Measurement of Respiratory Volume for Virus Retention Studies in Mice,

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A pressure plethysmograph for measuring respiratory volume in mice during exposure to virus aerosols is described. The respiratory frequency and tidal volume were measured, and from these data the minute ventilation was calculated. The mean respiratory frequency of adult, male mice was 255 per min; the mean tidal volume of 0.18 ml was inversely related to respiratory frequency. The standardized mean minute ventilation rate was 1.46 ml per g of body weight. The respiratory frequency and tidal volume of CD-1 and HA/ICR strains of mice of the same age were similar. The respiratory retention rate for a 2.7-μm aerosol of vesicular stomatitis virus was 41%, and 58% of the virus retained was found in the trachea and lung.

The pathogenesis of aerosol-induced respiratory infection in animals is influenced significantly by the number of microbes retained in the respiratory tract and by their anatomic site of deposition. These factors are influenced by a variety of respiratory parameters, particularly the total respiratory volume, respiratory frequency, and tidal volume during exposure. Ordinarily, in the past when mice were used in studies in which the exposure dose of microorganisms was estimated, either the respiratory volume was assumed to be that reported in a previous investigation or it was disregarded. In either instance, an experimental error was introduced because of interanimal variability of respiratory parameters which may make the interpretation of the experimental results more difficult.

Most studies requiring information on respiratory volumes in mice utilize the data reported by Guyton in 1947, which he obtained by an oscillographic respiriograph method (4). Guyton's technique, however, is not appropriate for concurrent exposure to dynamic viral aerosols. Other less commonly used techniques for measuring respiratory volume, including valve (4) and stroboscopic methods (6), have been adapted to mice, but they have not been widely employed.

A technique for measuring respiratory volume in mice during exposure to viral aerosols was developed at this laboratory. The technique utilizes a pressure plethysmograph which is similar to the plethysmograph that has been used extensively on guinea pigs, rabbits, and man for aero-toxicological studies (1, 7). This paper describes this apparatus and its use for measuring respiratory frequency and tidal volume of mice; thus, a method of calculating the respiratory volume per unit of time is provided. The respiratory retention rate and anatomic site of deposition of virus particles were also determined in the mice exposed to virus aerosols.

MATERIALS AND METHODS

Animals. The mice used were male, random bred, albino, Swiss-Webster strain (Charles River Mouse Farms, Wilmington, Mass.) or the HA/ICR strain (A. R. Schmidt, Madison, Wis.). Their ages and weights ranged from 5 to 10 weeks and 28 to 39 g, respectively.

Plethysmograph. The plethysmograph was essentially a tube 3 cm in diameter by 9 cm long consisting of a stainless-steel frame supporting an open wire-mesh screen which was encompassed by a glass cylinder 5 cm in diameter (Fig. 1). Two taps were inserted in the posterior end of the tube for connection to a pressure transducer and calibration of the apparatus. After the mouse was coaxed into the inner tube, his head was made to protrude through a snug-fitting opening of a latex rubber diaphragm. An open wire cage, and a cotton plug and rubber stopper inserted behind the animal, ensured that proper positioning was maintained during the experimental
procedure. The plethysmograph could accommodate mice weighing between 25 and 40 g. The volume of the plethysmograph was approximately 100 ml. Therefore, the dead space with a mouse in the apparatus ranged between two and four times the volume displacement of the mouse. Respiratory frequency and tidal volume measurements determined with this apparatus were not different from those data obtained when the dead space of the plethysmograph was increased to 500 ml and copper gauze was inserted in an attempt to insure isothermal conditions.

The pressure changes within the plethysmograph induced by respiration were sensed by a Sanborn 288B pressure transducer and recorded on a 7700 Packard-Hewlett thermal oscillograph.

Tidal volume was calculated from a calibration curve that was produced by injection of a known volume of air over a wide range of simulated respiratory frequencies into the plethysmograph; the latter contained an object equivalent in volume to a mouse. The amplitude of the pressure signal was unaffected by frequencies of 60 to 300 per min. Frequencies of greater than 300 per min could not be produced by the manual method used.

