observed that the unfed flies in another cage kept at a distance of 3 cm moved at random in all directions with unsheathed proboscis for a period up to about 60 sec. The experiments were repeated a sufficient number of times for the summated records to be statistically reliable and are given in figure 2. Flies with intact antennae or with the arista removed responded to the sounds of other individuals of the same species, but flies with no flagellum to their antennae did not do so. This seems to indicate that the organs for perceiving the sounds are situated in the flagellum. These experiments were performed at 25 °C and at a relative humidity of 80%.

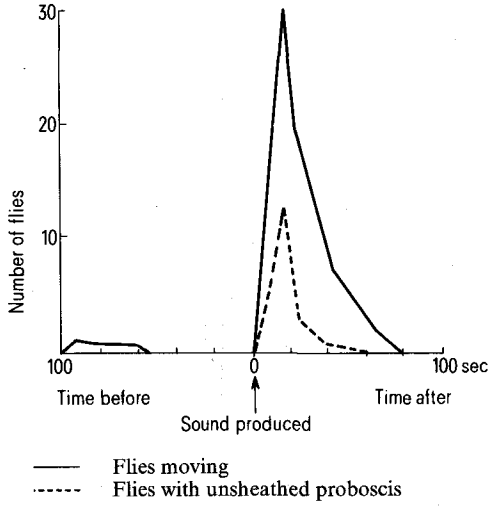


Fig. 2. Graph of response of tsetse flies to sounds produced by *Glossina morsitans*.

The tsetse flies failed to respond to the sounds of another species. The facts show that tsetse flies hear and respond to sounds produced by members of their own species.

It has been observed that tsetse flies are attracted to a host by radiant heat emitted from the skin, but feeding may be inhibited when the fur exceeds a critical length[2]. It would follow, therefore, that if a tsetse fly gorged successfully on an area of skin, the postfeeding sound could stimulate other flies which have failed to become gorged, to move away from those areas of skin where feeding is difficult. This would result in unfed flies eventually accumulating in those areas where blood could easily be obtained and/or where they could find a mate.

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## Dithiols simulate endotoxin in the *Limulus* reaction[1]

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*Summary.* Dithiothreitol, dithioerythritol and bacterial lipopolysaccharides increase optical absorbance and clot *Limulus* lysate. Purification of dithiothreitol from possible endotoxin contamination by vacuum sublimation or chromatography does not abolish the reaction with lysate. The dithiols reported active here represent the smallest molecules capable of simulating endotoxin in the *Limulus* test.

The *Limulus* amebocyte lysate (LAL) method is the most sensitive assay for the measurement of endotoxin in serum, radiopharmaceutical and biological products[2-9]. A number of proteolytic enzymes, synthetic polynucleotides and peptidoglycans have the ability to mimic endotoxin in the *Limulus* crab assay procedure[10-12]. Here we report that certain low mol. wt thiols have the same property.

*Materials and methods.* LAL was reconstituted from lyophylized material obtained from Sigma Chemical Co. Endotoxin-free water was prepared by distilling water over $KMnO_4$ in an allglass apparatus, or purchased from Abbott Laboratories. Whenever possible, disposable plasticware was employed and glassware was carefully washed and heated overnight at 180 °C. Oxidation of DTT was done according to Cleland's method[13]. LAL test was performed according to the method described by Watson et al.[14].

P-200 chromatography. In order to show that the active material was not in the high mol. wt fraction, and thus not contaminated with endotoxin, solutions containing thiols sublimed and not sublimed, were chromatographed on acrylamide P-200, BioRad Corp. 0.2 ml of the sample was chromatographed on an endotoxin-free 1.0 × 25 cm column of BioGel P-200, 50-100 mesh, which had been equilibrated with endotoxin-free 0.5 M NaCl. Fractions of 0.5 ml were collected, using the same eluant. All fractions were checked for reactivity with LAL. The void volumes were determined, using dextran blue obtained from Pharmacia.

We eliminated the possibility of contamination of thiols by degraded or retarded endotoxin by chromatographing a mixture containing 1 ng LPS and 0.2 ml of $10^{-2}$ M DTT. After such separation all LAL-active material in the thiol fraction was volatile in vacuo at 80 °C (0.005 mm).

*Results.* The table shows that significant reaction of LAL occurs in the presence of $10^{-4}$ M DTT or DTE. Such concentrations determined the gelation of LAL in 24 h at 37 °C. Samples of thiols both before and after sublimation showed the same activity with LAL. No activity would be sublimed from LPS. Mercaptoethanol, monothioglycerol or L-cysteine showed no reaction at such concentrations. Figure 1 represents the dose-response curve for LPS and

Activation of *Limulus* lysate by dithiols

| Molarity | Absorbance at 360 nm | | | |
|---|---|---|---|---|
| | DTT | | DTE | |
| | Not sublimed | Sublimed | Not sublimed | Sublimed |
| $10^{-2}$ | 1.77 | 1.56 | 1.90 | 1.6 |
| $10^{-3}$ | 1.49 | 1.22 | 1.28 | 1.22 |
| $10^{-4}$ | 0.99 | 1.08 | 1.01 | 0.87 |
| $10^{-5}$ | 0.34 | 0.23 | 0.03 | - |

The table shows OD of *Limulus* lysate after 1 h incubation at 37 °C and after subtraction of a blank of 0.1-0.2 A 360. (1 ng/ml *E. coli* endotoxin gave 0.42 units increased absorbance.)

