Effect of Inhalation Anesthetics on Swimming Activity of Artemia Salina

Mitsuhiro TAKASAKI, Tsuneo TATARA*, Yukio SUEZAKI**, Keishiro SHIRAHAMA***, Hiroshi KAMAYA*, Issaku UEDA*, and Tadahide TOTOKI

The swimming movement of artemia salina in the artificial sea water was measured by using the video camera system in the absence and the presence of anesthetics, i.e. enflurane, halothane, and isoflurane. The movement of artemia looked random at a glance but the obtained distribution curve for the swimming speed was skewed toward the high speed side somewhat resembling a Maxwellian distribution curve seen in the statistics of ideal gases. When anesthetics were added, the distribution curve became sharpened and shifted to the low speed side, which is similar to a behavior of ideal gasses when they are cooled down. The mean swimming-speed was decreased eventually leading to an irreversible death with increasing the anesthetic dose. The activity was analyzed by using the hydrodynamic equation. The ED_{50}, which is a dose that causes a 50% reduction in the activity, of all anesthetics used in this study was quite similar to the MAC values for human. It was also suggested that an interaction between anesthetics and artemia was highly cooperative since the large Hill coefficients were obtained for all three anesthetics used. (Key words: inhalation anesthetics, artemia salina, motility, video monitoring, cooperativity)

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There is no accepted theory on the mechanism of anesthesia while various reports have been produced over a long time[1–4]. It is suggested that the anesthetic action should be considered as an integrated effect on many parts such as membrane, protein, ion channel and so on, but not on single part. Although it is useful to examine the nature of anesthetic action by using a living animal as a whole system, experiments using a living animal are apt to be affected by many factors, and difficult to obtain accurate and reproducible data. In order to cope with these difficulties, it is necessary to use many animals in the same condition. It has been reported that artemia salina is one of the primitive animals[5] and highly sensitive to a wide range of chemicals[5–7]. In this study, we have measured the swimming motion of single artemia in the artificial sea water by a video camera system, and analyzed the data by hydrodynamics and statistical mechanics of stochastic processes.

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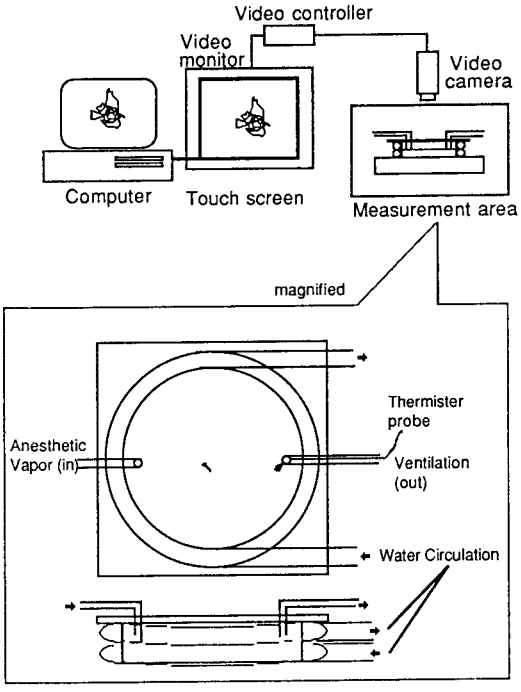

Fig. 1. Schematic diagram of measuring system.

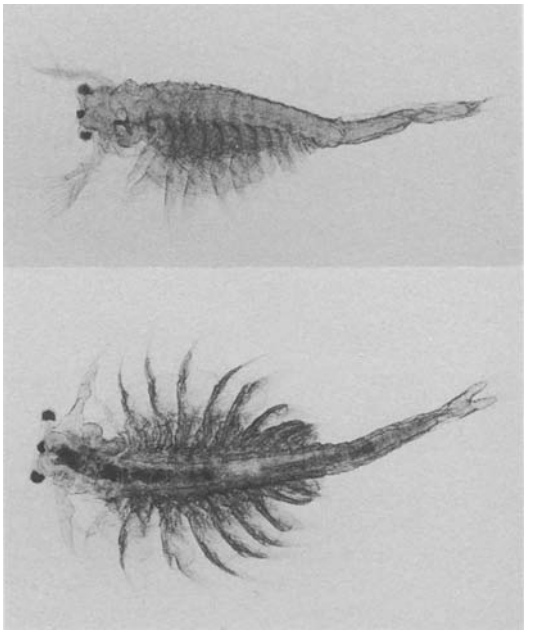

Fig. 2. Photograph of artemia salina (brine shrimp) aged three weeks.

