Table 1. Effects of tetrodotoxin (with four frog ventricles) and manganese ion (with two ventricles) on the rate of rise of the initial phase and the amplitude of overshoot of the plateau phase; means ± S.D. Numbers of measurements appear in parentheses.

Concentration	Overshoot (mv)	Rate of rise, maximum (volts per second)
Tetrodotoxin (g/ml)		
0 (9)	43 ± 5	36 ± 15
1×10^{-8} (19)	45 ± 5	20 ± 7
5×10^{-8} (8)	39 ± 6	6 ± 1
Manganese ion (mM)		
0 (19)	45 ± 6	50 ± 13
2 (12)	33 ± 5	48 ± 16
4 (14)	32 ± 3	47 ± 17
8 (18)	30 ± 6	33 ± 14

more, it has been shown that even at high concentrations (4×10^{-6} g/ml) tetrodotoxin has no effect on the action potential induced by Ca ions (Ca spike) in fibers of barnacle muscle (*6*). By contrast, the Ca spike is suppressed or abolished when manganese ions are added to the external solution (*6*); this agrees with the result of Fatt and Ginsborg (*7*). The effect of Mn^{++} becomes less marked with increasing external concentration of Ca, suggesting that Mn^{++} acts as a competitive inhibitor of the initiation of the Ca spike (*6*).

Brady and Woodbury (*8*) showed that the initial rapid rise of the action potential of the frog cardiac ventricle is due to increase in conductance by the membrane of Na ions. However, Coraboeuf and Otsuka (*9*) observed that, at the peak of the action potential, the membrane of the guinea pig ventricle did not behave as a sodium electrode; and the recent data of Orkand and Niedergerke (*10*) suggest that Ca-ion permeability also may be implicated in the plateau phase of action potentials of fibers of frog ventricle. Thus the action potential reached a peak in the plateau phase sometime after the initial rapid rise, and the maximum active membrane potential (overshoot) was essentially independent of the external Na-ion concentration over a considerable range when the Na ions were replaced by choline ions (*10*). If the initial rapidly rising phase of the spike potential in the fiber of frog ventricle were produced by increased permeability to Na ions, and if the plateau phase were related principally to permeability by Ca ions, the major effect of tetrodotoxin would be on the rate of rise of the spike potential, and the effect of manganese ions would be mainly on the overshoot.

Resting and spike potentials were recorded intracellularly by means of micropipettes filled with 3*M* KCl and inserted into a strip of isolated frog ventricle some distance from the stimulating electrodes; stimuli were delivered at an interval longer than one minute.

Application of tetrodotoxin (1×10^{-8} g/ml) decreased the maximum rate of rise of the initial phase to about 60 percent of the control value, but the amplitude of the overshoot of the plateau phase did not change at this concentration (Table 1). A higher concentration (5×10^{-8} g/ml) decreased the maximum rate of rise to about 17 percent of the control, whereas the overshoot was lowered by only 10 percent (Table 1). Plotting the relation between the rate of rise and the amplitude of overshoot obtained in each measurement (instead of grouping and averaging the values according to concentration) showed that there was essentially no change in the amplitude of the overshoot unless the rate of rise became less than 5 volt/sec—that is, about 14 percent of the control. When the rate decreased to below 5 volt/sec, the overshoot often became smaller, and in extreme cases, a spike of short duration, without the subsequent plateau phase, was observed. Fig. 1 shows typical examples of the marked effect of tetrodotoxin on the rate of rise and its relative lack of effect on the amplitude of overshoot.

