CHAPTER I

INTRODUCTION

The coagulation (gelation) of the horseshoe crab (*Limulus polyphemus*) was developed from the "basic research" that unexpectedly led to the discovery of the limulus amoebocyte lysate test by Howell in 1885 at John Hopkins University (1). In 1956 Dr. Frederick B. Bang was injected a heat stable extract of Gram negative bacteria (endotoxin) into the horseshoe crab causing intravascular coagulation and death of the animal (quoted from 1). In 1964 Bang and Levin demontrated that amoebocyte in haemolymph of the horseshoe crab was responsible for the gelation. The gelation was dependent on the concentration of endotoxin (quoted from 2). These works started an area of research in developing a sensitive endotoxin test which proved to be a very valuable test to many applications, especially to pharmaceutical industry. The test was sensitive and could detect endotoxin at the level as low as 0.1 or 0.05 ng/mL (3, 4).

Two reports in Thailand concerning the preparation of amoebocyte lysate from Thai horseshoe crabs have appeared (5, 6). The first report was from Klein et al (5). Their lysate could detect endotoxin in the level of 0.1 mg/mL. Usawattanakul (6) could detect the endotoxin at the level of 0.625 ug/mL which was nearly 200 times more sensitive than the work of Klein et al (5). Even then, these preparations were less sensitive when compared with the commercial

preparations. Usawattanakul stated that her lysate was opalescent in appearance whereas a clear solution was reported by other investigators who prepared the lysate from *L. polyphemus* (7, 8). Thus, this observation has stimulated an attempt to reinvestigate the preparation of amoebocyte lysate from Thai horseshoe crabs and some of their properties.

OBJECTIVE OF THE THESIS

1. Determination of protocol procedure for amoebocyte lysate preparation.

2. Determination of optimal incubation period of gelation reaction.

3. Determination of optimal magnesium ions concentration used in the amoebocyte lysate test.

4. Comparison of amoebocyte lysate test between tube method and micro-test method.

5. Determination of specificity of prepared amoebocyte lysate with natural endotoxins.

6. Determination of shelf-life of prepared amoebocyte lysate both lyophilized and liquid preparations.

7. Determination of endotoxin in some parenteral products.

LITERATURE REVIEW

ENDOTOXIN

Endotoxins are high-molecular-weight complexes associated with the outer membrane of gram-negative bacteria (GNB), and are the most significant pyrogen for the pharmaceutical industry. Although intimately associated bacterial cell membrane, these toxins are constantly shed into the environment of the bacterium, much like the daily shedding of the superficial layers of human skin. When the bacterium undergoes autolysis, all endotoxin is released from the cell. Unpurified endotoxins contain lipid, carbohydrate, and protein, but highly purified endotoxins do not contain protein and, therefore, are referred to as lipopolysaccharides (LPS) to emphasize their chemical nature (9). Several procedures have been developed for the extraction of LPS from whole GNB, but only the hot, aqueous phenol method of Wesphal *et al* .(1952) produces LPS free of protein and loosely bound phospholipid (quoted from 10). However, unpurified LPS is encountered in pyrogen testing of in-process and finished product pharmaceuticals (9).

Endotoxins are heat-stable compounds that can resist ordinary steam sterilization cycles; however, they are inactivated by extended dry heat cycles, alkaline conditions, acidic conditions, and polymyxin B, under certain conditions (9). Investigations have demonstrated that virtually all biological activity of endotoxin resides in the lipid portion of the molecule (9).

Because endotoxins are associated with GNB, they are ubiquitous, and like bacteria are found in air, water, and food. Due to their ubiquity, relative heat stability, and ability to cause profound physiological changes when administered parenterally, their detection and elimination is of paramount concern to the manufacturer of parenteral products to ensure the release of nonpyrogenic substances and devices (9).

Biological activities of endotoxin

Over 30 biological activities have been demonstrated to be induced by endotoxin or its lipid A moiety. A partial list of these activities is given in table 1 (11, 12). Although one of the mildest and most often studied effects of endotoxin is pyrogenicity, in sufficient doses, endotoxin has capacity to activate the coagulation system, alter carbohydrate and lipid metabolism, activate complement, modify hemodynamics, cause platelet aggregation, release vasoactive amines, and induce disseminated intravascular coagulation (DIC), shock, and ultimately death, in addition to many other pathophysiological aberrations.

Whole Gram-Negative Bacteria

Marcus et al. (quoted from 9) reported on an extensive study designed to evaluate the relative pyrogenicity of whole GNB using the

Table 1. Biological Activities of LPS.

A. Highly specific for LPS (in order of decreasing sensitivity)
Limulus lysate gelation *
Lethal toxicity in mice *
Pyrogenicity in rabbits *
Shwartzman reaction
Induction of tolerance to endotoxin
B. Characteristic but less specific (in alphabetical order)
Adjuvant activity
Bone marrow necrosis
Chick embryo lethality *
Complement activation *
Embryonic bone resorption
Enhanced dermal reactivity to epinephrine
Enhanced nonspecific resistance to infection
Enhanced phagocytosis
Hagemen factor activation
Helper activity for friend spleen focus-forming virus in mice
Hypoferraemia *
Hypotension
Hypothermia in mice
Induction of colony-stimulating factor
Induction of prostagladin synthesis
Induction of plasminogen activator
Induction of interferon production
Induction of IgG synthesis in newborn mice
Induction of tumor-necrotizing factor
Induction of mouse liver pyruvate kinase
Inhibition of phosphoenolpyruvate carboxykinase
Leukocytosis
Leukopenia
Platelet aggregation
Toxicity enhanced by adrenalectomy
Toxicity enhanced by BCG
Tumor necrotic activity
Type C RNA virus release from mouse spleen cells

* used as endotoxin assays.
Source: Modified from Ref. 11 and 12.

