Detection of Lipopolysaccharide (LPS): An Improved Method for Isolation of the *Limulus* Extract[1] (36897)

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Most of the bioassays for bacterial endotoxin (lipopolysaccharide, LPS) are time-consuming and expensive since they require the use of rabbits, large numbers of drug treated mice or chick embryos (1). This sharply limits the general application of any of these methods to the routine detection and quantitation of LPS. In the past few years a new method for LPS detection has been described. Taking advantage of the tendency of the extract from amebocytes of the horseshoe crab (*Limulus polyphemus*) to coagulate in the presence of as little as 5 ng LPS, the "*Limulus* assay" has been proposed as a rapid and sensitive detection system for LPS (1–4).

The *Limulus* assay seems simple and straightforward and by every indication it detects the same or similar biologically active forms of LPS that are measured in the other assay systems (1). One significant problem with the *Limulus* assay is the lack of a commercial source for the extract, making it necessary for the individual investigator to prepare his own extracts. In our numerous attempts to prepare *Limulus* extracts according to the procedure of Reinhold and Fine (4) we have been hampered by considerable variation in potency of extracts (ability to detect ng doses of LPS). Having made individual extracts from 950 crabs, Lindberg *et al.* (5) reported significant variations in extracts as a function of the crab donor. Furthermore, those authors have suggested that *Limulus* extracts vary in potency as a function of seasonal influences on the crabs.

In this report we describe a modification of the procedure of Reinhold and Fine (4) for obtaining the *Limulus* extract. In our experience this modified procedure results in manyfold increases in potency of *Limulus* extracts. Our experience further suggests that the considerable variation in potency of different *Limulus* extracts obtained by the Reinhold–Fine procedure may be a reflection of an inefficient and unpredictable extraction procedure.

Materials and Methods. For these experiments horseshoe crabs obtained from the Woods Hole Marine Biological Laboratory (Woods Hole, MA) were bled from the central dorsal sinus with siliconized needles and syringes, as described by Reinhold and Fine (4). The hemolymph was pulled directly into 50 ml plastic disposable syringes, each of which contained 25 ml 3% NaCl with 0.125% *N*-ethylmaleimide (Eastman Chemicals, Rochester, NY). Batches of hemolymph were centrifuged in siliconized plastic bottles at 800*g* for 20 min, following which the amebocyte pellets were washed in the same salt–maleimide solution described above. The third, and final, wash was in 3% NaCl lacking maleimide. The techniques to this point are the same as those of Reinhold and Fine. Beyond this point, however, the procedures diverge. Reinhold and Fine add to the amebocyte pellets a volume of distilled water 5× the volume of packed cells (amebocytes), allowing the suspension to sit overnight at 5°. The debris is then removed by centrifugation. Following this procedure, it has been our experience with at least 10 different extracts that *Limulus* extracts are very unpre-

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TABLE I. Coagulation of *Limulus* Extracts.[a]

Amount of LPS tested	Extract of Reinhold–Fine method (0.1 ml/0.2 ml)			Extract from modified method (0.1 ml)		
(μg) (min):	5	20	60	5	20	60
1	—/—[b]	—/—	—/±	+	+	+
0.1	—/—	—/—	—/±	+	+	+
0.01	—/—	—/—	—/—	—	+	+
0.001	—/—	—/—	—/—	—	—	±
None	—/—	—/—	—/—	—	—	—

[a] See text for details of preparation of the two extracts. Grading system: + is a clot that adheres to bottom of 15 × 72 mm glass test tube; ± is a clot not firm or completely adherent to glass surface; — is absence of clot formation in any recognizable form.

[b] Numerator = result using 0.1 ml *Limulus* extract; denominator = result using 0.2 ml *Limulus* extract. The assay was carried out by addition of 0.1 ml LPS preparation in phosphate buffered saline to 0.1 ml or 0.2 ml *Limulus* extract, followed by incubation at 37° in a stationary water bath, for the time indicated.

dictable in yield and activity.

Accordingly, the extraction procedure has been modified. As described above, the packed amebocyte volume is measured (after centrifugation) and 5× this volume of distilled water is added. Immediately thereafter 150 ml aliquots of the amebocyte suspension are placed in a Virtis vessel and submitted to cell rupture with a "Virtis 45" homogenizer. A blade diameter of 2 cm is used, with shaft speed of 40,000 rpm for 5 min, and the procedure is repeated once. During the homogenization, the vessel is surrounded continuously with ice water. This procedure results in rupture of all cells as judged by light microscopy. The homogenate is cleared by centrifugation at 1500*g* for 10 min. The supernatant is clear but opalescent and can be frozen in aliquots of 10 ml each at —70°. Extracts retain full potency for at least 1 yr when stored in this condition.

Results and Discussion. Data in Table I compare the potency of a *Limulus* extract prepared from an amebocyte pool isolated from 12 different crabs. The amebocyte suspension was divided into two parts, one being handled for extraction purposes according to the Reinhold–Fine procedure, the other extract being prepared with the modifications described above. In these tests LPS from *Salmonella typhimurium* (GBl, rfb, Ra) kindly provided by Dr. Mary Jane Osborn was used. When LPS isolated from *Escherichia coli* 0111:B4 (Difco Laboratories, Detroit, MI) was tested, the results were virtually identical. The *Limulus* extract obtained by the Reinhold–Fine method was relatively inactive (Table I). When incubation was carried out for 1 hr, incomplete coagulation (increased turbidity and gelation which was not adequate to cause adherence of the clot to the bottom of the tube) was seen with 100 ng LPS. In contrast to these results, the *Limulus* extract prepared according to the procedure described above was considerably more active. Within 5 min a good clot formed in the presence of 100 ng LPS; by 1 hr the cut-off point in the assay was between 1 and 10 ng LPS. By way of comparing the extracts in Table I, the protein content of the extract obtained by the Reinhold–Fine technique was 450 μg/ml whereas the extract obtained by the new method has a protein content of 960 μg/ml. The difference in total protein content does not completely account for the marked differences in potency of the two *Limulus* extracts. It should be pointed out that the data in Table I are similar to those obtained in the preparations of 10 other extracts. Representative data from a single experiment have been presented here.

The advantages of the new method for preparing *Limulus* extracts are several. Based on a homogenizing step, considerably larger yields of a more potent extract are obtained. The usual final volume of extract obtained

from 12 crabs varies from 300 to 500 ml. The entire procedure, from beginning to end, can be accomplished in 1 day. The extract itself is highly active, 0.1 ml being adequate to detect 1–10 ng LPS, with a reaction that can be read in 5–20 min, rather than 60–90 min.

In contrast to the reports of others who find considerable variation in the potency *Limulus* extracts (5), it has been our experience that the only significant variable is the yield of packed amebocytes. It may be that there are fluctuations in the resistance of amebocytes to hypotonic lysis; presumably, we have overcome this problem by mechanical disruption of the cells. While the modified procedure for obtaining potent *Limulus* extracts is not fundamentally different from that of Fine and colleagues, we believe that the procedure described above should enable interested investigators to isolate relatively highly potent and consistently active, rapid acting *Limulus* extract.

Summary. A modified procedure for obtaining the *Limulus* extract is described. The main change from the earlier described method involves homogenization of amebocytes. This results in a high yield of a potent extract useful in detection of bacterial lipopolysaccharide.

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