Haloganated methylethyl ether, such as enflurane, halothane, and isoflurane are the inhalation anesthetics most popularly used clinically. The effect of the above three anesthetics on the motion of artemia was investigated to elucidate the properties of anesthetic action.

Materials and Method

Artemia eggs and artificial sea water salt were purchased from Artemia Utah and Carolina Biological Supply Co., respectively. All materials and reagents were in the purest grade available and used as received.

Preparation of the artificial sea water

The salinity of artificial sea water was approximately adjusted to 7% for hatching and growing up larvae and 3.5% for growing up adults and measurements, respectively.

Hatching eggs

A spoonful of dried artemia eggs was put into distilled water and allowed to stand for 1 hr to separate the eggs sinking to the bottom of water from those floating. The sinking eggs were transferred into 7% of artificial sea water and illuminated continuously with a fluorescent lamp. Hatching resulted within 24 hrs. Artemia is apt to die especially at the stage of nauplii. This may be caused by either contamination by excess metabolites and excretion of artemia or various infection. Frequent replacement of the artificial sea water and the use of concentrated sea water could avoid these troubles. Artemia were fed by dried yeasts. They can grow up until 1 cm or more and survive several months.

Measurements of motility

The block diagram of the measuring system is shown in figure 1. A petri dish (10 cm diameter, 1 cm depth) in a glass container (4.5 cm depth) was filled with the artificial sea water (3.5%). Since some of artemia preferred to stay on the edge of the dish, they sometimes hampered the motility measurement. Using a petri dish, instead of a glass container only, artificial sea water heaped up by the surface tension which imparts homogeneous force inwards. This was effective to minimize the problem. A single

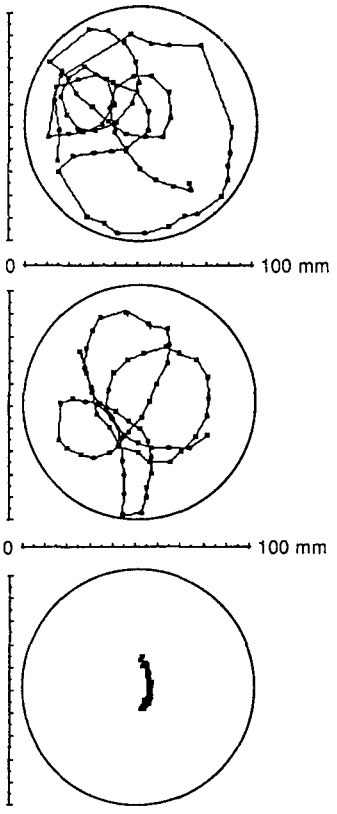

Fig. 3. Typical traces of location of artemia salina.
(a) 100% (control) (b) 50% and (c) 10% of activities.

artemia (fig. 2) which was 5 mm long, about 3 weeks old and was swimming actively, was transferred into the dish, covered with the clear acrylate plate supplied with two PVC tubings as an inlet and outlet of the air and gasses. The temperature was maintained at 25°C by circulating the constant temperature water outside the glass container. The swimming movement of the artemia was monitored from the top of the glass container by a video camera (Mitsubishi, IT-81-U). The video signal was transmitted to the digital video adaptor (Sony, XV-D300) via camera controller (Mitsubishi, PA-81-U) to be digitized and displayed as intermitted motion pictures every 0.5 second on the monitor screen (Apple computer Inc., A3M0039). The coordinates in x and y directions of the artemia position were taken every 0.5 seconds into the computer memory by pressing the touch screen system (NEC, PC9873) which was attached on the video monitor screen. The glass container was illuminated from the bottom by the homogeneous fluorescent light to minimize the phototactic side effect.

Administration of anesthetics

An inhalation anesthetic was vaporized by a copper kettle of an anesthesia machine (TEXAS MODEL, FOREGGER, New York) with air and introduced through the PVC tube. The flow of the vapor was kept constant and measured every 10 min. The measurement continued for 30 seconds at a run. The anesthetic gas concentration was measured immediately after the measurement of movement of artemia by using a gas chromatography (GC-MINI 2, Shimadzu).

