcium was to vary the effective level of plasma inhibitor, which resulted in varying antithrombic activity.

In the absence of inhibitor, calcium did not affect the antithrombic activity of the isolated antithrombin. Hence, calcium is neither essential for, nor inhibitory of, the action of antithrombin upon thrombin.

Other divalent cations had little or no cal-

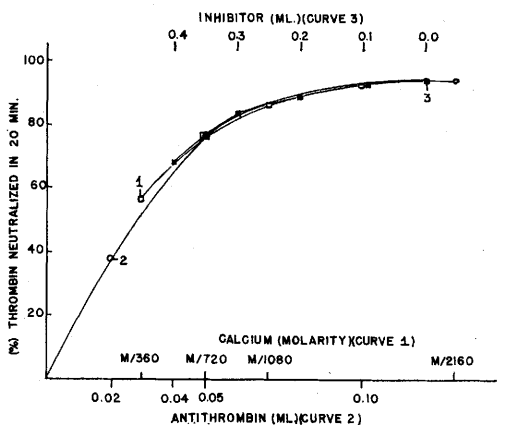

FIG. 3. Relationship of calcium concentration to antithrombin. Incubation mixtures consisted of: *Curve 1*: Plasma, calcium free .05 ml; $CaCl_2$ to give final concentration of 0-M/360 various ml; Thrombin, calcium free, .1 ml; Veronal buffer to .9 ml. *Curve 2*: Isolated antithrombin, 0-0.1 ml; Thrombin, calcium free, .1 ml; Veronal buffer, to .9 ml. *Curve 3*: Isolated antithrombin, 0.1 ml; Isolated inhibitor, 0-0.4 ml; Thrombin, calcium free, .1 ml $CaCl_2$ to give final concentration of M/360, .1 ml; Veronal buffer, to .9 ml. The reactions were started with addition of the thrombin.

cium substitutive effect. The chloride salts of the divalent cations Mg, Mn, Zn and Cu and of trivalent Fe were added to the calcium free plasma system in twice their physiologic concentrations. Only very slight inhibitor activity was detectable with each ion. The lack of effect of these various chloride salts suggests that the activation of the inhibitor is a specific function of calcium.

Discussion. These data indicate that while calcium does not influence the binding of thrombin by antithrombin, it is essential for the reaction between antithrombin and inhibitor. The schema proposed in the previous study(1) must now be modified to include calcium.

$$\text{Antithrombin} \leftrightharpoons \overset{\ulcorner \overset{+}{Ca} \urcorner}{\text{Inhibitor}} \rightarrow \text{Platelet factor}$$

In the resting state, antithrombin is bound by inhibitor in the presence of calcium, the divalent calcium perhaps acting as a bridge. When platelet disintegration furnishes platelet factor, it binds inhibitor releasing antithrombin. Whether calcium is required for the reaction between inhibitor and platelet factor is, for the moment, immaterial, since without calcium there is no binding of antithrombin by inhibitor, and consequently no platelet factor effect. Furthermore, it appears that calcium enters quantitatively into the reaction between inhibitor and antithrombin. The combined effect of calcium and platelets is exactly as anticipated from theory.

Summary and conclusions. 1. The reaction between antithrombin and inhibitor has a quantitative requirement for calcium. 2. Calcium is not the inhibitor of antithrombin. 3. Calcium is not required for neutralization of thrombin by antithrombin.

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Isolation and Characterization of a Bacterial Inhibitor from Human Throat Washings.* (31102)

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The antibacterial and virucidal activity of stimulated and unstimulated saliva in the human mouth has been confirmed by a number of investigators(1). The nature of the salival components that inhibit bacteria such as *Escherichia coli, Serratia marcescens,* and *Hae-*

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