

## Anniversary Issue Contribution

# Early studies on the coagulogen (clottable protein) of *Limulus polyphemus* (horseshoe crab)

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The first author of this short story presented his PhD thesis entitled “Fibrinogen as plasma and cell protein” at the University of Oslo in 1970. Four of the eight articles in his thesis had been published in *Thrombosis et Diathesis haemorrhagica*. Three of them were about bovine platelet fibrinogen as an intracellular clottable protein whereas the fourth was called “Some characteristics of the clottable protein of *Limulus polyphemus* blood cells” (1). Previously Levin and Bang had published a paper called “Clottable protein in *Limulus*: Its localization and kinetics of its coagulation by endotoxin” (2) in the same journal. Thus, *Thrombosis et Diathesis haemorrhagica* published some of the very earliest studies performed on the clotting system that paved the way for the introduction by others of the well-known Limulus test for endotoxin.

It should be stressed that our studies were done out of pure curiosity. Because of our interest in platelet fibrinogen we wanted to find out something about the “primitive” intracellular clottable protein of an ancient species like *Limulus polyphemus* often described as a “living fossil” (Fig. 1). In this animal all of the clottable protein is present intracellularly in the “blood cells” (i.e. the hemocytes of the hemolymph) until clotting takes place with degranulation of the cells after an encounter with endotoxin-bearing Gram-negative bacteria. After all, we also had some background in zoology. *Limulus*, which commonly, but erroneously, is called horseshoe “crab”, belongs to the arthropod subphylum Chelicerata which also includes animals like scorpions and spiders. Class and order are Merostomata and Xiphosurida, respectively. Today one may wonder why studies on such



**Figure 1: *Limulus polyphemus* photographed in our lab.** Left: From the dorsal side. Right: From the ventral side.

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**Figure 2:** A cartoon depicting Erik Mürer trying to sort out a kind of disagreement between the *Limuli* and himself as the animals wanted to go back to America when Erik wanted them to be transported to the aquarium near Oslo. Printed with permission from the artist Paul French who was another colleague of ours in Oslo at the time.

basal biological phenomena could be published in a “clinical” journal like *Thrombosis et Diathesis haemorrhagica*, but I think it tells us something about the broad scope of the journal, and about editors with academic merits and open minds already in the first decades of the journal’s existence. For us who worked with *Limuli* in Oslo, i.e. Randi Holme, Torstein Hovig and ourselves with much help and advice from Jack Levin of the Johns Hopkins Hospital in Baltimore, this was a highly fascinating experience. It is rather unbelievable today, but we managed to have

a few animals imported from the East Coast of the United States to our cold northern corner of Europe. Here we kept them at a marine biological station some forty kilometers south of Oslo. Thanks to Erik Mürer’s useful contacts, and a special gift to handle the animals (Fig. 2), we had them transported to Oslo every time we wanted to draw their hemolymph.

These studies were much extended during a period at Temple University in Philadelphia where we kept the animals in an aquarium in the laboratory, and where we were not that limited as to the amount of material. At that time contamination of all solutions by endotoxin, even the distilled water, was a great challenge trying to purify the coagulogen from extracts of the *Limulus* hemocytes because columns and equipment were so easily clogged by clotted material. We realized that the coagulogen was clotted after endotoxin had triggered a clotting system which produced a proteolytic enzyme that split the coagulogen to yield a “fibrin monomer analogue” that polymerized spontaneously to form the gel. Further, this endotoxin-mediated system could be short-cut simply by using trypsin as the proteolytic enzyme, as we interpreted our observations. Therefore, an important finding that advanced our studies was that the enzymatic system triggered by endotoxin could be inactivated by manipulating the pH of the hemocyte lysate (cell extract). After having realized this, we could purify the coagulogen, at least in part, by gel filtration, and study some of its characteristics (3). From amino acid analyses we had calculated a minimal molecular weight for the clotted form of the molecule of around 20,000 Da already in Oslo. This could now be shown by polyacrylamide gel electrophoresis as representing an approximate value for the true molecular weight, i.e. around 23,000 and 17,000 Da for the reduced non-clotted and clotted forms of the coagulogen, respectively. (3). Later, this was calculated to be 19,675 Da for the whole coagulogen after amino acid sequence determination (4).

Mürer, Levin and Holme (5) studied the *Limulus* hemocyte further. By exposure of the cells to propranolol they obtained intact granules from disrupted cells, which they then studied by electron microscopy, and proved that the granules were indeed the site of the clottable protein.

*Limulus polyphemus*, as well as the Japanese horseshoe crab *Tachypleus tridentatus*, has been studied extensively by Iwanaga et al., and the molecular basis of the horseshoe crab innate immunology has been reviewed by Iwanaga (6) both in relation to the clotting system and to other defense mechanisms.

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