Virus. The Indiana strain of vesicular stomatitis virus (VSV) was used to study virus aerosol retention. The virus was propagated on mouse fibroblast cell cultures (strain L929) in a growth medium of 10% agamma fetal bovine serum in minimal essential medium (MEM), 4 mM glutamine, 100 units of penicillin, and 100 mg of kanamycin.

A large pool of VSV was prepared, distributed in small glass ampoules, quick-frozen in a dry ice-ethanol bath, and stored at −70 C. The infectivity of this virus pool was determined before and at the time of each VSV aerosol by a plaque assay method. Serial twofold dilutions of virus in MEM were prepared within a range estimated to give 10 to 200 viral plaques. L929 monolayers grown in plastic plates 60 mm in diameter were washed free from growth media with two changes of MEM. Plates were inoculated with 0.2 ml of viral inoculum; three plates were used per dilution. The plates were incubated for 1 hr at 36 C, washed once with MEM, and overlaid with a medium of MEM, 2% fetal bovine serum, and 0.6% agarose. After a 48-hr incubation at 36 C in a humidified, 5% CO2 atmosphere, the plates were fixed with Formalin, the overlay was removed, and the cells were stained for several minutes with 1% crystal violet. The viral plaques were 2 to 4 mm in diameter and were readily counted. The infectivity of this virus pool was 2.5 × 105 plaque-forming units (PFU) per ml.

Virus aerosolization. A schematic of the aerosol apparatus is presented in Fig. 2. A Plexiglas-stainless steel tube 10 inches (25 cm) in diameter with ports for the plethysmograph and virus aerosol sampling was installed inside a stainless-steel chamber which serves as a hood. The volume of the aerosol chamber was 45 liters. Air flow through the apparatus was controlled by an exhaust fan and was maintained at 28 liters/min during the virus aerosol exposure. The air flow was under a slight negative pressure as a safety feature during the few seconds when the system was open to allow insertion and removal of animals. All contaminated air was passed through an absolute filter and exhausted to the outside air.

The virus aerosol was generated with a collision-type vaponephrin nebulizer operated under 10 psi of pressure and an air flow of 6 liters/min. This air was mixed with secondary filtered air whose temperature and relative humidity were controlled at 23 ± 1 C and 60% relative humidity ± 5%. The virus aerosol was generated for 3 min prior to animal exposure to allow the virus aerosol concentration to equilibrate.

The virus concentration was determined by an impingement method of aerosol sampling, and the virus aerosol size distribution was determined with an Andersen sampler by techniques described previously (2, 9).

Mice were exposed to the virus aerosol for 6 min. Immediately after virus exposure, the mice were killed rapidly with an intraperitoneal injection of sodium pentobarbital. The lung, trachea, nose, and head were obtained aseptically for virus isolation studies. The skin was removed from the head, and the anterior nares were separated from the skull at the median canthus of the eye. The remainder of the skull, less the brain, was regarded as the nasopharynx. All specimens were obtained within 15 min after cessation of virus aerosol exposure.

The specimens were ground in Ten-Broeck grinders in phosphate-buffered saline (PBS); the homogenate was chilled in an ice bath, followed by sonic treatment for 15 sec with a Bronwill sonic disintegrator microprobe operated at 30,000 cycles/sec. Preliminary experiments indicated that under these conditions VSV infectivity was not affected. The concentration of virus in each specimen was determined by a plaque assay technique in L929 mouse fibroblast cultures as described in the virus section above. Three plates were inoculated with a 0.2-ml volume of specimen. The total virus concentration in each tissue was determined after adjustment for total specimen volume.
RESULTS

Plethysmograph evaluation. A typical pattern of respiration in a normal mouse is shown in Fig. 3. This pattern of inhalation and exhalation is ordinarily maintained throughout an experimental procedure, although the respiratory frequency and tidal volume may change. The momentary irregularity in the respiratory pattern is a normal periodic occurrence which corresponds to movement of the head and apparent sniffing by the animal.

