

## Pressure Reversal of Halothane's Antiviral Effect

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The authors have studied the effects that hyperbaric pressure and halothane had on measles virus replication. Two per cent halothane at one atmosphere of air (ATA) reduced the number of infectious measles virus particles produced in cultured Vero cells by greater than four orders of magnitude ( $P < 0.001$ ) relative to virus produced in untreated cultures at 1 ATA. The same concentration of halothane at 100 ATA still reduced the amount of virus synthesized but only by 1½ orders of magnitude ( $P < 0.01$ ) as compared with control cultures. Pressure (100 ATA) alone reduced virus production insignificantly. Thus, it appears that the antiviral effects of halothane on measles virus replication in cultured Vero cells is reversible at least partially by pressure. (Key words: Anesthetics, volatile: halothane. Cells: virus particles. Hyperbaria: pressure reversal. Theories of anesthesia: pressure reversal.)

THERE IS A LONG-STANDING CONTROVERSY concerning the mechanisms by which general anesthetics act. Because of the strong correlation between lipophilia and anesthetic potency, it has been reasoned that general anesthetics interact with either the lipid bilayer of the cell membrane,<sup>1</sup> the hydrophobic regions of essential proteins within the membrane,<sup>2-4</sup> or both.<sup>5</sup> One of the strongest arguments implicating the membrane as the primary site of general anesthetic action has been the observation of numerous investigators that many effects of anesthesia are reversed at least partially after exposure to hyperbaric pressure. However, not all effects of anesthetics are reversible by pressure. For example, pressure fails to reverse the effect of halothane on oxygen uptake by isolated rat liver mitochondria.<sup>6</sup> Similarly, halothane's inhibition of nerve cell function<sup>7,8</sup> and the depression of the ciliary beat in *Tetrahymena pyriformis*<sup>9</sup> are not reversed by pressure. This has led us to question which part(s) of the cell are involved in the reversible and nonreversible aspects of general anesthesia.

Viruses are simple molecular systems that are totally dependent upon a few host cell functions. They replicate their own nucleic acids but require the protein-

synthesizing apparatus of the host cell. Viruses also use host membranes and intracellular structures for their assembly. Thus, they provide a convenient way of isolating and studying a few cellular functions for study.<sup>10</sup> We have demonstrated recently that the degree of sensitivity that a virus has in replicating in the presence of halothane correlates with the amount of involvement that the virus has with the outer membrane of the host cell.<sup>11</sup> Accordingly, we have performed the studies described below, where we show that the inhibition of a virus whose replication is highly sensitive to halothane<sup>12</sup> was reversed at least partially by pressure.

### Materials and Methods

Monolayers of approximately  $10^6$  Vero cells were grown in 60-mm glass petri dishes or 8 oz (ca 240 ml) prescription bottles.<sup>12</sup> Control experiments demonstrated that the number of virus particles produced per cell was the same, whether prescription bottles or petri dishes were used. Cell cultures were grown to confluency in sealed bottles containing 10 ml media. Plastic caps fitted with Teflon®-coated silicone rubber septa were used to seal the flasks. Halothane vapor was provided by directing a 95% air/5% CO<sub>2</sub> mixture at 5 l·min<sup>-1</sup> through a Dräger vaporizer. The vaporizer output was connected to a 20-gauge needle placed in the septum; a second 20-gauge needle provided an outlet from the flask. Each bottle was equilibrated for 20 min, and halothane concentrations were determined by gas chromatography. Preliminary experiments demonstrated that the culture medium was equilibrated with halothane vapor within 10 min. Halothane levels were checked routinely every 24 h, and the bottles were re-equilibrated by exposure to halothane for 10 min as described above. The final concentration of halothane in the sealed culture flasks after 24 h was within 5% of the initial concentration.

For each experiment, six petri dishes and two bottles were inoculated with  $10^6$  infectious particles of measles virus. Two of the petri dishes were equilibrated with 95% air/5% CO<sub>2</sub> (control), while the two prescription bottles were equilibrated with 2.0% halothane in 95% air/5% CO<sub>2</sub> for 20 min. The other petri dishes were placed in a Parr cell disruption bomb, which had the siphon tube removed. Two per cent halothane in 95% air/5% CO<sub>2</sub> was allowed to flow through one of the bombs for 20 min. The second bomb was prepared identically, except with the omission of halothane. Both bombs then were pressurized with helium to 100 at-