United Stated Pharmacopoeia (USP) rabbit pyrogen test as a measure of pyrogenic response. The ability to produce fever varied by two orders of magnitude, even in closely related genera of bacteria, such as the *Enterobacterioceae*. *Escherichia coli* was the most pyrogenic, requiring

only 1,000 bacteria per milliliter. *Enterobacter, Flavobacterium,* and *Achromobacter* required 10,000 organisms per milliliter whereas *Vibrio cholerae, Salmonella typhimurium,* and *Pasteurella multocida* required 100,000 bacteria per milliliter to elicit a significant pyrogenic response. Based only on the relative potency of endotoxin isolated from these bacteria, it is somewhat surprising that significantly fewer *Flavobacterium* and *Achromobacter* cells were required to produce a pyrogenic response than was true with *S. typhimurium.* Such wide variations in test results may reflect, in part, the biological variation inherent in the rabbit fever test (13) and colony-counting procedures.

Studies conducted by Travenol Laboratories, monitored the pyrogenicity of increasing doses of GNB using the LAL assay and the rabbit pyrogen test. *Klebsiella pneumoniae, Pseudomonas putida, E. coli, Serratia marcescens,* and *Pseudomonas aeruginosa* were suspended in sterile distilled water that contained no demonstrable endotoxin as measured by the LAL assay. The rabbit pyrogenic response was uniform, and all bacteria that were evaluated induced a rabbit test failure at approximately 10^5 bacteria per milliliter when tests were performed just after suspension was made. Representative data are presented in table 2. When the same solutions were tested using rabbit and LAL assays 1 year later, rabbit test failures and LAL test failures showed significantly altered thresholds (quoted from 1, 14).

Table 2. Pyrogenicity of whole Gram-Negative Bacteria.

Bacterial strains	Viable count ($x10^5$)	Sum of temperature increase in three rabbits.	LAL test (ng/mL)
K. pneumoniae	2.0	3.10	>0.2
P. putida	1.7	2.35	>0.2
E. coli	3.6	3.20	0.106
S. marcescens	1.0	2.30	0.106
P. aeruginosa	1.0	2.75	0.114

Lipid A

When free lipid A is complexed with bovine serum albumin (BSA), or human serum albumin (HSA), lipid A causes pyrogenicity comparable to that of intact endotoxin according to rabbit pyrogen tests, thereby demonstrating that the portion of the endotoxin molecule responsible for pyrogenic activity is lipid A (9). Intravenous injection of 0.01 ug lipid A per kilogram to rabbits leads to a biphasic fever response with peak fevers appearing at 1 and 3 hours after injection (9,15).The mechanism by which lipid A-induced fevers occur is thought to be the same as that for intact endotoxin which is a direct effect on thermoregulatory center (9).

Other biological effects of endotoxin

To the pharmaceutical industry the single most important biological property of endotoxin is its pyrogenicity, but endotoxin causes other diverse and profound pharmacological alterations, directly or indirectly, on all body systems. Endotoxin is known to interact with formed elements of blood, including platelets, red blood

cells, granulocytes, monocytes, and macrophages. Some of these interactions can lead to profound physiological changes. Endotoxin also activates complement and coagulation cascades. In sufficient doses and under appropriate conditions, endotoxin causes disseminated intravascular coagulation, generalized Shwartzman reaction, liver toxicity, lung pathology, abortion, shock, and death. It has a profound effect on the immune system and on carbohydrate and lipid metabolism, as well as neurological and endocrinological effects (9).

PYROGEN TESTING

There are many methods that can be used for pyrogen testing such as:

1. Rabbit Pyrogen Test (16),
2. Limulus Amoebocyte Lysate (LAL) Test (3, 17, 18, 19),
3. Mass Spectrometry for detection of B-hydroxymyristic acid (20),
4. Radioimmuno assay (RIA) technique of serotype specific antibody (21),
5. Radio-rocket immunoelectrophoresis of anti-core of LPS (22), and
6. Enzyme-linked immunosorbant assay (ELISA) of anti-lipid A (23).

But the rabbit pyrogen test and LAL test are the most widely used. The mass spectrophotometry method is not used although it can detect as little as 200 fmol of B-hydroxymyristic acid, the amide-linked long-chain fatty acid most frequently associated with GNB endotoxin, particularly *Enterobacteriaceae* (20). The RIA method of serotype-specific antisera, which are directed against O-specific side chain, is less sensitive than LAL test in most cases (21). The use of radio rocket immunoelectrophoresis takes advantage of antigen (LPS) and antibody (IgG) interaction and amplified end point by using [^{125}I] protein A which has an ability to bind with Fc fragment of IgG (22). In this method, endotoxins from many strains of GNB can readily be

detected in buffer solution but inhibition occurs in normal human serum. Although, ELISA of anti-lipid A can detect at 10 ng/mL of lipid A by using antibody (anti-lipid A)-antigen (lipid A)-antibody (rabbit anti-lipid A IgG) or sandwiched fashion technique, immunological cross-reaction with endotoxin from closely related bacteria cannot be detected (23).

Rabbit Pyrogen Test

The first official rabbit pyrogen test was included in the twelfth edition of the USP in 1942 (16). The test procedure as it stands today in USP follows the same basic format as the original official assay (24). The test is far from perfect, but it has provided the pharmaceutical industry with as effective means of ensuring non-pyrogenicity to the consumers of intravenous solutions for nearly 50 years (16). The procedure of rabbit pyrogen test involves measuring the rise in temperature of rabbits following an intravenous injection of a test solution. The test is designed for products that can be tolerated by the rabbit in doses not to exceed 10 mL/Kg body weight per intravenous injection within a period of not more than 10 minutes (16).