When $MnCl_2$ (2 to 4 m*M*) was added to the Ringer solution by replacing the osmotically equivalent NaCl (Ca concentration was kept constant at 1.8 m*M* throughout), there was no significant change in the rate of rise (Table 1). This fact, however, caused a characteristic change in the plateau potential (Fig. 2). The potential tended to rise more slowly after the initial rapid rise, the development of the plateau being delayed (Fig. 2*B*). At the same time, the potential became lower —that is, the overshoot became smaller. With 8 to 10 m*M* $MnCl_2$, the initial rapid rise of the potential was often followed by a dip before it rose to the peak of the plateau (Fig. 2*C*), and finally the plateau almost disappeared (Fig. 2*D*). At these concentrations there was also some decrease in the rate of rise of the spike (Table 1), but even the rate of rise in this condition was much larger than that associated with any observable change of the plateau of the spike under tetrodotoxin. The effects of tetrodotoxin and Mn^{++} were usually reversible.

These findings, if considered in the light of data obtained on fiber of barnacle muscle, are in accord with the idea that the initial rise of the action potential in ventricle fiber of the frog is related to the increase in permeability to Na, while the plateau phase is related to the increase in permeability to Ca. However, this does not exclude the possibility that the changes in permeability to Na are also implicated in the plateau phase.

SUSUMU HAGIWARA
SHIGEHIRO NAKAJIMA
Department of Zoology and *Brain Research Institute, University of California, Los Angeles*

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Anesthesia of Artemia Larvae: Method for Quantitative Study

Abstract. *Potency of anesthetics is quantitatively measured with laboratory-hatched larvae of the brine shrimp* Artemia salina. *Statistical fluctuations are minimized in that 100,000 animals are used to determine a single median anesthetic dose value. The technique was developed to study molecular mechanisms of general anesthesia.*

Accurate values for AD_{50} (the dose necessary to produce anesthesia in 50 percent of animals) are needed to assess the relative merits of the recent molecular theories on general anesthesia of Pauling (*1*) and Miller (*2*), and of the theory advanced 65 years ago

by Meyer (*3*) and Overton (*4*). Pauling proposed that general anesthetics act by stabilizing clathrate-like structures in the encephalonic fluid of the neural system. This theory implies that anesthetic potency should correlate not only with the electric polarizability of the molecules of the anesthetic agent but also with their size and shape. However, the contribution to anesthetic potency attributable to different steric arrangements of the atoms in anesthetic agents of similar molecular composition has not yet been accurately demonstrated.

To obtain anesthetic potencies accurate enough to test these theories, the biological system should have the following characteristics:

1) The system should permit measurement of anesthetic potency at very near true thermodynamic equilibrium. At equilibrium the activity of each component is the same in all parts of a system, so that measurements in the environment of the organism should also be applicable to sites within the organism. Therefore no assumptions are needed with regard to transport of the anesthetic agent to its site of action in the organism (*5*).

2) The system should permit measurements of great precision in order to show the small differences in potency in a series of anesthetic molecules with dissimilar spatial configurations. Variations in the susceptibility of individual organisms, variations caused by environmental and time factors, and statistical fluctuations produce probable errors of more than 10 percent in most pharmacological studies.

3) It must be possible to control accurately the parameters of temperature, pressure, and anesthetic concentration in the external and internal environments of the organism (the internal environment is the chemical system that makes up the organism itself). Variation of these parameters over wide ranges should not cause irreversible changes in the organism; otherwise, phenomena such as toxicity instead of anesthetic potency may be measured.

4) Criteria for anesthesia should represent a real depression of motor activity and sensory thresholds.

5) The technique should be convenient and fast as well as reproducible, so that the effects of widely ranging conditions may be investigated.

With these goals in mind, we carried out reversible anesthesia on several small organisms, including the *Alga dunaliella*, the flagellate *Tetrahymena*, the embryo of the sea urchin *Lytechinus pictus*, and larvae of the brine shrimp *Artemia salina*. Ten times the concentration of anesthetic agent required to suppress movement of the shrimp is needed to similarly affect *Alga*, *Tetrahymena*, and the sea-urchin embryo. The AD_{50} values for shrimp are near those for fish (*6*); the values for primates are still lower. These comparisons illustrate the correlation between organism simplicity and increased AD_{50}.