DTT. Over the range of 0.1 ng–1.0 ng/ml the increase in optical density (OD) was a linear function of LPS-concentration. A proportionate increase with log (thiols concentrations) was observed between 1 µg and 141 µg DTT/ml. No reaction of the LAL with concentrations below 1 µg DTT/ml was recorded. Figure 2 presents a time course for LPS (1 ng/ml), $10^{-3}$ M DTT and DTE. The increased absorbance of lysate in the presence of LPS or thiols follows a curvilinear path. The kinetics of absorbance change during the LPS test are known to be complex[15]. When DTT, sublimed or not sublimed, was passed through a P-200 column, only one peak emerged in the area of the low mol.wt product (fractions 38–46). When a mixture of DTT and LPS was applied to the same column, 2 peaks active with LAL were obtained; one emerged in the first ml after the void volume (LPS-fraction) and the 2nd one eluted with 19–24 ml of the eluant (DTT-fraction). After chromatography no active substance could be sublimed for the LPS-peak while all the activity could be sublimed from the 2nd peak. These experiments were necessary to exclude the possibility of LPS-contamination of DTT, as well as the possibility of degradation or retardation of contaminating LPS by thiols. For the same reason the heat stability of DTT- and DTE-solutions was tested, since LPS is very stable. When $10^{-3}$ M DTT or DTE was heated at 100 °C for 20 min most of the ability to activate *Limulus* lysate was lost (82% and 63%, respectively), although no change in the activity of LPS was noted. When DTT was oxidized by the method of Cleland and purified by vacuum sublimation, the product was inactive in the *Limulus* test.

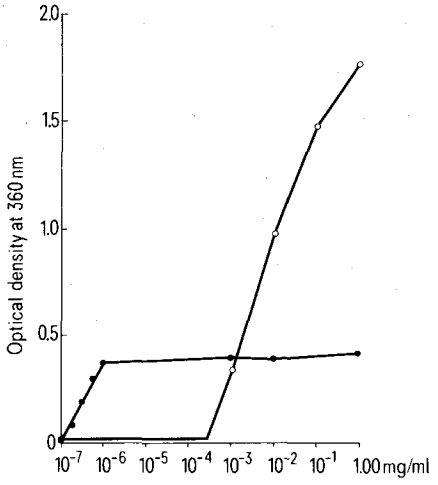


Fig. 1. Dose response curve for LPS and DTT: Between 0.1 ng and 1 ng/ml the increase of $OD_{360}$ is a linear function and LPS-concentration. Above 0.0005 mg/ml the increase in $OD_{360}$ is proportional to log (DTT-concentration). ●——● LPS, ○——○ DTT.

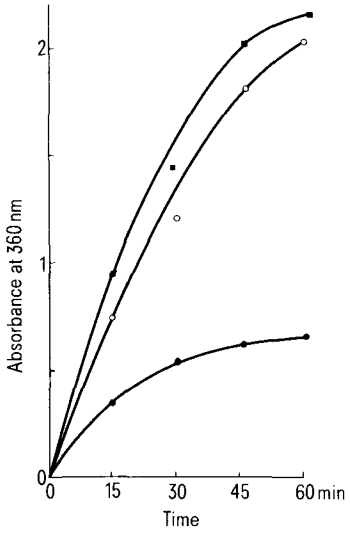


Fig.2. Time course for 1 ng/ml *E.coli* endotoxin (●——●), $10^{-3}$ M DTT (■——■) and $10^{-3}$ M DTE (○——○).

*Discussion.* The LAL-test is the most sensitive method presently available for the detection of LPS[5,6,16,17]. The specificity of the method is questioned. Products other than endotoxin can give a positive reaction with LAL[10–12]. The present demonstration that substances containing dithiol groups give the reaction with LAL is important. These are the smallest molecules known to affect the *Limulus* reaction. Contamination with dithiols would produce a false positive for LPS. These substances are frequently added to buffers to stabilize enzymes, receptors and other proteins. Therefore, for any new assay chromatography is required in order to eliminate the possibility of contamination of the sample with nonspecific material. The demonstration of activity of thiols in the LAL-test may bear on the enzymatic mechanism for the detection system which has been described by other authors. LPS apparently activates an enzyme contained in the lysate[19,20], which converts a coagulogen[10,21] to a polymerized substance which then either clots or shows an increased OD. The reaction of dithiols and LPS with LAL follows a curvilinear path. The complex reaction mechanism probably accounts for this curvilinear time course. The current work suggests that the *Limulus* enzymatic reaction may have, in part, some chelation or cross-linking by means of dithiol compounds. Studies are in progress to answer this question.

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