The acclimation to the plethysmograph, as reflected in frequency of breathing, tidal volume, and minute ventilation, is shown in Fig. 4. Respiratory frequency was unchanged in CD-1 mice during a 60-min observation. In HA/ICR mice, however, respiratory frequency increased immediately after insertion into the plethysmograph, but stabilized within 20 min. Most of the variability in frequency of respiration occurred as interanimal variation. For example, after 30 min the respiratory frequency ranged between 190 and 345 for CD-1 and between 160 and 300 for HA/ICR mice.

The pattern of tidal volume during the acclimation to the plethysmograph is shown in Fig. 3. This pattern of inhalation and exhalation is ordinarily maintained throughout an experimental procedure, although the respiratory frequency and tidal volume may change. The momentary irregularity in the respiratory pattern is a normal periodic occurrence which corresponds to movement of the head and apparent sniffing by the animal.

Fig. 2. Schematic of virus aerosol exposure apparatus.

Fig. 3. Pattern of normal respiration in the mouse.
mation to the plethysmograph was characterized by an initial decline in both groups of mice during the first 20 min; this decline was followed by stabilization (Fig. 4b). The interanimal variability in tidal volume was substantial. For example, after 30 min in the plethysmograph, the tidal volume ranged between 0.15 and 0.26 ml and 0.14 and 0.26 ml for CD-1 and HA/ICR mice, respectively.

The time course of minute ventilation in these mice, standardized for their body weight, clearly indicates that the values declined for a short time after insertion of the mice into the plethysmograph, but stabilized after 20 min (Fig. 4c). The lower values for the HA/ICR mice reflect their lower respiratory frequency.

Respiratory function values for mice of different ages (and body weight) determined after 30 min in the plethysmograph are presented in Table 1. No difference in respiratory frequency was observed between the various age groups of mice. As was anticipated, the tidal volume was inversely correlated with respiratory frequency. Despite an attempt to standardize minute ventilation based upon body weight, mean values ranged between 1.07 and 1.81 ml/g of body weight.

Based upon the time course of change in

![Graph Image](image-url)

**Fig. 4.** Time course of respiratory function parameters in mice. The CD-1 mice were 55 to 56 days old and had a mean weight of 31.3 g. The HA/ICR mice were 63 to 64 days old and had a mean weight of 29.8 g.

Table 1. Respiratory function values in normal mice

| Strain of mice | No. of mice | Age (days) | Body weight (g) | Respiratory frequency (per min) | Tidal volume (ml) | Minute volume per g of body weight (ml) |
|---------------|-------------|-----------|----------------|-------------------------------|-----------------|----------------------------------|
| CD-1          | 19          | 37       | 26.2          | 261 ± 74                      | 0.16 ± 0.07     | 1.46 ± 0.53                       |
|               | 9           | 55-56    | 31.3          | 252 ± 72                      | 0.16 ± 0.07     | 1.61 ± 0.40                       |
|               | 10          | 72-76    | 37.4          | 229 ± 45                      | 0.30 ± 0.06     | 1.81 ± 0.40                       |
| HA/ICR        | 8           | 69       | 29.9          | 248 ± 94                      | 0.17 ± 0.05     | 1.34 ± 0.42                       |
|               | 8           | 90       | 33.8          | 284 ± 104                     | 0.13 ± 0.04     | 1.07 ± 0.38                       |

*The respiratory function values were determined after a 30-min acclimation period in the body plethysmograph. These values are means ± SD.*
these respiratory function parameters, subsequent experiments on respiratory retention of aerosolized virus were begun after a 30-min acclimation period in the plethysmograph.

Respiratory retention of aerosolized vesicular stomatitis virus. Nine mice were exposed to seven virus aerosols. The amount of virus retained in the upper and lower respiratory tract and the total retention rate are shown in Table 2.