Artemia salina was studied because: (i) Brine shrimp eggs are commercially available and the homogeneity of several lots of dried eggs can be assured by pooling and mixing. (ii) The environment of the larvae from hatch until the end of the experiment can be easily controlled. (iii) Large populations of brine shrimp of uniform vitality can be obtained by selecting larvae that swim toward light from a darkened beaker to an illuminated beaker. (iv) Loss of the brine shrimp's ability to swim toward light provides a convenient end point of anesthesia for two reasons: it is visibly manifest, and it permits separation of the sample into two portions—those anesthetized and those not anesthetized—without requiring a subjective decision on each animal. (v) Use of a photoelectric brine-shrimp counter makes possible accurate and objective counts of large populations to determine the percentage anesthetized. (vi) The brine shrimp may be completely revived after anesthesia by evaporation of the anesthetic agent. (vii) Potency of anesthetics at different temperatures may be measured, since the brine shrimp adapts easily to a wide range of temperatures.

Table 1. Characteristics of five anesthetics.

AD_{50} (g/liter)	Partial pressure, calc. (mm-Hg)	Mole refraction (cm^3/mole)
	Chloroform	
0.0682	1.4	21.2
	Ethyl ether	
1.14	6.8	22.3
	Halothane	
0.0415	2.3	24.0
	n-*Pentane*	
0.0120	100	25.0
	Cyclopropane	
0.292 atm	220	14.6

Several pints of dry *A. salina* eggs (*7*) were pooled and thoroughly mixed to provide a homogeneous source of shrimp. For each experiment, 3 g of dry eggs were added to 2 liters of artificial sea water (*8*); no nutrients were added. This water was used throughout the experiments. The sea water and the hatching eggs were vigorously aerated at a controlled 20°C. The effective age of a shrimp is markedly affected by the temperature at which it is raised. The time schedule was scrupulously observed during the experiments, since the density of the individual shrimp decreases with age.

At 24 hours the shrimp were transferred to a darkened 4-liter beaker connected to a similar beaker by a horizontal tube that was partially obstructed by a number of grids made of plastic window screen. The second beaker was brilliantly illuminated by a 200-watt photo-enlarger bulb placed so that some light shone through the horizontal tube into the darkened beaker (Fig. 1). Viable brine shrimp swam along the beam of light through

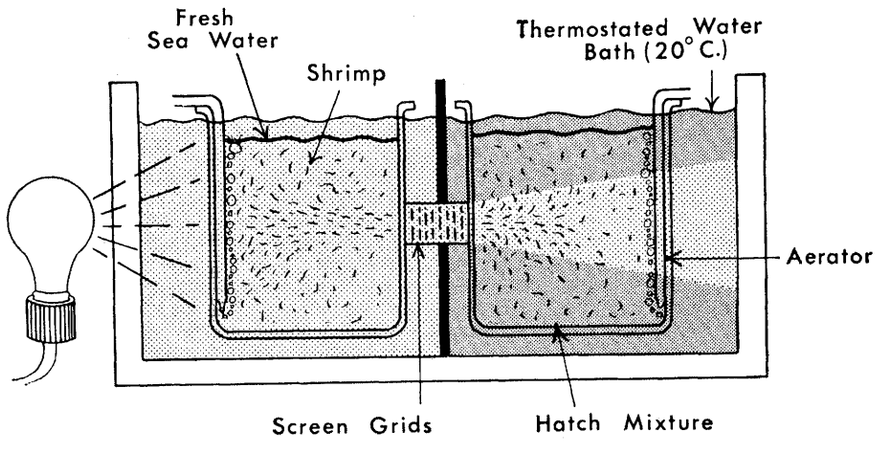

Fig. 1. Apparatus for separating active from inactive shrimp and from refuse.

the maze in the connecting tube and into the illuminated beaker. Both beakers were aerated at 20.0°C; the grids in the connecting tube prevented mixing by currents caused by the aerators.

