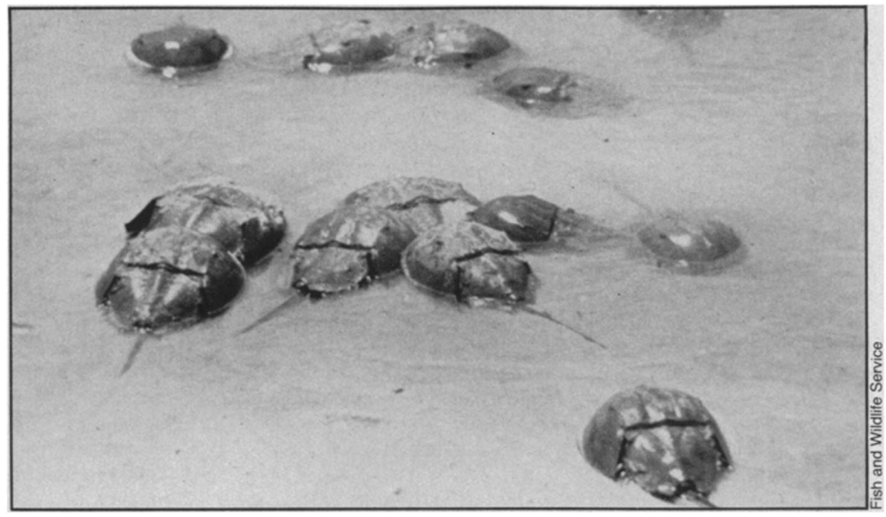
...with the blood of a horseshoe crab



A simple test for the presence of potent bacterial toxins saves time, money and a lot of bunnies

BY JANET RALOFF

It sounds like an ingredient from a witch's brew. But to the pharmaceutical industry and increasingly to other branches of medicine also, a product manufactured from the blood of the horseshoe "crab" is becoming a divining rod, par excellence, for a ubiquitous and elusive type of fever-causing bacteria.

The culprit under siege is endotoxin, a pyrogen — fever-producing product — shed by gram-negative bacteria (those that do not hold the purple dye when stained by Gram's method). Found in the outer cell membrane of gram-negative bacteria, endotoxins form a complex breed of compounds known as lipopolysaccharides (containing a lipid and carbohydrate). Although they are seen throughout the environment, they cause harm — occasionally death — only upon entering the circulatory system of animals, including humans (SN: 2/7/76, p. 93).

Because of this fever-producing capability, endotoxins must be barred entrance to the bloodstream, particularly in persons who are ill or whose immune systems are weaker than normal. It's not surprising, then, that one of the major applications for endotoxin detection is in the pharmaceutical industry, where every drug, intravenous solution and medical device that comes in contact with the blood must be screened for endotoxins prior to marketing.

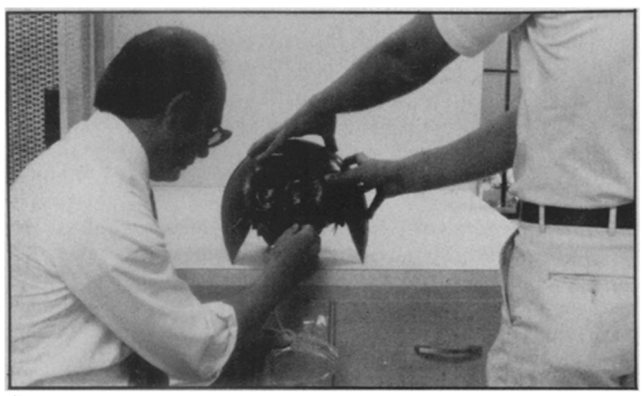
For many years, rabbits played "guinea pig" for the pharmaceutical world. To screen a new batch of some intravenous solution, such as glucose, blood, plasma or human albumin for endotoxin contamination, sample doses of the substance were injected into rabbits. If the animals developed a fever, the batch was reworked. Shortly thereafter, the bunnies were retired. An immune reaction caused by their unwitting exposure to endotoxins in human-serum protein, for example, would necessitate a quick finale to their screening career.

But a find by Frederick Bang in the mid 1960s ushered in a revolution in pyrogen screening. He showed that the blood of horseshoe crabs *(Limulus polyphemus)* clotted following *in vivo* injections of live or heat-killed gram-negative bacteria. Further investigation revealed that the congealing was a reaction by the animal's amoebocytes — the only cells circulating in its blue blood. By the early 1970s, an aqueous extract made from those cells, known as Limulus amoebocyte lysate, was developed to understudy the rabbit. But so successful was the Limulus lysate during its initial screening debut that it promises to upstage the rabbit test if not eliminate it altogether.