Results

When the artemia was placed into the artificial sea water, it swam at random with changing both direction and speed frequently. There seemed to be no favorite direction for swimming except that some of artemia tended to stay on the edge of the glass ware. The measurement was stopped if the artemia stayed on the edge for a long time, or the artemia made the continuous rotational motion with smal radii. The movement of the artemia was slowed down as anesthetic gas was introduced into the system. Significant differences in the onset times were not seen for all three anesthetics used. Typical tracings of the movement change at three concentrations of an anesthetic are shown in figs 3(a)–(c), where (a) was for control, or without anesthetic, (b) and (c) were movements for the moderately affected by the anesthetic (about 50% of activity obtained as a ratio of square velocities with and without anesthetic), and for the highly affected by the anesthetic (about 10% of activity), respectively.

The changes in the velocity with time and its distribution curves corresponding to the cases in fig. 3 are shown in fig. 4 top and bottom, respectively.

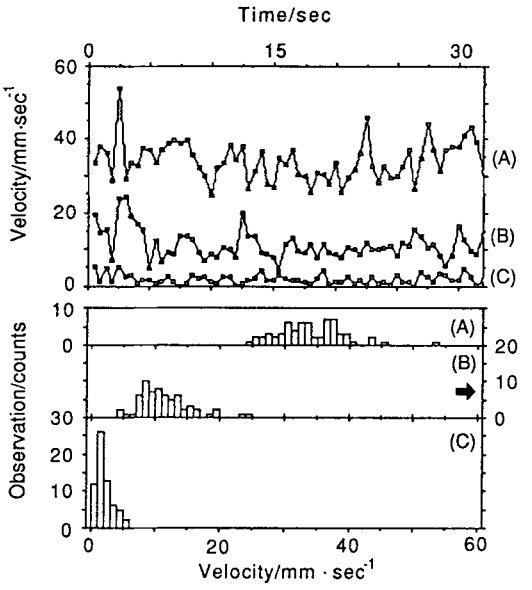

Fig. 4. (TOP) Typical traces of velocity with time.
(BOTTOM) Velocity distribution curves.
(a) 100% (control) (b) 50% and (c) 10% of activities.

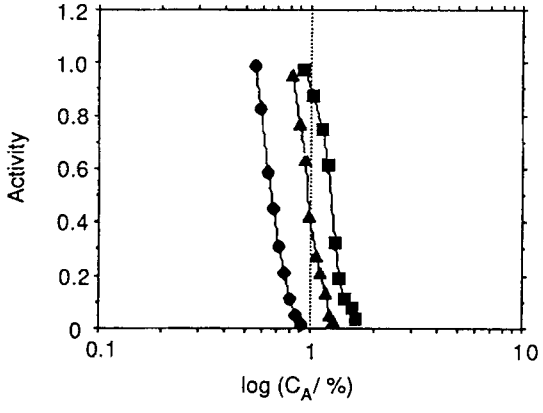

Fig. 5. Dose responce curves for (■) enflurane, (●) halothane, and (▲) isoflurane.

Activities at various anesthetic concentrations were calculated from the velocity data, and plotted against the logarithmic concentration of anesthetics in fig. 5. The sigmoidal decrease in the activities were observed for three anesthetics used. The anesthetic concentration at which the activity was decreased by 50%, i.e. ED_{50}, was in good agreement with the MAC (minimum alveolar concentration) for human for all three anesthetics (fig. 6).

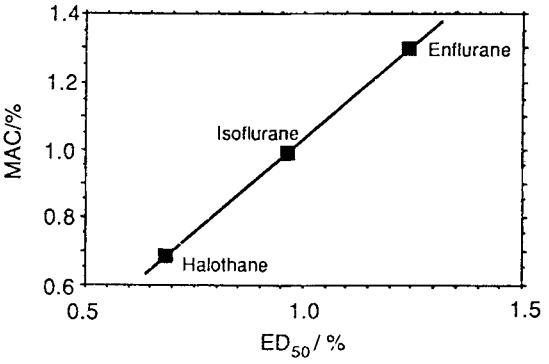

Fig. 6. Relationship between MAC and ED_{50}.

Discussion

The distribution curve of the swimming speed in the absence of anesthetics was skewed toward the high speed side resembling a Maxwellian distribution curve as seen in the gaseous statistics. When anesthetics were added, the distribution became sharpened and shifted to the slower speed side, which is similar to a behavior of gasses when they are cooled down.