Forty-eight hours after their first wetting, unhatched eggs and hatch debris in the darkened beaker were discarded, and the 4 liters of sea water containing 100,000 to 150,000 actively swimming larvae in the illuminated beaker was divided into 16 approximately equal portions. Each portion was transferred to a standard 500-ml separatory funnel that had been fitted with a detachable tube below the stopcock, into which anesthetized shrimp would fall. Each separatory funnel was surrounded by an opaque, matte-black cylinder, so that only the top 6 cm was exposed to light (Fig. 2).

Concentrated solutions were prepared by injecting a measured volume of anesthetic agent into sea water in a 250-ml iodine flask. The agent was injected below the surface of the water, and a slight excess of water was immediately expelled by inserting the glass stopper so that no air bubbles were left in the flask. The contents of the flask were placed in a water bath at 20.0°C and were stirred continuously for 8 hours. A measured volume of the concentrated solution was pipetted under *positive* pressure into each of the 16 separatory funnels, which were already three-fourths full. Each funnel was then quickly filled with sea water and stoppered so as to expel and exclude air. Analyses (by vapor-phase chromatography) of samples of anesthetic solution taken at various stages of an experiment with cyclopropane showed that the losses of anesthetic agent during the preparation, transfer, and anesthesia were negligible (*9*).

The 16 funnels were arranged in two rows in a large water bath at 20.0°C. The top 6 cm of the funnels was equally illuminated by "warm white" fluorescent lamps, shining almost horizontally (Fig. 2). At first the shrimp congregated in the lighted part of the funnels; later, as the phototactic swimming activity became depressed by the anesthetic, some sank to the bottom of the funnels, through the stopcocks, and into the bottom tubes. Ten hours were allowed for each experiment to ensure equilibrium; the change in the percentage of shrimp anesthetized was negligible after 5 hours.

Ten hours after addition of the anesthetic agent the stopcocks were

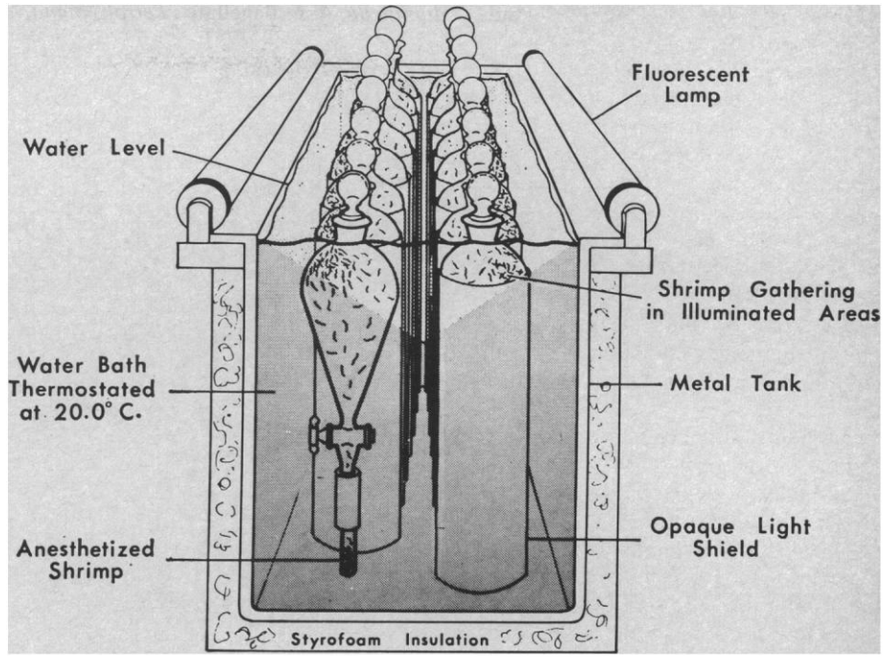

Fig. 2. Arrangement of the 16 separatory funnels as anesthesia chambers.

closed, the funnels were removed from the water bath, the upper portions were separated from the lower portions, the shrimp were killed by addition of acetone, and the number of shrimp in each portion was counted (Fig. 3). A suspension of shrimp, kept dispersed by aeration, was passed through a capillary tube on which a beam of light from a 6-volt d-c bulb was focused. The shadow of each passing shrimp caused a change of resistance in a cadmium sulfide cell placed behind the capillary. The pulse caused by the momentary change of resistance was made acceptable to a Nuclear-Chicago model 151A counter by a pulse amplifier and a Schmidt trigger-pulse-shaping circuit. Thus we were easily able to count 20,000 shrimp per hour.