Factors influencing the test

Animals Pyrogen test rabbits must be both healthy and mature. Young rabbits, from 4 to 7 weeks of age, are 50 times less susceptible to the lethal action of endotoxin than are older animals. The most practical weight range for albino New Zealand white appears

to be 2,000 to 4,000 g. Rabbits are individually housed in an area with a uniform temperature between 20 and 23°C and free from disturbance. The pyrogen test area must be separated from the housing area but maintained under similar environmental conditions. Stranger should not be allowed into the area during testing, because their presence often results in false-positive rabbit temperature responses. In any group of test rabbits, control temperature may not vary from rabbit to rabbit by more than 1°C, and no rabbit to be used if its temperature exceeds 39.8°C (16).

Nature of the test Sample

Drugs or solutions that cause, decrease, or arrest fever are obviously ill suited to rabbit pyrogen testing. Examples of products that arrest the fevers produced by pyrogens are acetanilid, acetophenetidin, and acetylsalicylic acid. Drugs that mask pyrogenicity by decreasing fever include chloropromazine and related phenothiazine derivatives, hypnotics, anesthetics (such as procaine), and strophanthidin. Phosphate buffer solutions have been shown to elicit pyrogenic responses in rabbits if sufficient phosphate ions are infused intravenously, even though the buffers are free of bacterial endotoxin (16). Some substances, such as steroids or the antibiotic amphotericin, are also known to elicit fever in mammals; others may be intrinsically toxic to the rabbit. It is therefore extremely important to understand complete pharmacological effects of the new test samples before performing the rabbit pyrogen test (16).

Degree of restraint

It is necessary to restraint pyrogen-test rabbits if electrical thermometer probes remain in place in the rabbit's rectum through out the test period. However, rabbits become hypothermic when they are restrained (16), with the degree of hypothermic related to the degree of restraint (25).

Tolerance

Endotoxin tolerance develops in two distinct phases, early and late. Early tolerance is the most easily demonstrated in rabbits continuously infused with endotoxin. Within hours, the animals will be totally unresponsive. Early tolerance is specific for all endotoxins as a class and is not associated with increments in antibody. Late-phase tolerance is seen about 72 hours after a single injection of endotoxin. It generally increases over the next several days and may persist for several weeks. In contrast to early tolerance, the late phase is unrelated to the initial febrile response but is related to the antigenicity of the immunizing endotoxin injected into the rabbit (16).

The USP requires rabbits that have been tested with pyrogenic solution to be rested for 2 weeks before they are reused (24). Because the incidence of pyrogenic reactions in most test laboratories is rare, most rabbits do not receive the repeated injection of the same endotoxin pyrogen necessary to develop immunological tolerance. Unfortunately, the effect of repeated sub-febrile doses of endotoxin

is unknown, but could produce some degree of tolerance to marginally pyrogenic solutions. It is possible that this unsuspected tolerance could explain the wide range of rabbit colony febrile responses to endotoxin standards employed in intraindustry and international collaborative studies (16).

Amoebocyte Lysate Test

Biology of Horseshoe Crabs

Horseshoe crabs are classified in phylum Arthropoda and possess chitinous outer skin. The body is divided into 3 parts: cephalothorax, abdomen, and tail. The general characteristic of horseshoe crab is shown in figure 1 and its transverse section is in figure 2 (26, 27).

There are only four species of horseshoe crabs available today, namely *Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas,* and *Carcinoscorpius rotundicauda. L. polyphemus* lives in north-american water, whereas *T. tridentatus* is populated in japanese sea. There are two species of horseshoe crab found in gulf of Thailand; namely *T. gigas* and *C. rotundicauda.* A comparative characters of the four species of horseshoe crabs are depicted in table 3 (27).

Figure 1. General outer appearance of horseshoe crabs.