It was found that 58% of the virus was retained in the trachea and lung. The remainder was divided between the anterior nares and nasopharynx. Attempts were made to isolate virus from the stomach, but in all cases this specimen was toxic for the tissue cell cultures.

The total retention rate of vesicular stomatitis virus was calculated by the formula:

\[ \text{retention rate (\%)} = \frac{R}{\text{AVC} \times V} \times 100 \]

where \( V \) is the respiratory volume (milliliters during viral exposure), \( \text{AVC} \) is the aerosol virus concentration (PFU per liter of air), and \( R \) is the total virus retained (PFU in all tissues sampled).

A virus retention rate of 41% was observed based upon the minute ventilation values determined by the plethysmograph technique during virus exposure. The retention rate, which is based upon an assumption of minute ventilation derived from the data of Guyton (4), is 51%.

This overestimate of the actual rate occurred because of the 20% underestimate of the actual minute ventilation rate in these mice.

Virus aerosol sampling. The particle size distribution of the virus aerosol was determined during three experiments because droplet size in which virus is contained is an important factor that influences the site of deposition in the respiratory tract. The virus aerosol size distribution shown in Fig. 5 is log normal. The mean droplet size in which virus was contained was approximately 2.7 \( \mu \)m.

DISCUSSION

A pressure plethysmograph for mice described here has been used successfully to measure respiratory frequency and tidal volume. From these data, the respiratory volume per unit of time can be calculated. The pattern of respiratory frequency and tidal volume of mice during a 60-min observation period suggests that the technique is not unduly stressful. Respiratory frequency was not appreciably altered in CD-1 mice; after an initial increase in respiratory frequency in the HA/ICR mice, the rate stabilized within 20 min in both strains of mice. The elevated values observed within the first few minutes after introduction of the animal into the apparatus presumably reflect transient anxiety. This transient increase in respiratory frequency and tidal volume observed in the HA/ICR mice is consistent with our empirical observations that these mice become more excited than the CD-1 mice when

### Table 2. Vesicular stomatitis virus in the respiratory tract of mice after aerosol exposure in a body plethysmograph

| Virus aerosol concn (per liter of air) | Mouse body weight (g) | Percentage of total amount of virus in each tissue | Total virus retained (PFU) | Per cent retained in lower respiratory tract | Retention rate | Plethysmograph method | Avg m.v. method |
|---------------------------------------|-----------------------|---------------------------------------------------|---------------------------|---------------------------------------------|---------------|----------------------|----------------|
| 5.16 \( \times 10^3 \)                | 29.1                  | 9.9                                              | 9.9                       | 71.0                                        |               | 509                  | 80.2           | 37.5             | 45.8            |
| 4.72 \( \times 10^3 \)                | 28.4                  | 21.6                                             | 48.5                      | 10.0                                        |               | 291                  | 29.9           | 48.6             | 29.4            |
| 4.72 \( \times 10^3 \)                | 28.4                  | 18.8                                             | 12.3                      | 44.4                                        |               | 309                  | 68.9           | 21.3             | 31.2            |
| 5.60 \( \times 10^3 \)                | 33.0                  | 13.6                                             | 10.7                      | 31.2                                        |               | 709                  | 75.7           | 49.3             | 51.5            |
| 1.00 \( \times 10^4 \)                | 37.5                  | 15.6                                             | 44.5                      | 4.1                                         |               | 1,139                | 39.9           | 27.5             | 40.5            |
| 1.00 \( \times 10^4 \)                | 35.8                  | 24.1                                             | 13.0                      | 4.1                                         |               | 3,466                | 56.9           | 40.9             | 76.7            |
| 1.43 \( \times 10^4 \)                | 39.0                  | 28.4                                             | 17.5                      | 6.4                                         |               | 3,473                | 54.1           | 67.0             | 84.6            |
| 1.13 \( \times 10^4 \)                | 37.5                  | 17.0                                             | 8.4                       | 3.7                                         |               | 1,147                | 74.6           | 24.4             | 36.0            |
| 1.28 \( \times 10^4 \)                | 37.0                  | 10.9                                             | 49.7                      | 13.2                                        |               | 2,297                | 39.3           | 55.2             | 65.0            |
| Mean                                  | 17.8                  | 24.5                                             | 11.4                      | 46.3                                        |               | 57.7                  | 57.7           | 41.3             | 51.2            |
| SE                                    | 2.9                   | 5.8                                              | 3.1                       | 5.9                                         |               | 6.1                   | 6.1             | 5.1              | 6.7             |

*The lower respiratory tract is regarded as the trachea and lung.