We assume that the distribution function for the anesthetic sensitivity of individual brine shrimp is a Gaussian function of the log of the concentration of the anesthetic agent (Fig. 4). Therefore, the conversions to the AD_{50} are conveniently made on log-probability paper ("probit paper") (*10*) (Fig. 5). The slope of the straight line is a measure of the width of the Gaussian curve; it was reproducible in ex-

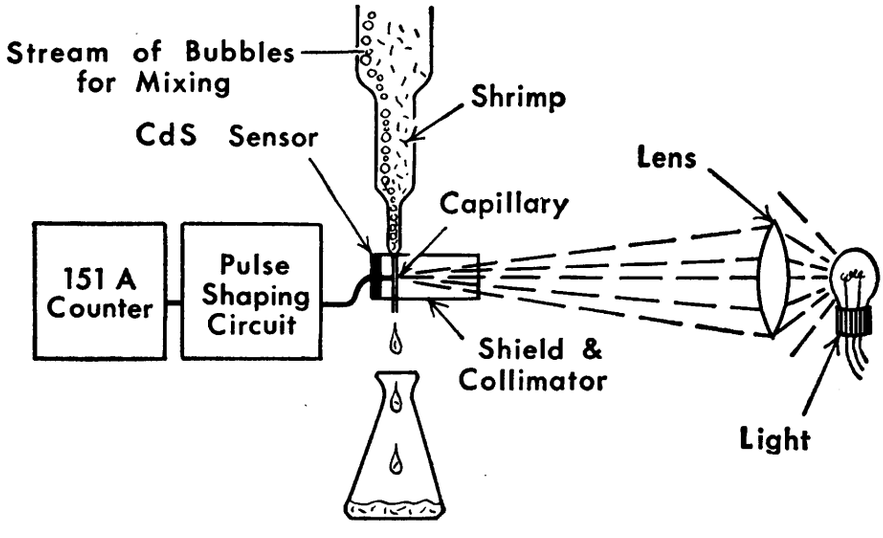

Fig. 3. The shrimp counter.

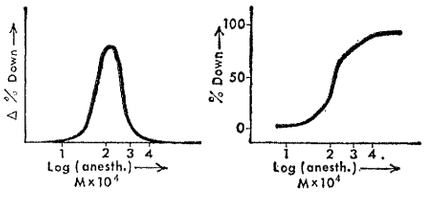

Fig. 4. Statistical behavior of a population of *Artemia salina* as a function of concentration of anesthetic.

periments with a given temperature and anesthetic agent, but varied with change of temperature or anesthetic agent.

Before equilibrium is reached (during the first 5 hours) the points plotted are best fitted by a curved line. In all results reported the shrimp were exposed to the anesthetic for 10 hours to ensure equilibrium. Even after 10 hours the shrimp could still be revived by allowing the anesthetic agent to evaporate; on recovery they were normal in behavior.

We performed a series of five experiments with Halothane ($CF_3CClBrH$) at 20.0°C. Each AD_{50} value was determined with 12 concentrations of anesthetic agent. The values were 207, 211, 196, 214, and 224 μmole of Halothane per liter of sea water. The mean is 210; the unbiased standard deviation of a single determination is 10 μmole/liter (5 percent). The standard deviation of the mean of five replicate determinations is 2.1 percent. Corresponding probable errors are 3.2 and 1.4 percent, respectively. At the end of each experiment 1 or 2 percent of the shrimp were always down in the four control