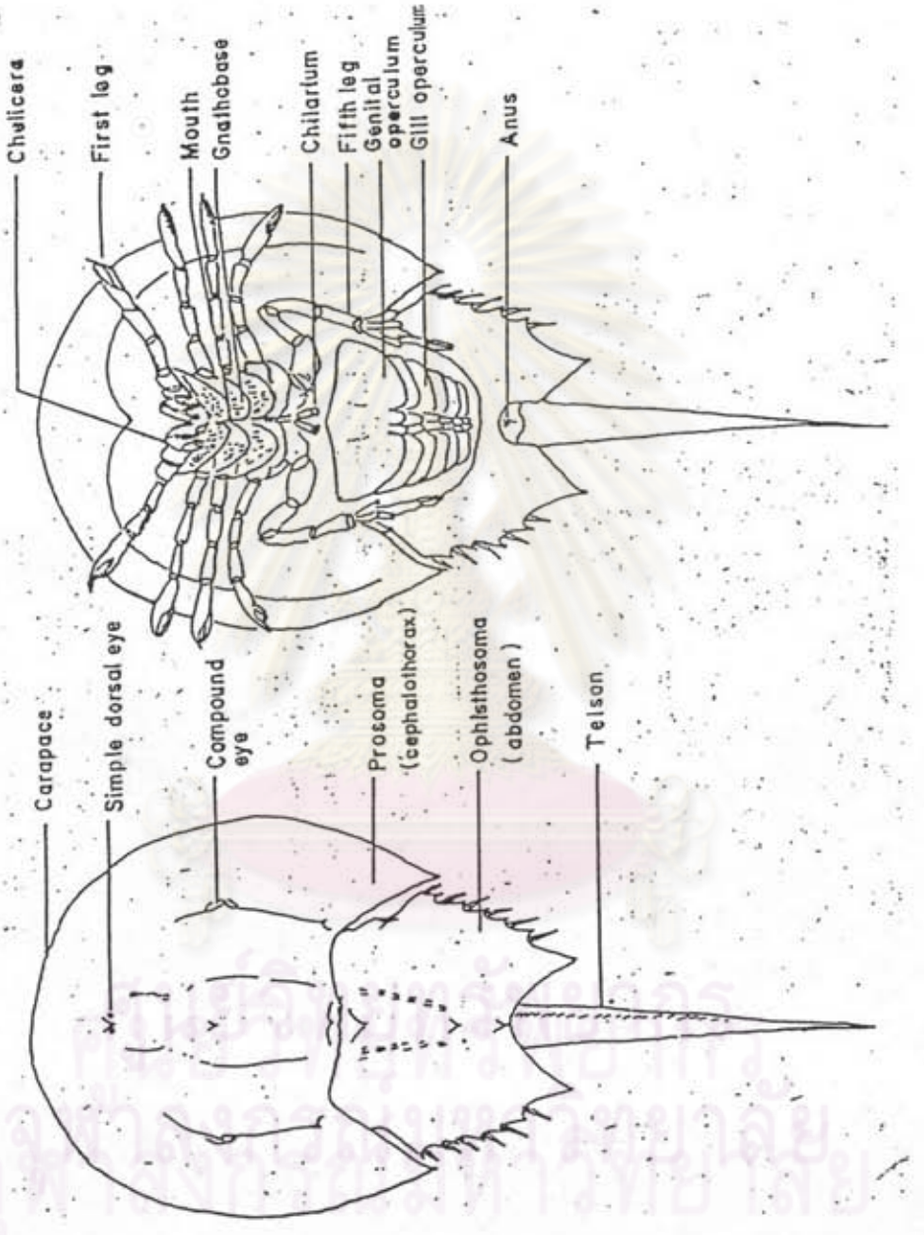


Figure 2. Transverse section of horseshoe crabs.

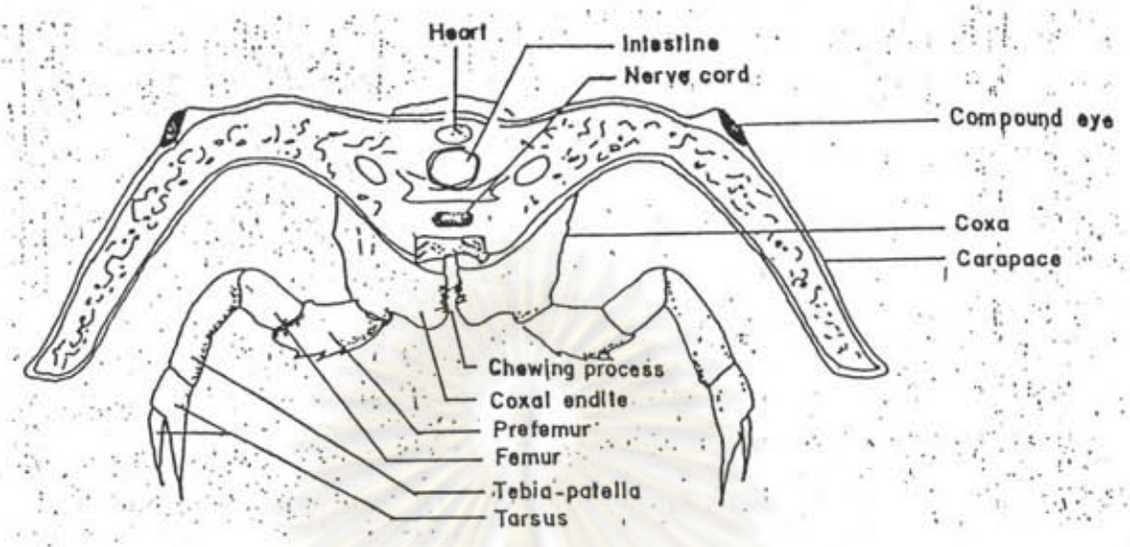


Table 3. Comparative characters of four species of horseshoe crabs.

	Limulus polyphemus	Tachypleus tridentatus	Tachypleus gigas	Carcinoscorpius rotundicauda
male: Frontal margin				
male: Frontal view				
male: 2nd prosoma app.				
male: 3rd prosoma app.				
male: Telson (cross section)	△	△	△	◔
female: Genital operculum				
female: Marginal spines				

History of Amoebocyte Lysate

In 1885, Howell (quoted from 1) observed that the blood of *L. polyphemus* formed a solid gel clot when withdrawn from the animal. Loeb (Quoted from 1) reported that, when the blood cells of *Limulus* were collected and exposed to a foreign substance, they underwent liquefaction followed by coagulation. Shirodkar reported that an unidentified marine gram negative bacterium (GNB) caused a fulminating disease of the horseshoe crab characterized by extensive intravascular clotting and death. A heat-stable derivative of this bacterium, as well as a number of heat-stable derivatives from other GNB, caused intravascular clotting in otherwise normal *Limuli*. Using an *in vitro* maintenance system for amoebocytes, the degranulation and destruction of cells occurred when the cells were exposed to pathogenic *Vibrio* or their thermostable toxins (17).