The hydrodynamic equation was employed to analyze the motion of artemia[8]. The shape of artemia is coarsely regarded as spherical for simplicity. Because the depth of the measuring container was not so deep the motion of artemia was considered to be confined in the (x, y) plane. The equation of the motion of artemia can be written as

$$m\, du/dt = -6\,\pi\mu a u + f(t) \qquad (1)$$

in which m is the mass of artemia, u the velocity, a the radius of artemia, μ the viscosity of aqueous media, and f(t) the fluctuating force which causes the motility of artemia itself. It is necessary to examine whether Eq. (1) is adequate or not, prior to analyzing the actual data. While the term for the viscosity appears explicitly in Eq. (1), there exists a resistant force due to inertia beside the resistance due to viscosity of aqueous media. The force of inertia makes the uniformity of the flow disturbed, on the other hand the viscous force makes the uniformity of the flow increased. The Reynolds number, R, or

the ratio of the resistance force due to inertia over that due to viscosity is a good index to characterize the hydrodynamic properties of the motion of the particle, and calculated by Eq. (2).

$$R = 0.2\,\rho a u/\mu \tag{2}$$

When this number is not large, the flow will be laminar. If the Reynolds number exceeds several hundreds, turbulent flow accompanied with the random fluctuation must be considered. Because the Reynolds number was coarsely estimated to be about 15 by using the mean velocity obtained for our control case ($u = 2.5$ cm·sec^{-1}) and the realistic values for other parameters[8] ($\rho = 1$ g·cm^{-3}, $\mu = 0.01$ poise, $a = 0.3$ cm), Eq. (1) was applicable to analyze the motion of artemia as the first approximation[9].

Eq. (1) can be solved and written in the following form

$$u = u_0 \exp(-\beta t) + \exp(-\beta t)\int_0^t \exp(\beta t')f(t')dt' \tag{3}$$

where $\beta = 6\pi\mu a/m$ and u_0 represents the initial velocity of artemia. By taking the average, Eq. (3) becomes

$$\langle u\rangle_{u_0} = u_0 \exp(-\beta t) + \exp(-\beta t)\int_0^t dt' \exp(\beta t')\langle f(t')\rangle \tag{4}$$

Because the average of the random force is zero ($\langle f(t)\rangle = 0$), final from of $\langle u\rangle$ becomes

$$\langle u\rangle_{u_0} = u_0 \exp(-\beta t) \tag{5}$$

Next, from Eq. (5), the following equation is derived for mean square velocity

$$\langle u^2\rangle = u_0^2 \exp(-2\beta t) + \exp(-2\beta t) \times \int_0^t dt'dt'' \exp(\beta(t'+t''))\langle f(t')f(t'')\rangle \tag{6}$$

Due to the nature of the fluctuating force, f(t), the ensemble average of the second term of the right hand side of Eq. (6) can be rewritten as

$$\langle f(t')f(t'')\rangle = \phi(t'-t'') \tag{7}$$

By changing the variables of t' and t'' to $v = t' + t''$ and $w = t' - t''$, we have

$$\langle u^2\rangle_{u_0} = u_0^2 \exp(-2\beta t) + (1/2)\exp(-2\beta t) \times \int_0^{2t} dv \exp(\beta v)\int_{-\infty}^{+\infty}\phi(w)dw = u_0^2 \exp(-2\beta t) + (\tau/2\beta)[1 - \exp(-2\beta t)] \tag{8}$$

where $\tau = \int_{-\infty}^{+\infty}\phi(w)dw$.

In the case of passive small particles such as molecules or small colloidal particles, the mean square velocity is related to the thermal energy kT (k and T are the Boltzmann constant and absolute temperature, respectively) in the form $\langle u^2\rangle/m = kT$. In our case, artemia is not a passive particle but an active shrimp which exerts random force f(t) by consumption of its own nutrient energy. The absolute value and direction of the individual motion of artemia are observed to be random when we look at their motion.

On the other hand, the mean square velocity relates to the average kinetic energy consumption per unit time, E, of an artemia as shown below. When an artemia moves small distance dx in small time dt, the increment of energy dW is represented by

$$dW = 6\pi\mu a u^2 dt \tag{9}$$

where we have utilized the fact that $dx = udt$. By definition, E becomes

$$E = \langle dW/dt\rangle = 6\pi\mu a\langle u^2\rangle \tag{10}$$

Thus, we have arrived at the conclusion that observed mean square velocity is related to the energy consumption of artemia exerted due to the frictional force of the suspended solvent. If we take the typical set of numerical values used in the derivation of Eq. (4), the value of E turns out to be

$$E = 0.35 \text{ erg/sec} = 35 \times 10^{-9} W \tag{11}$$

The value of E might be controlled by some neural system of artemia and an anesthetic could be considered to control the value of E.

