citrate gradient (*13*) two opalescent bands separated (Fig. 5); continued centrifugation for 20 hours did not affect the appearance of the upper band but the lower band showed splitting into multiple diffuse bands (Fig. 6). All of the above observations indicate that prolonged exposure of the Rauscher virus to concentrated potassium citrate or tartrate causes a lysis of the virus, which is aggravated by the centrifugation conditions, and a simultaneous release of subviral components which then separate in the density gradient.

The behavior of the Rauscher virus on centrifugation for short periods in potassium citrate or tartrate gradients, or on centrifugation for prolonged periods in cesium chloride, rubidium chloride or sucrose gradients, is characteristic of that expected of a "membrane virus" as shown by previous studies on influenza (*7*), Newcastle disease (*7, 14*) and Rous sarcoma (*12*) viruses. In view of the reported chemical composition of the influenza (*15*) and the avian myeloblastosis (*16*) viruses, it appears probable that "membrane" viruses in general have outer lipoprotein membranes, which comprise significant fractions of the total viruses and which enclose more dense nucleoprotein cores. Electron microscopy of the materials recovered from the prolonged centrifugation of the Rauscher virus in potassium citrate gradients suggests that dissolution of the viral membrane is concomitant with the viral lysis. Accordingly it was of interest to determine whether the behavior of the Rauscher virus on these gradients was characteristic of "membrane viruses" or a peculiarity of this etherlabile virus. The Moloney leukemia virus separated as a single band (density: 1.16 g/cm^3) in potassium citrate gradients after 3 hours centrifugation but gave multiple bands on centrifugation for 22 hours. The Rous sarcoma virus (Bryan strain), on the other hand, separated as a single band (density: 1.16 g/cm^3) in the potassium citrate gradient after centrifugation for 18 hours (*17*). Thus the susceptibility to lysis in potassium citrate gradients may reflect constitutive differences between viruses which show similar density behavior.

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N-Ethylmaleimide Inhibition of Horseshoe Crab Hemocyte Agglutination

Abstract. *The sulfhydryl inhibitor* N-*ethylmaleimide* (*NEM*) *inhibits the agglutination of horseshoe crab hemocytes. The inhibitory action is neutralized by adding cysteine to the NEM before it is mixed with the hemolymph. This new method for arresting horseshoe crab hemocyte agglutination suggests the participation of sulfhydryl groups in the process.*

Loeb (*1*) and Copley (*2*) have described two phases in the clotting of hemolymph (blood) of the horseshoe crab (*Limulus polyphemus*): hemocyte agglutination and subsequent gelation of the hemolymph. The study reported here concerns the first phase, the rapid and apparently irreversible clumping (agglutination) of the cellular elements of the shed hemolymph. In agreement with a previous report (*2*), low temperature, nonwettable surfaces, and heparin were largely ineffective in preventing hemocyte agglutination. The cation binding agents (oxalate, citrate, and ethylenediamine tetraacetate) were also ineffective. Recently, certain sulfhydryl inhibitors were shown to prevent the agglutination of mammalian platelets (*3*). We now report that under certain conditions, agglutination of *Limulus* hemocytes is inhibited by *N*-ethylmaleimide (NEM). This material alkylates sulfhydryl groups in a specific manner (*4*); the reaction is difficult to reverse (*5*).

Hemolymph was collected by inserting an 18-gauge needle into a clean leg joint and allowing the hemolymph to flow into a Krogh-Keys syringe pipet. Hemolymph appears to be an appropriate term for the fluid thus collected since there is an open circulation in this area of the *Limulus* (*6*). In these studies the hemolymph was always diluted ten times in the collection step by having in the syringe pipet a sufficient volume of one of the following test solutions: buffered NaCl, NEM, cysteine, and NEM-cysteine solutions. The diluent for *Limulus*

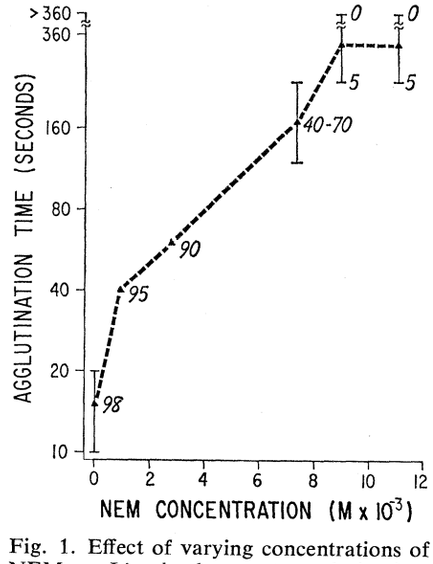

Fig. 1. Effect of varying concentrations of NEM on *Limulus* hemocyte agglutination. The points on the curve represent the time interval between the puncture of the joint and macroscopic agglutination. Included beside the points are the microscopically estimated percentages of agglutinated cells after 5 minutes of observation. Agglutination was absent for points greater than 360 seconds.

hemolymph and the solvent for NEM and for L-cysteine hydrochloride was 0.51*M* NaCl solution buffered at *p*H 7.8 with 0.01*M* tris(hydroxymethyl) aminomethane. The electrical conductivity of this diluent was approximately that of hemolymph from a horseshoe crab kept in artificial sea water (*7*). L-Cysteine HCl solutions were adjusted to *p*H 7.8 with 0.51*M* NaOH. The NEM concentrations of standard solutions were checked spectrophotometrically (*8*). All materials were maintained at 22°C. The diluted hemolymph was transferred immediately to a glass tube, agitated, and observed. The time required for macroscopic agglutination was recorded. Five minutes after collection an estimate of the relative number of agglutinated cells was obtained microscopically. Inhibition of agglutination was considered complete when the hemocytes, packed by centrifugation, could be suspended again as free cells 6 minutes after collection.

