THE MODE OF ACTION OF THE LAKI-LORAND FACTOR IN THE CLOTTING OF FIBRINOGEN.

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The thrombin-induced clotting of fibrinogen does not lead to hemostasis (Duckert et al, 1960). The fibrin molecules in the clot are connected only with hydrogen bonds, and are readily dispersed in concentrated urea solutions (Laki and Lorand 1948; Laki and Chandrasekhar, 1963).

It was discovered by Laki and Lorand (1948) that the intervention of a plasma component (called: Laki-Lorand Factor = LLF, Fibrin Stabilizing Factor = FSF, Fibrinase) in the presence of Ca-ions was needed to render the clot insoluble in urea solutions. The plasma component introduced bonds between the fibrin molecules so that urea could not disperse the clot. A few years ago, Loewy et al (1961) succeeded in purifying this plasma component and showed that it was an enzyme. The purpose of this communication is to show that it is now possible to describe the mechanism by which the primary bonds are introduced between the fibrin molecules.

The release of carbohydrate during clotting. Fibrinogen contains about 3% carbohydrate material, consisting of glucosamine, mannose, galactose and sialic acid residues (Blomäck, 1958). These sugar residues probably form 7 oligosaccharide chains which on the

average contain 2 acetyl-glucosamine, 2 mannose, 1 galactose and 1 sialic acid residues.

After digestion with pronase, 80% of the carbohydrate content of fibrinogen could be isolated in the form of 3 different glycopeptides having a molecular weight of about 2300 (Mester et al, 1963 a, b). About half of these fibrino-glycopeptides are amino acids. Since the total sugar content in these peptides represent a molecular weight of about 6,900 (80% of the total sugar in fibrinogen) and since the carbohydrate content in the glycopeptides is about 1,150, the implication is that the 3 different glycopeptides represent 6 oligosaccharide chains that occur in 3 pairs. However, at this stage of our knowledge, we can not exclude the possibility that the 3 different glycopeptides are still not homogenous and we may be dealing with a mixture of glycopeptides containing oligosaccharide chains of varying length.

The peptide portion of these glycopeptides is about 10 amino acid residues long. Among these amino acids, aspartic-acid which was found common to all three could be implicated to carry the carbohydrate residues. Since the glycopeptides on hydrolysis yielded more ammonia than expected, it is quite likely that the sugar residue is bound to asparagine rather than to aspartic acid [1]. This is a situation similar to the binding of carbohydrate in ovomucoid (Montgomery and Wu, 1963; Marks et al, 1963), (Fig. 1).

It was first reported by Laki (1951) that bovine fibrin contained about 20% less carbohydrate than fibrinogen. This implied that in the clotting process about one fifth of the carbohydrate was released. These observations were confirmed by Bagdy and Szára (1955) and by Blombäck (1958). We have re-

[1] The simultaneous release of carbohydrate and ammonia as shown in this paper further supports the conclusion that at least some of the sugar residues are linked to amide through their reducing carbon atoms.

cently observed that fibrinogen from other species also lose about the same amount of carbohydrate material on clotting (Chandrasekhar and Laki).

We can now demonstrate that the release of carbohydrate (1.2 residues of sialic acid and 3.6 residues of neutral sugars in the case of bovine fibrinogen) takes place only in the presence of LLF. Fibrinogen free of LLF when clotted with thrombin does not release any of its carbohydrate residues whereas, the addition of LLF to fibrinogen and subsequent clotting with thrombin produces splitting of the carbohydrate moiety (Chandrasekhar and Laki, 1964). If we now consider that one oligosaccharide chain in fibrinogen represents about 13-14% of the carbohydrate moiety, then the release of 20% carbohydrate corresponds to the cleavage of about 1.5 chains per fibrin molecule. If some of the oligosaccharide chains are shorter than pictured above, then the release of 20% of the carbohydrates may represent more than just 1.5 bonds split; at the most 3.6 bonds could be cleaved.

At first LLF looked as if it might be an enzyme capable of splitting glycosidic bonds between carbohydrate residues. This, however, does not seem to be the case because the liberation of the carbohydrate stops after about 20% of the carbohydrate has been released. Thus, it appears that the release of carbohydrate is indirect but is related to the bond formation between fibrin molecules.

Disappearance of N-terminal residues during clotting. Lorand et al. (1962) found that when LLF participated in the clotting process, some of the α-amino groups of the N-terminal glycine residues disappeared. They suggested that a transpeptidation involving these α-amino groups took place in the reaction. This proposed mechanism, however, had to be discarded because the peptides appearing in the supernatant during clotting were the

same irrespective whether LLF operated or not (Laki and Chandrasekhar, 1963). On the other hand, the disappearance of the α-amino groups could be explained by a transamidation reaction involving the β-carboxyl of asparagine or the γ-carboxyl of glutamine.

We propose on the basis of our data, that such a transamidation actually takes place at the β-carboxyl of asparagine which contains carbohydrate residues bound to it (see Fig. 1). The glycosylamine liberated in this transamidation is very unstable and would immediately decompose to sugar and ammonia (Isbell and Frush, 1951). Thus, the release of ammonia parallel with the release of sugar residues would provide a strong argument in favor of such a mechanism.

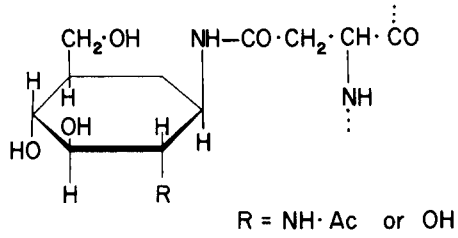

Fig. 1

Schematic representation of the binding of carbohydrate residue to asparagine.

The release of ammonia during clotting. Loewy (1963) recently reported that when LLF participated in the clotting reaction, ammonia was liberated. We can now confirm the finding of Loewy that the liberation of ammonia is correlated to the action of

LLF. Our experiments show that LLF catalyzes the release of 2.7 moles of ammonia per one mole of fibrinogen [2].

When fibrinogen free of sialic acid was clotted in the presence of LLF and calcium, no carbohydrate or ammonia was liberated and the clot remained soluble in urea. This experiment demonstrates that the three processes discussed above are inter-related. The simultaneous liberation of carbohydrate and ammonia, the disappearance of α-amino groups, and the appearance of the insoluble clot, strongly suggest that LLF is indeed a transamidase, specified to direct the reaction to the β-carboxyl group of asparagine bound to carbohydrate residues.

The exact quantitative relationship between the release of the carbohydrate and ammonia and the disappearance of the N-terminal glycine residues in the cross-bonding process remains to be established. It appears from the data of Lorand et al. (1962) that more N-terminal groups disappeared than could be expected from the amount of ammonia or carbohydrate released. This would indicate that some of the carbohydrate liberated may become rebound to N-terminal groups in a mechanism similar to the browning reaction of carbohydrate containing proteins. Such a process could lead to additional cross-bonding between fibrin molecules.

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The reaction mixture contained 2 ml fibrinogen (56 mg) + 1 ml borate buffer (0.2 M, pH 6.0) + 1.7 ml water + 0.05 ml human thrombin (50 units). 0.2 ml 0.15 M $CaCl_2$ and 0.1 ml LLF (0.3 ml water in controls) were added to the appropriate tubes. Clots were allowed to remain at 25° for 2 hours after which the fibrin was removed to collect the clot liquor. 1 ml of this clot liquor was taken up and the ammonia present in it assayed by the Conway method.

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