Here the activity, a, is defined as

$$a = E/E_0 \tag{12}$$

where E and E_0 are the energy consumptions of an artemia in the presence and absence of anesthesia, respectively.

The activity decreased from unity to zero in very narrow concentration range as shown

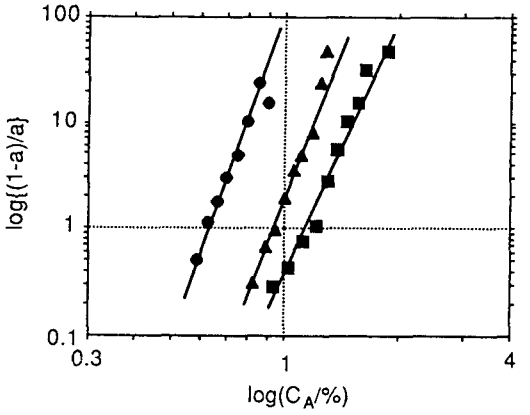

Fig. 7. Hill plots for (■) enflurane, (●) halothane, and (▲) isoflurane.

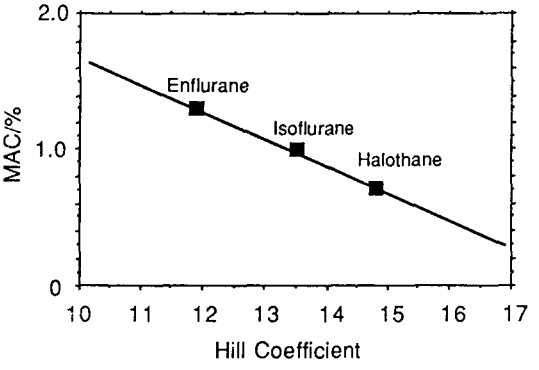

Fig. 8. Relationship between MAC and Hill Coefficients.
(■) enflurane, (●) halothane, and (▲) isoflurane.

in fig. 5. It is interesting to notice that the ED_{50}s for artemia were similar to MAC values for humans. It might suggest some common factors in the molecular action of anesthetics. The steep sigmoidal curves can be explained by assuming the cooperative reaction between anesthetics and the binding sites of artemia as follows:

$$S + nA \rightarrow SA_n \ldots\ldots$$
Anesthetized artemia (13)

where S represents anesthetic binding site of artemia, A the anesthetic molecule. According to this reaction scheme, the equilibrium constant can be calculated by

$$K = [SA_n]/[A]^n[S] \quad (14)$$

In this equation, $[SA_n]/[S]$ represents the ratio [sites occupied by anesthetics] to [sites available]. If we assume the activity, a, equals to the ratio $[S]/([S] + [SA_n])$, Eq. (14) becomes

$$K = (1 - a)/a[A]^n \quad (15)$$

$$(1 - a)/a = K[A]^n \quad (16)$$

By taking the logarithm of Eq. (16), we obtain

$$\log\{(1 - a)/a\} = n \log [A] + \log K \quad (17)$$

This equation is usually called Hill's equation[9]. In case of n=1, there is no cooperativity and resulted in the Langmuir-type adsorption or the first order chemical reaction. Hill plots for anesthetics used in this study are shown in fig. 7. Hill coefficient, n, obtained from the slope of Hill plot, were 11.9, 14.8, and 13.5 for enflurane, halothane, and isoflurane, respectively. A cluster of n anesthetic molecules is considered to be bound at a time. The value of n represents the degree of cooperativity but not the number of binding sites. All anesthetics used in this study shows the large value of n. A clinically stronger anesthetic has a larger Hill coefficient as shown in fig. 8. The mode of anesthetic binding to artemia was significantly cooperatiye. The nature of anesthetic binding to lipid membranes has been investigated by many researchers[10-14]. This result is consistent with the report that the binding of anesthetics to lipid membrane showed atypical Langmuir type adsorption[13,14]. The cooperativity obtained from the electrophysiological study using an isolated biological cell, however, is very small. The values of n are no more than 2[15,16]. It has also been reported[17,18] that there is no or little effect on the fluidity of the lipid membrane by the clinical concentration of anesthetics, although our result showed obvious cooperativity. It is generally important to compare the results from the study using living animal as a whole system with those from the study using the model system. The purpose of this study is not to judge the discussion on the anesthetic action so far, but the following

conclusion can be drawn. The living animals including human being can be anesthetized even at concentration lower than the clinical concentration. The cooperativity on the anesthetic action may appear only in an integrated system in harmony with the functions of the parts such as membranes and proteins.

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