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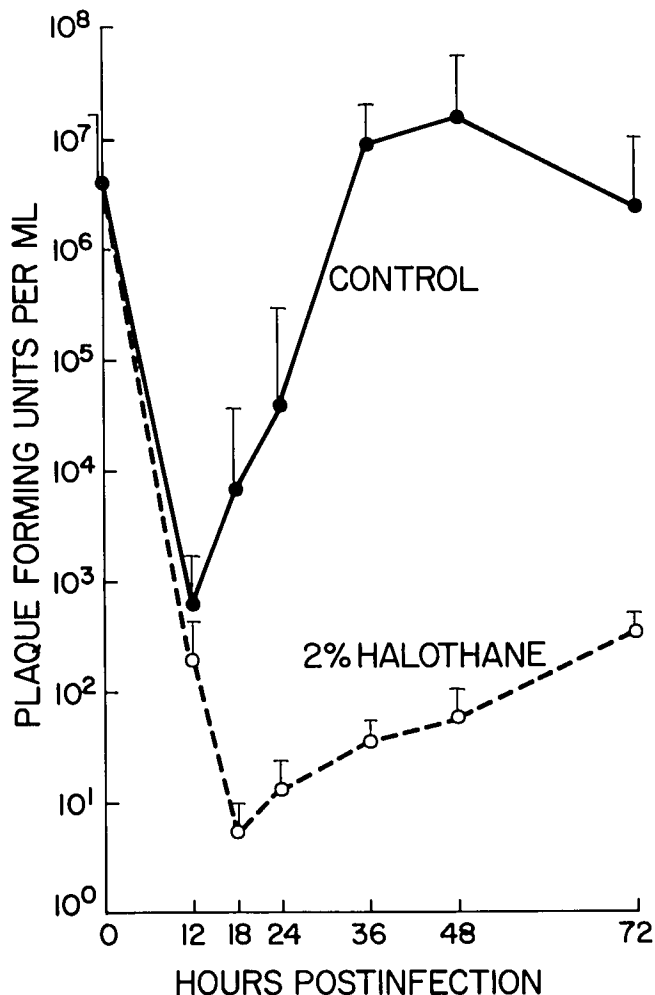


FIG. 1. Comparison of the replication of measles virus in the presence of 0% (●) or 2% (○), halothane. The presence of virus was assessed by its ability to create localized lesions (plaques) in a monolayer of cultured Vero cells as a function of time post virus infection. Error bars represent standard deviations.

mospheres of air (ATA) over a period of 10 min. The cultures remained under 100 ATA of pressure until the infected control (without halothane) demonstrated virus-specific cytopathologic characteristics in 100% of the cells (approximately 43 h). Next, the pressurized cultures were allowed to decompress over a 10-min period. Before the harvest, cells were examined with phase contrast microscopy to establish gross morphologic changes. All cultures then were harvested for infectious virus, and the titers were determined by plaque assay in the following manner.

Vero cells grown in 60-mm<sup>2</sup> plastic petri dishes were inoculated with 0.4 ml of the measles virus suspension to be assayed. After virus adsorption for 1 h at room temperature in the dark, the Vero cell monolayers were overlaid with Eagle basal medium containing 10% fetal

calf serum, 1% agar, 0.23% NaHCO<sub>3</sub> and antibiotics. Infected cultures were kept in 95% air/5% CO<sub>2</sub> at 37° C for 4 days, after which time, 2 ml of 0.025 μ tris(hydroxymethyl)aminomethane-buffered saline (pH 7.4) containing a 1:20,000 dilution of neutral red was added. Plaques were counted 5 days after initial virus adsorption. Each assay was performed in triplicate.

Mean values for duplicate cultures were determined, and a total of six experiments were performed. Statistical analysis was performed using an analysis of variance to assess changes within the total population. Specific comparisons were made using Student's *t* test for unpaired data. Values were considered significant when *P* < 0.05.

### Results

Noninfected Vero cells possess a typically fibroblastic shape when observed through phase contrast optics. Cells exposed to halothane retain their shape for the most part, although prolonged treatment (≥48 h) tends to make the cells more refractile and slightly spherical.<sup>12</sup> Similarly, pressure does not alter grossly Vero cell morphologic features, although a somewhat more stellate appearance did occur in one or two experiments. Vero cells treated with a combination of pressure and halothane do not have their appearance altered significantly either. Thus gross cellular alterations apparently have not occurred under the conditions of these experiments.

Measles virus typically grows to a titer of 1–3 × 10<sup>6</sup> plaque forming units/ml in untreated Vero cells,<sup>12</sup> but the addition of 2% halothane (1 ATA) inhibits virus replication. Figure 1 demonstrates that in these experiments, the production of measles virus barely was detectable in the presence of 2.0% halothane. Virus titers dropped by more than four orders of magnitude, and what little virus was produced was synthesized slowly. When identically infected cultures were treated with halothane at 100 ATA, only a 1½ order of magnitude drop in the infectious virus produced was observed. Thus, the decrease in virus titers observed with halothane plus pressure, although significant when compared with nonhalothane treated controls, was markedly less than those observed with halothane alone (table 1).