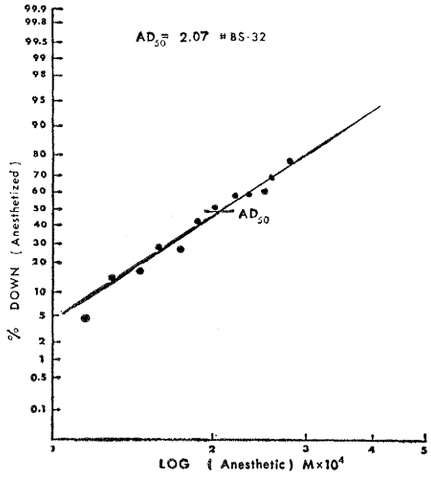

Fig. 5. Data from a single experiment with Halothane.

funnels that were included with each set of 12 doses. This percentage, as a normal death rate, was deducted from the percentage down in each experimental funnel.

The AD_{50} values for four other anesthetic agents were determined by single runs (12 concentrations per experiment) (Table 1). Solubility values in fresh water were used to convert the AD_{50}'s into partial pressures of the anesthetic agent, by use of Henry's law; correction for difference in solubility of the anesthetic in sea water from that in fresh water has not been made.

We can now determine the potency of anesthetic agents near thermodynamic equilibrium with a greater precision than hitherto possible. Thus the hypothesis that potency is related to the size and shape of the molecules of the anesthetic agent may be tested; possible synergistic behavior of anesthetic agents of different size may also be investigated. Synergism is suggested by the proposal of a clathrate structure with two cavities of different sizes (see *1*).

Temperature is an important parameter throughout. In addition to its direct effect on anesthesia, temperature affects the physiological age and buoyant density of the brine shrimp. The more lethargic behavior of the unanesthetized controls in trials at 10°C may be due to the anesthetizing effect of the low temperature itself.

The method can be made even more precise than this report indicates. We present absolute AD_{50} values obtained at different times on different populations of shrimp. If a standard anesthetic agent were run with each population, or if anesthetic agents to be compared were run on the same population of shrimp, higher precision could be expected; use might also be made of the extremely reproducible slope of the lines in Fig. 5. All variation reported in the Halothane experiments was due to shifts in the intercept; the slope was constant.

Our results with several anesthetics of similar electric polarizability agree with the hydrate-microcrystal theory of general anesthesia. For instance, the results in Table 1 show that, of the four anesthetic agents with similar electric polarizabilities, chloroform, the most spherical and the best fit for the hexakaidecahedral cavities of the type II hydrate structure, is by far the most potent. Halothane, the next most potent, is not such a good fit into the hydrate cavity because its molecule is slightly larger and less spherically symmetrical than the chloroform molecule. Diethyl ether is a linear molecule that would be a poor fit, but conceivably it might stabilize the hydrate by making use of its hydrogen-bonding capability. *n*-Pentane has the unfavorable geometry of diethyl ether and does not form hydrogen bonds. Cyclopropane, although it has a roughly spherical molecule and forms hydrates, is the least potent of this series, presumably because it has an electric polarizability only half as great as that of the other four substances.

With anesthetic-potency values accurate to 1 or 2 percent, one may adequately test the proposal (*1*) that a combination of anesthetic agents of different molecular size may act synergistically by occupying both the larger and the smaller cavities of the hydrate, thus enhancing its stability; this should be an interesting test of the hydrate-microcrystal theory.

ARTHUR B. ROBINSON
KENNETH F. MANLY
MICHAEL P. ANTHONY
JOHN F. CATCHPOOL
LINUS PAULING
Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena

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9. We thank Louis Newman for the cyclopropane analysis.
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11. Work supported by a grant from the Ford Foundation. California Institute of Technology communication 3260. Authors' present addresses: A.B.R., Chemistry Department, University of California at San Diego, La Jolla; K.F.M., Biology Department, Massachusetts Institute of Technology, Cambridge; M.P.A., Physics Department, California Institute of Technology, Pasadena; J.F.C., University of California Medical Center, San Francisco; L.P., Center for the Study of Democratic Institutions, Santa Barbara, Calif.

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