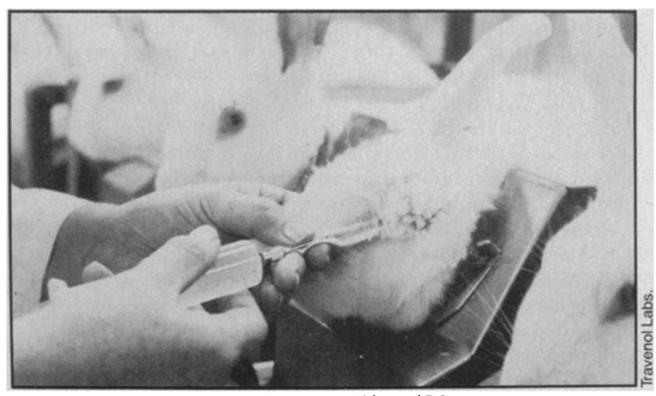
Not only does the Limulus lysate assay cost 15 to 30 times less than the standard rabbit pyrogen test, says Frederick Pearson, manager of pyrogen research at Travenol Laboratories in Morton Grove, Ill., but it also is generally 5 to 10 times — sometimes even 1,000 times — more sensitive than the rabbit tests, depending on the substance tested. And whereas the rabbit test requires a rabbit, carefully controlled laboratory conditions and a long waiting period, the simplest lysate test requires only a few test tubes, a small quantity of lysate and test material, and a one-hour wait. What's more, the lysate test appears more quantitative, more standardized (due to individual variability in rabbits' responses) and more applicable (some of the materials to be tested for pyrogens are toxic if not lethal to rabbits).

The lysate test routinely detects endotoxin contamination on the subpyrogenic level, that is, at levels below which any response would be detected in rabbits — or, presumably, in humans. Although some rabbits will begin exhibiting signs of a fever around the range of 100 picograms (trillionths of a gram) of endotoxin per milliliter of injected material per kilogram of body weight, Pearson says his firm will not release a product contaminated at greater than 50 picograms of endotoxin per milliliter as demonstrated by lysate testing.

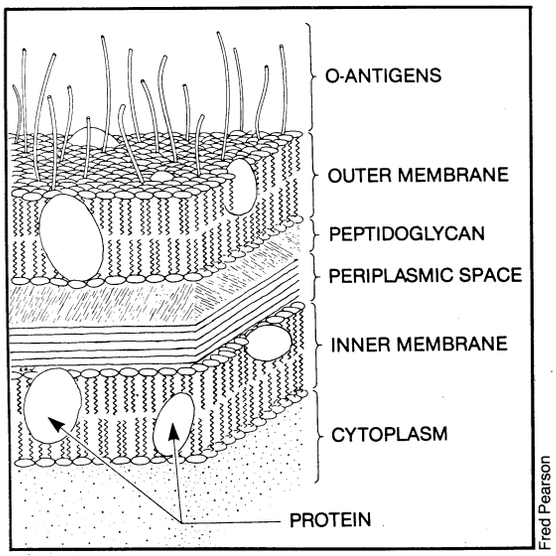
In fact, expansion of the limits for detecting endotoxins through lysate testing has opened a Pandora's box of questions regarding the true role endotoxins play in



Experimental bleeding of "crab" for amoebocytes.



Pyrogen assays using rabbits cost $10 to $76 per test.



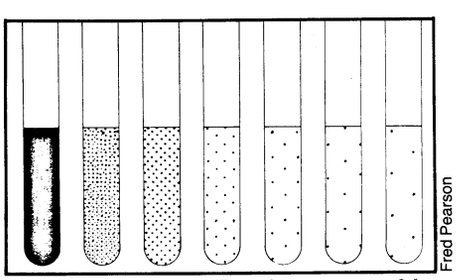
Fred Pearson

Cell wall gram-negative bacterium: Endotoxin forms in the outer membrane.

disease. For instance, a 1977 paper by D. Fumarola in the Italian research journal IL FARMACO found that six anticancer drugs supposedly free of endotoxin failed the Limulus lysate test. Two, Bleomycin and L-asparaginase, showed endotoxin contamination on the order of 50 nanograms (billionths of a gram) per 50 milliliters of the drug.

"Several antitumor chemotherapeutic agents exhibit a high incidence of adverse effects in which pyrogens are likely to play a role," Fumarola writes. Indeed, he adds, since many of these drugs are the product of microbial fermentation, "pyrogens are an expected contaminant in some agents (e.g., L-asparaginase because this enzyme is prepared from *Escherichia coli* or *Erwinia* organisms [all gram negatives])." He goes on to speculate that some of the adverse effects reported previously to have been caused by the drugs may actually be due to or enhanced by the presence of endotoxin contamination.

Firms making parenteral pharmaceutical materials (those that are injected into the bloodstream) and medical devices, such as Travenol, must test each batch of solutions or devices they produce for endotoxins before it can be released for sale; all test results must be forwarded to the U.S. Food and Drug Administration. For a firm running 150,000 tests a year, the savings in switching from rabbit tests to lysate tests could be as much as $1 million annually.