*Retention rate based upon the formula presented in the text.

*Average minute volume method is based upon 1.25 ml per g of body weight observed by Guyton (4).
handled. As a result of this time course of respiratory frequency and tidal volume, a 30-min acclimation period in the apparatus is used before experimental procedures are begun.

Considerable interanimal variability in respiratory frequency and tidal volume was observed in both strains of mice, although the mean values and variance estimates for groups of mice of different ages (body weights) were similar. When the respiratory volume was standardized for body weight by expressing it as milliliters per gram of body weight per minute, a substantial difference between the groups of different weights remained, particularly between these two strains of mice. Guyton observed a minute ventilation of 1.24 ml/g of body weight in 19.8-g mice (4), a value commonly accepted for the respiratory volume of mice in general. The standardized minute volume for HA/ICR mice was in close agreement with the values observed by Guyton. Although the higher values in CD-1 mice may reflect a real difference in minute ventilation characteristics of this particular strain of mice, the difference could be explained by interanimal variation. The difference between Guyton’s values and those observed in the present study suggests that it is of value to measure respiratory volume during experiments when accurate calculation of inhaled dosage of an agent is desired, whether it be a microorganism or any other inhalant.

Very few data are available concerning the respiratory retention of microbes in mice. The observed respiratory retention rate for mice of 41% for VSV aerosols compares with retention rates of 30% for 1-μm aerosols of 32P-labeled Pasteurella pestis (3) and 27% for 32P-labeled Bacillus subtilis spores of the same size (5). These retention rates are not, however, directly comparable to the rate observed in this study, because the mean particle size of the VSV aerosols was 2.7 μm. A greater retention rate for particles of this size would be expected because, at least in other animals including the guinea pig, monkey, and man, the total retention rate of viable or nonviable particles increases as particle size is increased between 1 and 3 μm (5, 8). The retention rate of 2.5-μm B. subtilis particles in the guinea pig is 54% (5). Therefore, the total retention rate in mice of viable particles of approximately the same size appears to be similar to that in this animal.

The retention rate of VSV in the upper respiratory tract of mice (42%) is also similar to the rate previously reported (54%) for 2.5-μm nonviable particles in the same anatomic site in guinea pigs (5). The retention rate determined on the basis of the actual respiratory volume measured during the virus aerosol exposure was less than that retention rate determined on the basis of the minute ventilation values observed by Guyton (4). This overestimate is explained because the respiratory volume of our mice was approximately 20% greater than for Guyton’s mice.

This plethysmograph provides a method whereby the respiratory volume of the mouse can be determined during exposure to infectious aerosols. The inhaled dose of infectious aerosols can be accurately determined, and an important source of variability caused by substantial differences in respiratory volume can be recognized and taken into account. In our experience, the interanimal variation in minute ventilation in mice may be as great as a factor of 4. In infectious disease models such as murine influenza, in which severity of infection, measured in terms of pneumonia, is closely related to inhaled dose of influenza virus (G. A. Fairchild and J. Roan, Arch. Environ. Health, in press), interanimal variation in respiratory volume may be a large source of experimental variability in the quantity of pneumonia which develops in groups of mice exposed to the same viral aerosol. To the extent that variations in respiratory volume can be taken into account, a reduction in this source of experimental error will increase the sensitivity of the infectious disease model system. Similar limitations apply when the effects of other inhaled agents are investigated in mice; this apparatus may prove to be useful in those studies as well.

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