The experiments are summarized in Fig. 1. As previously reported (*2*), agglutination occurred spontaneously in 10 to 20 seconds in the absence of inhibitors. By the end of 5 minutes, more than 98 percent of the hemocytes were agglutinated. With concentrations of NEM greater than $0.9 \times 10^{-3}M$, agglutination time was prolonged and the number of agglutinated cells was decreased. Inhibition of agglutination was complete in three-fourths of the studies when either $9 \times 10^{-3}M$ or $11.1 \times 10^{-3}M$ NEM was used. In the other studies at these concentrations, less than 5 percent of the cells were agglutinated after centrifugation and suspension.

Several reagents for arresting *Limulus* hemocyte agglutination have been described. These include neodymium chloride, lanthanum nitrate, ammonium sulfate (*2*) and formaldehyde (*9*). Although effective, the use of these materials sheds little light on the mechanism of hemocyte agglutination. The inhibitory action of NEM on hemocyte agglutination indicates a possible role of sulfhydryl groups in the process. The specificity of NEM could be questioned because of the relatively high concentrations required. However, when hemolymph was collected in the test mixture which contained equimolar concentrations of $9 \times 10^{-3}M$ cysteine and NEM, hemocyte agglutination occurred. Cysteine and NEM react stoichiometrically (*8*). Cysteine alone had no effect on the hemocyte agglutination. Thus these experiments support the concept that NEM inhibits hemocyte agglutination by reacting with sulfhydryl groups.

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Daily Rhythm in the Susceptibility of an Insect to a Toxic Agent

Abstract. *Adult boll weevils exhibited a daily rhythm in their susceptibility to standardized doses of the insecticide, methyl parathion. The mortality produced by the insecticide was intimately related to the time of day at which the toxicant was applied. The rhythm appeared to be photoperiodically entrained and, regardless of the length of day or "clock time-of-day of treatment," a period of greatest resistance always occurred at dawn and recurred at 6-hour intervals throughout the 24-hour cycle. The greatest difference in response occurred in a photoperiod having 10 hours of light per 24-hour cycle. Here, the same dose of methyl parathion killed approximately 10 percent of the weevils treated at dawn but almost 90 percent of those treated only 3 hours later.*

A daily rhythm in the susceptibility of mice to certain toxic agents has been reported by Halberg and associates (*1*). In one series of experiments, these authors observed differences in mortalities ranging from 3 to 85 percent in standardized groups of mice injected with equivalent dosages of *Escherichia coli* endotoxin. The differences in mortality were related to the time of day during which the various groups of mice were injected.

The possibility that a similar phenomenon might occur in the reaction of insects to insecticides was a subject of discussion between us and C. S. Pittendrigh, of Princeton University, during his visit to our laboratory in 1963. In this conversation, as in his publications concerning circadian rhythms (*2*), Pittendrigh stressed the importance of the photoperiod in the entrainment of daily rhythms. Consequently, the following experiments were conducted.

The adult boll weevil, *Anthonomus grandis* Boh., was selected as the test animal, and methyl parathion (O, O-dimethyl-O-*p*-nitrophenylphosphorothioate) as the toxicant. Methyl parathion, a powerful cholinesterase inhibitor, is extremely toxic to the boll weevil even at low concentrations. The compound is an excellent contact insecticide, kills quickly, and has short residual properties. These qualities made this toxicant an appropriate choice for our study.

Adult weevils were reared from flower buds of infested cotton, growing from nearby fields. Buds containing the immature stages of the insect were collected and placed in special cages and held at room temperature under the day and night conditions of midsummer at College Station, Texas. On the day of emergence, the adult weevils were collected and transferred to incubators that automatically maintained particular photoperiods. Three photoperiods, having light phases of 10, 12, and 14 hours per 24-hour cycle, were used. Temperature in all cases was maintained at 27°C. Homogeneous groups of weevils were entrained to each of the three photoperiods for 10 days prior to insecticidal treatment.

Insecticide treatments were made by placing the weevils on glass surfaces coated with standardized amounts of methyl parathion. To accomplish this, the inner surfaces of glass vials (30-ml capacity) were uniformly coated with 0.85 ml of an acetone solution containing 0.0025 percent methyl parathion (technical grade, 80 percent purity). The vials were coated 30 minutes before use, and the solvent was evaporated. At 3-hour intervals beginning with "dawn," or lights on, and continuing throughout the day and night,