In 1964, Levin and Bang (2) reported that thermostable endotoxin induced the extracellular coagulation of Limulus hemolymph. Although endotoxin did not cause coagulation of cell-free serum, clotting activity could be restored by the addition of amoebocytes to serum. These studies could be concluded: amoebocytes were required for coagulation, disruption of amoebocytes enhanced the reaction, and quantities of endotoxin were probably inactivated by the coagulation reaction. The endotoxin-mediated effect to gelation of LAL was closely tied to the biologically active or "pyrogenic" portion of the molecule since it has been shown that "detoxified" endotoxin yielded a negative LAL test (9, 28). In 1973 Jorgensen and Smith developed a method for

preparation of LAL for detection of endotoxin and could detect endotoxin in a level as low as 0.1 ng/mL (3).

In Thailand, 1974 Klein and his group failed to use the lysate from both species of the Thai horseshoe crab to detect endotoxin at a concentration below 0.1 mg/mL (5). Then in 1979, Usawattanakul submitted a thesis of using Tachypleus lysate for detection of GNB infections and her lysate could detect endotoxin at a minimum of 0.625 ug/mL (6). It can be seen that both of these studies could not be obtained the lysate from the Thai horseshoe crabs for detection of endotoxin at a level comparable to avialable LAL test kit which has sensitivity of 0.1 ng/mL with a tube method (3).

Endotoxin Standard

With the increasing use of the LAL assay in the pharmaceutical industry over the past decade, a concerted effort has been launched by several laboratories to produce a suitable endotoxin standard that will relate pyrogenicity to an appropriate LAL end point. The use of a standard endotoxin is also mandatory for routine LAL testing and comparison of test results from one laboratory with those from another (29). There are four widely used endotoxin (pyrogen) preparations:-

1. *Shigella dysenteriae*,
2. *Escherichia coli* O113:H10:KO (EC),
3. *Escherichia coli* O55:B5, and
4. *Salmonella abortus equi*.

Escherichia coli O55:B5 is extracted by Westphal or Boivin

method (trichloroacetic extraction) and is commercially available. The Health Industry Manufacturers Association (HIMA) selected *E. coli* 055:B5 as its standard after conducting a collaborative study in a cross section of rabbit pyrogen test laboratories in the United States (30). The study concluded with 95% confidence that a 50% pass-fail result at some concentration above 98 pg/mL (approximately 0.1 ng/mL), or at approximately 1.0 ng/kg when administered at 10 mL/kg (USP), will attained with a USP rabbit pyrogen test. Therefore, the HIMA task force recommended establishing 0.1 ng/mL of endotoxin as the standard reference against which the LAL test could be compared. Comparable pyrogenic activity of EC-2, the Office of Biologics reference endotoxin, and *E. coli* 055:B5 has been reported by Tsuji and coworkers (31). Because of its homogeneity from lot to lot based on LAL results, and its commercial availability, *E. coli* 055:B5 appears to be a potential primary standard if EC lot variation is not resolved as well as an excellent secondary standard for routine LAL testing (29).

Identification of Pyrogenic Level

When the rabbit pyrogen test was included in USP, no attempts were made to define the levels of endotoxin that were pyrogenic in rabbits or humans (9). Keene et al. (32) demonstrated that purified endotoxin from *S. marcescens* administrated at a dosage of 30 ng/kg to four human subjects gave threshold fever responses, which were comparable in human and rabbits (33).

Threshold Pyrogenic Doses

Although the threshold pyrogenic doses (TPD) per kilogram body weight are virtually identical for human and rabbit, the dose-response relationship for the human is steeper than it is for the rabbit. At doses considerably higher than threshold doses, humans respond to endotoxin more vigorously than do rabbits. However, this does not change the basic fact that the USP rabbit pyrogen test is a valid predictor of pyrogenic risk for humans, particularly at threshold levels. The TPD in human and rabbit was shown to be between 0.1 and 0.14 ng/kg for *S. typhosa*, 1.0 ng/kg for *E. coli*, and 50 to 70 ng/kg for *Pseudomonas* (33). It should be noted that the low pyrogenic level reported for *S. typhosa* was due to the use of a highly purified endotoxin preparation and bears little, if any, resemblance to the muted pyrogenicity encountered with naturally occurring endotoxins found in pharmaceutical manufacturing (9).

The HIMA collaborative study (30) concluded that it was "reasonable to set 0.1 ng/mL, which is slightly above the lower confidence limit, as the **standard of reference pyrogenic activity** against which the LAL test is to be compared." The suggested end point is equivalent to 1.0 ng/kg when test material is delivered at a USP dose of 10 mL/kg. Furthermore, the study concluded that if a laboratory could demonstrate an LAL test failure rate significantly greater than 50% at 0.1 ng of Difco's *E. coli* O55:B5 endotoxin per milliliter, the test could be considered qualified for use as equivalent to the USP rabbit test. A 0.1 ng/mL end point provides a significant safety factor against human pyrogenicity because the value

was calculated at the lower 95% confidence level and is comparable to the TPD for humans (9).

Parenteral Drug Association (PDA) Limulus Amoebocyte Lysate Task Force provided valuable data confirming that, under optimal conditions, the sensitivity of the USP rabbit pyrogen test approaches 1 ng of commercial *E. coli* endotoxin per kilogram, and this value could serve as needed reference point for endotoxin limits in small-volume parenterals (SVP) (9).