Fred Pearson

Limulus lysate test: Equal amounts of lysate and test sample, usually 0.1 ml, incubate an hour at 37°C in pyrogen-free test tubes. Proclotting enzyme in lysate is activated by endotoxins. Amount of precipitate indicates contamination.

At present, FDA permits only medical devices to be released for market solely on the basis of Limulus lysate testing, Pearson says. The agency's Division of Biologics, which oversees development and production of things such as albumins, antihemophilic agents, blood, and drugs derived from animals, such as insulin, is considering substitution of rabbit tests with those using lysate. For the time being, firms using lysate endotoxin assays must duplicate their findings with rabbit tests until a sufficiently large body of data exists to validate that the lysate results are at least as accurate as the rabbit data. Pearson said his laboratory forwarded data to FDA in mid December that he hopes will validate that claim.

But the "real hornet's nest" is FDA's Bureau of Drugs, he says, which oversees regulations for producing injected drugs such as antibiotics. It has proved the most difficult bureau to convince regarding the efficacy of lysate testing, although there are signs that it too may eventually sanction lysate testing.

But the story doesn't necessarily end there. In fact, Pearson says, "We hope that's just the beginning." For example, the lysate test may be adaptable to clinical tests.

At present there are no reliable diagnostic tests for endotoxemia (endotoxins in the blood), he says. With rabbits or lysate one can find endotoxins in pharmaceutical preparations, but once those pyrogens enter the body, one is left to infer from symptoms whether a patient has developed the blood disease. For infants, the mortality rate from hospital-acquired infections, most of which Pearson says are gram-negative, can be as high as 80 percent. The elderly face similarly high risks.

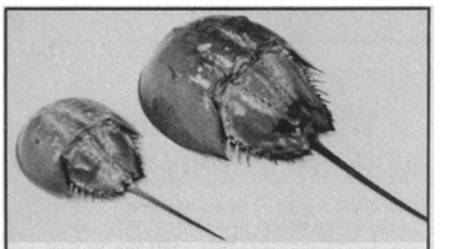
The single largest bacterial problem is from gram-negative species, Pearson claims, particularly among patients recovering from surgery. And for those whose immune systems have been suppressed — such as organ-transplant or chemotherapy patients — their ability to fight off the infection is virtually eliminated. What he hopes to see growing out of basic research on Limulus lysate is a clinical, diagnostic test.

"The raging controversy is in the blood," Pearson told SCIENCE NEWS. "If the endotoxin isn't in the blood it's because of the nature of the way it's metabolized. It isn't there to detect." But if it is there and can be detected, proper diagnostic testing and immediate treatment could probably save around 250,000 lives a year, some experts contend.

Pearson says it might be possible to prevent a patient from undergoing septic or surgical shock, or at least to mitigate the consequences of it, if infected individuals can be identified immediately, perhaps by administering the lysate test to everyone exhibiting fever following surgery. "We're virtually there in terms of diagnosis," Pearson says. Although factors that prevent use of the lysate test have been found in human blood, research is pointing toward mechanisms to remove those problem factors.

Stanley Watson at the Woods Hole Oceanographic Institution in Massachusetts agrees. Based on work he's doing there, Watson says there seem to be two inhibitors in human blood that biologically inactivate the lysate. But, said the scientist whose private firm has been a national supplier of Limulus lysate for eight years, "I think we're [at Woods Hole] as close as anybody to solving the problem." In fact, his laboratory has already begun clinical tests in conjunction with patients at the Memorial-Sloan Kettering Cancer Center in New York. The aim is a reliable Limulus amoebocyte lysate test to diagnose endotoxemia in the blood. Results, however, are not yet in.

But the ultimate goal challenging tomorrow's researchers is isolating the toxic portion of the lipopolysaccharide and manufacturing an antibody to help patients fight it. □



NOT A CRAB

The horseshoe crab is not a crab at all but an arthropod somewhat distantly related to the spider. Having evolved little during the past 300 million years, it is often called a "living fossil."

There are only four species. Ancestors of the American one appear to have migrated to North America during the period when the Alps were created and the Mesozoic seas of Europe were disappearing. Today they frequent shallow Atlantic coastal waters from Canada to Mexico's Yucatan Peninsula.

Barely visible when born, the young "crab" molts frequently. Adults can grow to several feet in length (if one includes their spiny tail), live 15 years and weigh more than eight pounds. Except for one accidental family born last year to animals in Fred Pearson's (see story) lab, horseshoe crabs have never been known to reproduce in captivity. That explains why the animals must be harvested in large numbers for lysate production. But once caught they can be bled repeatedly without harm, much like human blood donors.