### Discussion

The results presented here demonstrate that the ability of halothane to block measles virus replication in cultured cells<sup>12</sup> is decreased by 100 ATA. Although the pressure reversal of halothane's antiviral effect was not complete, more than 100 times as much virus was produced in the presence of 2.0% halothane at 100 ATA than at 1 ATA. These results are not likely to result from halothane- or pressure-induced toxicity to Vero

cells. Halothane concentrations used in these experiments did not alter Vero cell morphologic characteristics, nor do they affect protein and RNA synthesis.<sup>12</sup> Furthermore, pressure (100 ATA) alone did not significantly affect measles virus replication or cellular morphologic characteristics, indicating that Vero cells are functional under the conditions of these experiments.

The fact that 100 ATA of helium had no effect on virus replication is interesting. Most biologic and biochemical systems ranging from humans to membrane model systems are affected by high pressures of inert gases.<sup>13</sup> For example, humans exposed to 15 ATA of helium and greater, may develop a condition known as high-pressure nervous syndrome,<sup>14</sup> while application of high-pressure helium generally has been shown to raise the phase transition temperature of pure phospholipids as demonstrated by electron paramagnetic resonance techniques.<sup>15</sup> One notable exception to this latter example recently has been reported.<sup>16</sup> These authors have shown that dipalmitoyl phosphatidic acid bilayer membranes are not as sensitive to 100 ATA as are membranes comprised of less charged molecules, such as dipalmitoyl phosphatidylcholine. Their hypothesis is that membrane bilayers with highly condensed charges are more resistant to compression than are membranes comprised of lipids of lower charge density. They have further shown that a volatile anesthetic, methoxyflurane, disorders the membrane and that the anesthetic-induced alterations are reversible by pressure. These findings<sup>16</sup> are in perfect agreement with those observed in our experiments.

In light of these findings, it is tempting to speculate the following. There may be localized areas within the cell's outer membranes that are vital for virus replication. Although sensitive to anesthetic-induced alterations, these areas would be refractile to compression by 100 ATA of helium, because they exhibit a relatively dense negative charge. Anesthetic-induced alterations would be reversible, however, because the altered membrane could be compressed<sup>13</sup> or the anesthetic extruded<sup>16</sup> by 100 ATA of helium. However, one must be cautious when trying to compare results from systems as different as virus-infected culture cells and chemically defined membrane models. Further studies are needed to determine if a common mechanism is shared by these two systems.

The fact that the reversal was not complete may further reflect the mechanism by which halothane blocks virus replication. Currently it is believed that the cell membrane is the primary site of action for general anesthetics.<sup>17</sup> We have observed recently that the ability of halothane to inhibit virus replication correlates with the degree of involvement that viruses have with the

TABLE 1. Mean Virus Titers for Measles Virus Cultures Grown in Vero Cells Exposed to Air, Halothane Pressure, or Halothane Plus Pressure

Treatment	Pressure (ATA)	Virus Titers*	P versus Control	P versus Halothane
Air (control)	1	$2.62 \times 10^6$ ( $7.09 \times 10^5$ )	—	—
Halothane	1	$2.22 \times 10^2$ ( $1.60 \times 10^2$ )	<0.001	—
Pressure	100	$1.20 \times 10^6$ ( $4.06 \times 10^5$ )	>0.10	—
Halothane + Pressure	100	$7.08 \times 10^4$ ( $2.67 \times 10^4$ )	<0.01	<0.05

\* Plaque-forming units/ml, means with SE in parentheses.

cells' outer membrane during the course of their replication.<sup>11</sup> Many, but not all viruses require a portion of the host cell membrane to form an outer envelope. This completes the virion assembly and enables subsequent virus infections. For example, herpesvirus, which packages part of its host cell membrane, is quite sensitive to the inhibitory action of halothane, while poliovirus, which is not enveloped, is totally resistant to halothane's antiviral properties.<sup>11</sup> Measles virus, whose replication is inhibited totally by all general inhalational anesthetics studied,<sup>18</sup> not only requires the host outer membrane for its envelope but also requires an outer membrane fusion step in its replication.<sup>19</sup> It is possible that the failure to observe a total reversal of halothane's antiviral effect with measles virus may be a reflection of the dose of halothane or the degree of pressure utilized in this study or both. Alternatively, the lack of total reversibility may be seen because some noncompressible structures in the cell, vital to virus replication, also are affected by halothane. For example, Nunn and Allison<sup>20</sup> have reported that microtubules, which are disassembled in response to hyperbaric pressure *in vitro*,<sup>21</sup> also are disrupted by anesthetic treatment. Similarly, Cohen<sup>6</sup> has shown that the halothane-induced inhibition of oxygen uptake in isolated rat liver mitochondria is not pressure-reversible.

In conclusion, we have shown that a virus whose replication is highly dependent upon a functional and intact host cell outer membrane is inhibited by halothane<sup>12</sup> and that this inhibition is at least partially reversible by pressure. Ongoing research in our laboratory is aimed at determining which virus membrane components are affected by anesthetic treatment, in an attempt to better understand the mechanism of action of general anesthetics at both the molecular and cellular level.

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