Biochemistry of Amoebocyte Lysate

After Levin and Bang (2) demonstrated that the clotting activity of *Limulus* haemolymph resided in the amoebocyte, work by Young et al. established the enzymatic nature of the endotoxin-induced reaction (34). Using Sephadex G-50 and G-75 column chromatography, these workers isolated three peaks. One fraction contained a clottable protein that had a molecular weight of 27,000 and was heat stable. The second fraction was comprised of a high-molecular-weight, heat-labile substance that was activated by endotoxin and formed a gel with the clottable protein. Concentration of both the heat-labile fraction and endotoxin indicate that the rate of reaction is dose dependent. The authors concluded that the reaction of *Limulus* amoebocyte lysate with endotoxin is dependent on endotoxin activation of the high-molecular-weight enzyme, which in turn gels the lower molecular weight clottable protein. This reaction is critical for providing an end point in the conventional LAL gel clot test.

Tai and Liu demonstrated that activation of the clotting enzyme zymogen (proclotting enzyme) depended not only on endotoxin but also on Ca^{2+} (35). The proclotting enzyme had a molecular weight of at least 150,000, and appeared to consist of a single peptide chain. Exposure of the reduced and carboxymethylated enzyme to 6 M guanidine hydrochloride failed to disassociated it into subunits. Because the enzyme was affected by soybean trypsin inhibitor, this study also suggested that the proclotting enzyme was a serine protease, confirming the finding of Young et al. (34). Trypsin, a serine protease, had been shown to induce the gelation of LAL (35, 36).

The low-molecular-weight clotting protein has been studied by Solum, who named it coagulogen (36). He concluded that the molecular weight loss was caused by the proclotting enzyme splitting a portion of coagulogen polypeptide. This conclusion was consistent with the observation that trypsin promoted clot formation of untreated as well as acid-treated preparations.

Nakamura and coworkers (37) demonstrated that together coagulogen and the activated proclotting enzyme obtained from the blood of *T. tridentatus* produced a gel clot protein consisting of two peptide chains and provoked the release of low-molecular-weight peptide C. Amino acid sequences were determined for peptide C as well as for the A chain of the gel protein; partial sequencing was reported for B chain as shown in Table 4.

The C-terminal octapeptide sequences were significantly homogeneous with primate fibrinopeptide B, suggesting that coagulogen

and the fibrinopeptide are derived from a common ancestor or that coagulogen is a prototype of primate fibrinogen (37). A general schematic of endotoxin induced gelation of coagulogen by endotoxin is in figure 3 (17).

Table 4. Partial amino acids sequencing.

Fragments	
A chain	H-Ala-Asp-Thr-Asn-Ala-Pro-Ile-Cys-Leu-Cys-Asp-Glu-Pro-Gly-Val-Leu- + Th-4 ++Th-2 ++ Th-1 ++ Th-6
	Gly-Arg-OH +
Peptide C	H-Thr-Gln-Ile-Val-Thr-Thr-Glu-Ile-Lys-Asp-Lys-Ile-Glu-Lys-Ala-Val- + T-1 ++ T-III ++
	Th-6 +
	Glu-Ala-Val-Ala-Gln-Glu-Ser-Gly-Val-Ser-Gly-Arg-OH + T-II
B chain	H-Gly-Phe-Ser-Ile-Phe----------Phe-OH

[a]The peptides obtained from digests with trypsin (T) and thermolysin (Th) are shown by solid lines.

Figure 3. General schematic of gelation.

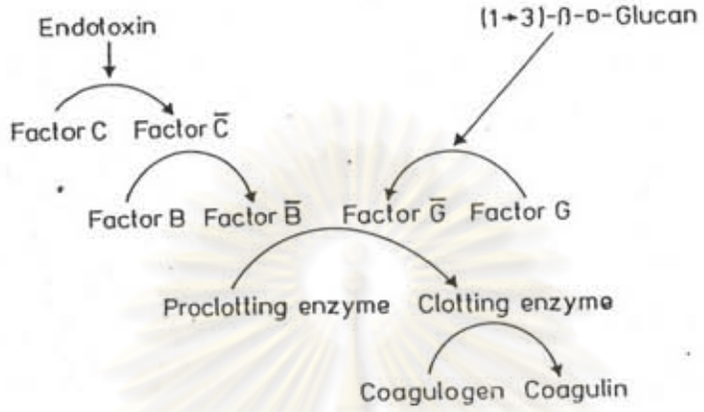
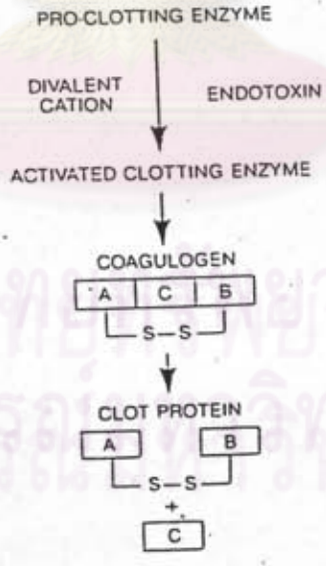


Figure 4. Endotoxin-mediated proteolytic conversion of coagulogen.



Mosesson's group and that of Tai findings (38, 39) suggested that coagulogen was composed of two identical chains joined by a noncovalent bond. Endotoxin-mediated proteolytic conversion of coagulogen to clot protein (coagulin) was characteristic of cleavage at a minimum of two sites on each chain. Both the proclotting enzyme from *Limulus* and bovine trypsin will induce the gelation of coagulogen by effecting the cleavage of the Arg- Lys bond to form coagulin, which reacts in a noncovalent fashion to form a clot (40). Ohki and coworkers isolated an additional clotting factor (41). This factor is activated by endotoxin and in turn converts the proclotting zymogen to activate proclotting enzyme, which then acts on coagulogen. This reaction is depicted in figure 4.

