PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 497th Meeting of the Society was held at the Queen's University, Belfast, on Thursday and Friday 4 and 5 September 1969, when the following papers were presented:

COLLOQUIUM ON 'RECENT ADVANCES IN THE STUDY OF SOME LESS FAMILIAR PROTEOLYTIC ENZYMES'

Enzymology and Blood Coagulation

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Much of the recent advances in the ideas on blood coagulation have been stimulated by the proposal of the Cascade (Macfarlane, 1964) and the Waterfall (Davis & Ratnoff, 1964) hypotheses of blood coagulation. In both these propositions the mechanism of blood coagulation is viewed as a sequence of zymogen activations, in which the enzyme produced in one stage catalyses the activation of the zymogen in the following stage.

Macfarlane (1964) further pointed out that such a sequence of interactions would serve as a biological amplifier. The idea of the amplifier may also provide a basis for explaining the evolution of a large number of clotting factors. The greater the number of stages there are in the amplifier, the more effective will be the response. One of the most attractive aspects of the sequential hypothesis is that it can be tested experimentally and the results of some of these experiments will now be discussed.

The last zymogen transformation in the clotting sequence is the conversion of prothrombin (Factor II) into thrombin (Factor IIa), a reaction that was first postulated by Morawitz (1905). In the sequential hypothesis it was suggested that prothrombin was converted by an enzyme, Factor Va. However, Papahadjopoulos & Hanahan (1964), Barton, Jackson & Hanahan (1967), Jobin & Esnouf (1967) and Hemker, Esnouf, Hemker, Swart & Macfarlane (1967), using purified reagents, synthetic substrates and from kinetic studies, have come to the conclusion that Factor Va is not formed, and that the enzyme responsible for prothrombin conversion is Factor Xa. Factor V seems to act in a novel way in this reaction, in that it accelerates the rate of thrombin formation only in the presence of Factor Xa.

Factor Xa has recently been shown to be a protease containing a reactive serine (Leveson & Esnouf, 1969), as does thrombin (Gladner & Laki, 1958). Factor Xa in the absence of Factor V will slowly convert prothrombin into thrombin (Milstone 1964; Jobin & Esnouf, 1967). It will also activate

chymotrypsinogen and it possesses slight caseinolytic activity (Milstone, Oulianoff & Milstone, 1963). Factor Xa also has slight esterase activity on synthetic substrates such as toluene-*p*-sulphonyl-arginine methyl ester, for which, compared with thrombin, it has a low affinity (Esnouf & Williams, 1962; Jackson & Hanahan, 1968).

The addition of Factor V and metal ions to mixtures of Factor Xa and synthetic substrates does not alter the rate at which the substrates are hydrolysed. However, in mixtures in which prothrombin is the substrate, the rate of thrombin formation is increased at least 1000-fold.

Factor Xa is derived from its parent zymogen by agents other than those of the intrinsic or extrinsic coagulation mechanisms. These include trypsin (Hanahan & Papahadjopoulos, 1965), a specific fraction of Russell's-viper venom (Williams & Esnouf, 1962) and 25% sodium citrate (Lechner & Deutsch, 1965). There is no evidence available as yet that suggests that these different agents give rise to the same molecule, even though the biological activity of Factor Xa is the same, however produced.

At the present time very little is known of the chemistry of the extrinsic clotting system, and such evidence as there is implies that the extrinsic Factor X activator consists of a complex of Factors III and VII and phospholipid, which will convert Factor X into Factor Xa.

The intrinsic clotting system refers to the generation of Factor X activator as the result of the exposure of plasma to a foreign surface. Both Hemker & Khan (1967) and Barton (1967), as the result of preliminary experiments, have suggested that Factor VIII behaves similarly to Factor V and that Factor IXa is the enzyme that converts Factor X. This idea would be more attractive if it could be shown that Factor IXa possessed enzymic activity.

Factor IX, on the sequential hypothesis, is activated by Factor XIa, which in turn is derived from Factor XI. Very little is known about this stage of the clotting process except that Factor XI undoubtedly exists; its activation and subsequent interactions are a matter of conjecture.

The first factor in the sequence is Factor XII,

which is converted into Factor XIIa on exposure to a foreign surface, has been purified in the activated form by Shoenmakers, Matze, Haanen & Zilliken (1965), who have found that it has estereolytic activity that, like its coagulant activity, is inhibited by di-isopropyl phosphorofluoridate.

Thus at the present time it seems that there are two types of coagulation factors: those that are zymogens and become converted into enzymes, and those factors that potentiate the activity of the enzymic factors.

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The Chemistry of the Activation of Prothrombin

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Bovine prothrombin prepared from oxalated plasma by precipitation of euglobulins at low ionic strength (Mellanby, 1930) followed by adsorption on magnesium hydroxide and elution with carbon dioxide (Fuchs, 1930), ammonium sulphate fractionation, isoelectric fractionation (Seegers, 1952) and equilibrium chromatography on cation-exchange resin (Amberlite IRC-50) (Miller, 1958) appears to be homogeneous during ultracentrifugation (Lamy & Waugh, 1958) and

has a molecular weight about 68 000. One mole of *N*-terminal alanine/mole (Magnusson, 1958; Miller, 1958) and about 11.5% carbohydrate, namely 4.3% sialic acid, 5.4% neutral sugar (galactose and mannose in a 1:1 ratio) and 1.8% glucosamine (Magnusson, 1965b), have been found. Neuraminidase degradation (Schwick & Schultze, 1959; Tishkoff, Pechet & Alexander, 1960) shows that sialic acid is terminal. Partial acid hydrolysis (Magnusson, 1965b) indicates that the oligosaccharide sequences are sialic acid-galactose-mannose-glucosamine-peptide. Autocatalytic activation in 25% sodium citrate (Seegers, 1949) and activation in the presence of thromboplastin, calcium chloride and Factor V have been studied by *N*-terminal amino acid analysis (Magnusson, 1958, 1965a) and both found to be proteolytic in nature. In both types of activation mixture 2 moles of *N*-terminal isoleucine/mole of *N*-terminal alanine were found at the peak of thrombin activity. Carboxypeptidase A and B degradation released only arginine and lysine from citrate-activation fragments, indicating that the activating enzyme has trypsin-like specificity (Magnusson & Steele, 1965). Miller & van Vunakis (1956) showed that di-isopropyl phosphorofluoridate inhibits thrombin. When they attempted citrate activation of prothrombin in the presence of di-isopropyl phosphorofluoridate no thrombin was formed and no prothrombin consumed. They also found that a mixture of thromboplastin, calcium chloride and dilute serum could be preincubated with di-isopropyl phosphorofluoridate without losing its prothrombin-activating capacity. When activation of prothrombin with thromboplastin, Factor V and calcium chloride was attempted in the presence of di-isopropyl phosphorofluoridate (Magnusson, 1965a) no thrombin was formed and no new *N*-terminal residues appeared. It is thus clear that both types of activation depend on a serine proteinase with trypsin-like specificity that is present in an inactive form in this type of prothrombin preparation. It seems unlikely at present that this enzyme is thrombin. A second enzyme activity is formed during citrate activation (Kowarzyk & Marciniak, 1961). It has esterase activity towards α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester but no thrombin activity and it can accelerate prothrombin activation. The stoichiometric relation of this activity to prothrombin is not clear. If, on the one hand, it turns out to represent only a very small amount of protein relative to the amount of thrombin it could either represent impurity in the prothrombin preparation, which seems unlikely, or it could be the product of a small proportion of prothrombin molecules, activated in an alternative way to that which produces thrombin. In the latter case one would expect it to contain the same active-