Preparation of Amoebocyte Lysate

In principle, the preparation of amoebocyte lysate consists of the collection of haemolymph and amoebocytes in anti-clotting solution and then separation of haemolymph and amoebocytes before washing in isotonic solution of the cells to remove the anti-clotting solution. The amoebocyte is subjected to lysis with hypotonic solution and/or mechanical methods.

In 1964, Levin and Bang (2) using sterile pyrogen-free 20 gauge needle inserted on the dorsal junction of the cephalothorax of the *Limulus polyplemus*, horseshoe crabs. The area is previously cleaned with 70 % alcohol. The haemolymph and amoebocytes were collected into anti-clotting solution containing N-ethylmaleimide (NEM) in tris buffer pH 7.28. The final concentration of NEM was 5 mM.

The solution had been warmed to 40°C before use. Then the mixture was centrifuged at 600 rpm for one minute. The packed amoebocytes were resuspended in 10 mM NEM at 40°C. The procedure was repeated twice before they were washed two times with buffered artificial sea water. The amoebocytes were lysed by freezing and thawing 4 times with dry ice and acetone. A clear-colourless liquid (lysate) was obtained after centrifugation at 2,000 rpm for 5 minutes. In 1969, Rojus-Corona et al. (42), prepared the lysate by using 15 gauge needle and 0.125% NEM in 3% NaCl. They allowed the amoebocytes to settle down by standing for 1-4 hours without centrifugation and washed 4 times with the NEM solution. The final wash was done with artificial sea water. The lysis of the cells was carried out with 7.5 mL pyrogen-free distilled water per 100 mL of total blood collected. Then clearification was obtained by centrifugation at 2,500 rpm for 15 minutes. Prior to use, 0.05 M phosphate buffer saline (PBS) pH 7.2 was added equal volume. The sensitivity of the lysate was found to be $4X10^{-6}$ mg/mL. In 1970, Solum (36), carried on an experiment using 0.01 M NEM and centrifugation at 750 g for 10 minutes and washing with 0.15M NaCl. Apart from above, he followed similar procedure as described by previous workers (2, 42). In 1971 Reinhold (7) performed the same experiment with some exceptions. The needle used was 13 gauge, and 0.125% NEM in 3% NaCl buffered with Tris pH 7.4 was used. The ratio of packed cells to distilled water was 1:6. The lysate had a sensitivity of 0.002 ug/mL. Pearson (8) investigated a spiny spider-crab, *Maia squinado*, using NEM at a final concentration of $5X10^{-3}$ M and the lysate established activity of gelation as that of horseshoe crab. Jorgenson in 1973 (3)

prepared the lysate of horseshoe crab similar to the previous ones (7, 36, 42) with the exception that centrifugation was at 50 g, and the ratio 1:3 of packed cells to distilled water was used. The sensitivity was 0.1 ng/mL. Nandan 1977 (18) obtained the lysate from horseshoe crabs with some different in procedure and chemical used. EDTA at the concentration of 0.025 M in 3% NaCl was used. The ratio of 1:5 of packed cells to distilled water was recorded.

In Thailand, Kien et al. (5) was the first to report using both *T. gigas* and *C. rotundicauda* for the preparation of lysate. They found that the lysate could form a gel generally on the order of 10^{-1} mg/mL of endotoxin, eventhough varieties of pH and electrolyte composition in NEM collection solution were performed. Many lysis techniques were carried out including osmotic lysis using distilled water, mechanical lysis using a tissue homogenizer, freeze-thaw lysis, and lysis by sonication. None of the techniques could improve the sensitivity of the lysate however. In 1979, Usawattanakul (6) used *Tachypleus gigas* lysate for detecting endotoxin in patients with GNB infections. Her method of lysate preparation utilized 0.25% NEM in 3% NaCl as anti-clotting solution and the cell lysis was done with homogenizer to obtain an opalescent supernatant. Her lysate could induce solid gelation at 5 ug/mL of endotoxin from *E. coli* O111:B4.

Methods of endotoxin detection

A. Clot End Point

The simplest and most widely used procedure for the detection of endotoxin in solutions is the clot end point (3, 43). An equal volume of lysate and test solution (0.1 mL of each) are mixed in depyrogenated test tubes. The mixture is then agitated gently and incubated in a water-bath at 37°C for one hour. Because the clot end point of some lysates is modified by handling, the reaction mixture is usually left undisturbed during incubation. The end point is read easily by carefully withdrawing each tube from the water-bath and inverting it 180°. If a solid clot is formed and remains solid through inversion, the test solution is said to be positive for endotoxin. Quantitatively, a number of two-fold serial dilutions of the test solution are made and the clot end point determined. The level of endotoxin is calculated by multiplying the reciprocal of the highest dilution of the test solution giving a positive end point by the sensitivity of the lysate preparation. A positive control consisting of a known concentration of endotoxin and a negative control using non-pyrogenic water is used in all the test procedure. Jorgensen 1973 (3) had graded degree of positive result to apply in the determination of quality of the test materials as in table 5.

B. Micro-test Method

In the micro-test system 10-uL volumes of test material are mixed with the lysate in a sterile petri dish and incubated undisturbed at 37°C for 30 minutes. A positive reaction is characterized by gelation, whereas in a negative one the mixture remains liquid. The end-point has been interpreted either by tilting

the plate, for the positive reaction the gel is immobilized, or using

Table 5. Grading of positive gelation from Jorgensen, 1973.

Reaction	Description
4+	Firm gel with considerable opacity
3+	Soft gel with moderate to considerable opacity
2+	Weak gel with slight to moderate opacity and adhesion of starch-like floccules to sides of the tube when tube is slanted
1+	Very weak gel with slight opacity and with some starch-like adhering to sides of tube
Negative	No visible increase in viscosity or opacity

0.1% methylene blue to visualize the end point (44). The problem of subjectivity in interpreting end points of less than complete gelation cannot be detected by the above end point techniques. The use of the cumbersome enzymic method which measures cleavage of a synthetic chromogenic substrate by the clottable enzyme has been reported (45). A simple and rapid method for the visual confirmation of a positive reaction by use of fresh, defibrinated sheep blood has been described recently (46).

Application of amoebocyte lysate test

There are some important application of the test which divided into three main headings.

Pharmaceutical industries The use of amoebocyte lysate

test is for quality control of endotoxin which in most of the cases, implies to pyrogenic of the products particularly in the area where rabbit pyrogen test is limited.

In biological products such as vaccines and sera or blood derivatives products, the test is used in-process control of pyrogen or the final cross-check pyrogen test (47,48). Influenza virus vaccine which is produced by inoculation of virus to large number of embryonated chick eggs to yield a single lot of vaccine by pooling allantoic fluid containing virus. It is not possible to detect and discard every egg that may contain bacteria. Therefore some amount of bacterial endotoxin may be present. Hence there is a routine use for the amoebocyte lysate test in this product (49).

In the area of parenteral products the amoebocyte lysate test is very useful since some of the products cannot be tested by rabbit pyrogen. With the help of ultrafiltration process at molecular weight cut off of 10^4 (50) or dilution of the products (4,7,51), antibiotic and radiopharmaceutical products can be tested.

Intravenous fluids such as dextrose solution and normal saline solution, there have been reported to have passed rabbit pyrogen test but failed to pass the amoebocyte lysate test and negative rabbit pyrogen test samples, positive by the lysate test showed positive by rabbit pyrogen test on re-examination (52).

Food industries There have been many reports using the basic principle of the high sensitive and rapid test of the amoebocyte lysate test for the detection of endotoxin in some food products. The quality of raw fish is usually determined by sensory evaluation indicating either fresh or a spoiled condition; otherwise more accurate, time-consuming chemical analysis is used. The criteria of estimate fish quality is due to: bacteria responsible for spoilage during refrigerated storage are principally gram negative, and amoebocyte lysate test is used to detect endotoxin from or associated with gram negative bacteria (53). With the same criteria the test is used for quality of ground meat. In the field of parenteral nutrition, fat emulsion for parenteral nutrition has made use of amoebocyte lysate test for detection of pyrogen since the test is more sensitive and elimination of false positive as compared to rabbit pyrogen test (54). Extraction of endotoxin from lipid using Folch's method, increase sensitivity of amoebocyte lysate test by testing the nonlipid phase (55).

Clinical application It has been attempt to use amoebocyte lysate test for diagnosing of the following :-

Bacterial meningitis diagnostic test was study (56,57). The result has concluded that amoebocyte lysate test alone could only be used to distinguish gram negative from gram negative meningitis, but the test used in parallel with C-reactive protein would further distinguish non-bacterial and bacterial meningitis.

The gram negative bacteriuria has been studied using

amoebocyte lysate test, since asymptomatic bacteriuria of pregnancy would increase risk of perinatal morbility and mortality. The amoebocyte lysate test was used by diluting mid-stream urea to approximately 1:10 and the end-point is $>10^5$ gram negative bacteria cells per mL having sensitivity 88.7% and specificity 98.7% (58,59).

In case of gonorrhea from male and female has been studied using amoebocyte lysate test (60). Gonococcal urethritis (61) was studied with 125 patients which consisted of gonococcal (67 patients) and nongonococcal (58 patients) urethritis. The exudates were collected and diluted 1:400 with water. The sensitivity and specificity of the test were 98.5% and 93.1% respectively as compared with gram stain method which showed to have sensitivity and specificity as 95.5% and 100% respectively. The gonococcal cervicitis was other promising area for a screening test (60). The study was carried out with 100 untreated gonococcal cervicitis with culture-proof, and 50 normal volunteers. The specimens were collected by removal of ectocervical mucous with a sponge, and a depyrogenated cotton-swab was used to collect endocervical specimens. The swab was placed in 1 mL of water and serial twofold dilution were made. At 1:256 sensitivity and specificity were 57% and 99% respectively, but at 1:8 dilution sensitivity and specificity were 100% and 78%. Therefore, at 1:8 dilution, negative predictive value was 100%. The gram stains and repeat cervical cultures of the same specimens were 53% and 93% respectively for positive group of patients and all negative for negative group of patients (62).

Sera and blood derivatives are tested for endotoxin by many investigators (47). The detection of endotoxin in plasma has been focused. It has been showed that there was a interfering substances in plasma which included α_1 globulin and α_1 lipoprotein esterases which degraded lipid A; IgM and IgG antibodies directed against lipid A and against polysaccharide O and R antigens; and protease inhibitors such as α_1 antitrypsin, α_2 macroglobulin and anti thrombin III (63). Methods used to treat plasma in an attempt to overcome these difficulties included chloroform extract (64), alkalinization (65), perchloric acid (66), pH shift (7,67), and various combinations of dilution and heating (68). The study was carried out with 98 patients whom septicemia due to gram negative bacteria was suspected, had given a good correlation between the lysate test and bacteria culture (69). Japanese investigations (70) using a chromogenic test were reported to obtain highly specific test for endotoxin and had no reaction with fungal polysaccharide. The test had a sensitivity in the level of 1 pg/mL. The samples used in the test were platelet-rich plasma.

The amoebocyte lysate test has been used officially in endotoxin testing of medical devices such as transfusion and infusion assemblies, haemodialyzer, intraocular lenses, catheters, and implant devices (52,71).