# An Apparatus for Airborne Infection of Mice. (19538)

GARDNER MIDDLEBROOK.* (Introduced by R. J. Dubos.)

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The continuous ventilation type of airborne infection apparatus first described by Wells (1) permits quantitative experimental infection of animals by inhalation of droplet nuclei bearing the panthogenic agent. The purpose of this report is to describe an apparatus of this type for the airborne infection of mice. This instrument† differs in the following respects from others of its kind which have been described in the literature(2-5): 1) It is small and compact, occupying less than 18 cubic feet of space, so that it can be placed on a laboratory bench or mounted on a table equipped with casters; it weighs less than 150 lb. 2) it is relatively inexpensive to build.

*The infection chamber* is 24″ in diameter and 14″ high, constructed of 21-gauge stainless steel, and covered with a removable UVA (impermeable to ultraviolet rays) plexiglass top, ½″ thick. (Fig. 1 and 2) It has a stainless steel bottom with a central opening onto which is soldered a short elbow pipe; on this pipe is slipped a 1″ i.d. "Tygon" hose. The hose is 6′ long and is attached at its other end to a nebulizer-venturi (N-V) apparatus. A removable steel baffle plate is located 1″ above the true bottom of the chamber; this baffle has a circular series of 24 round perforations ¾″ in diameter at 9½″ from its center. At 2″ above the baffle are located 5 projections from the wall of the chamber, and on these projections rests a removable basket-cage constructed of 12-mesh stainless steel wire mesh. The cage has a removable, flat, wire mesh top which can be rotated about a center post, and is divided by wire mesh partitions into five equal sectors so that five groups of mice (up to 20 mice each) can be kept separated in the cage and can be introduced and removed through a port located in the movable top. On the plexiglass top is mounted a 30-watt ultraviolet lamp‡ which consists of a horizontal circular tube (outside diameter of circle is 6″) with two vertical electrodes rising out of the chamber proper through a 3½″ diameter hole in the plexiglass. The electrodes are protected and the hole is covered by a round metal box, 4″ in diameter and 5″ high, with a flange at its

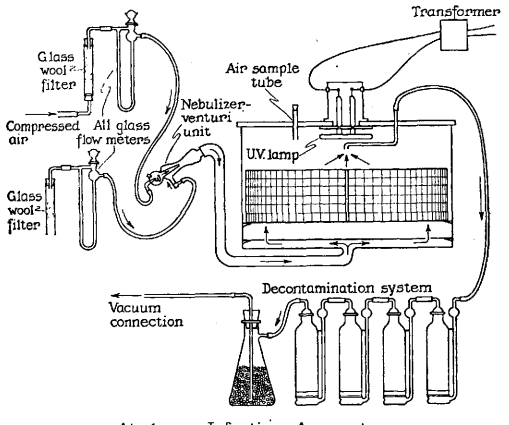


FIG. 1. A diagramatic representation (drawn by Ruth J. Mandelbaum).

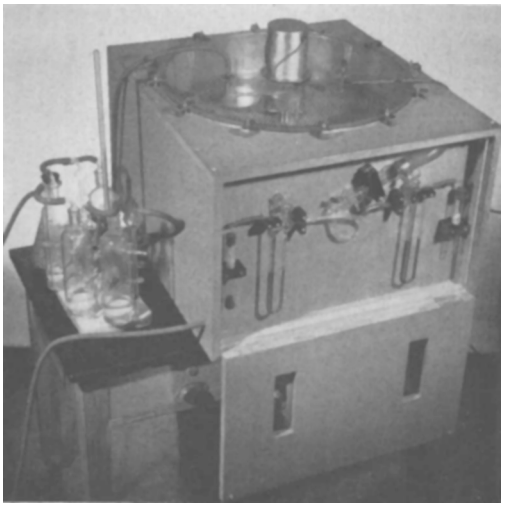

FIG. 2. Photograph of airborne infection apparatus (by Julian Carlile).

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† Designed with the assistance of Josef Blum and constructed by him.

‡ Available from Hanovia Co., New York City.

open end through which it is tightly screwed with a rubber gasket onto the plexiglass top. Two other openings are provided for 7/16″ copper tubing with appropriate flanges and gaskets by which they are secured to the top, one for attachment of tubing for ventilating the chamber air into the decontamination system of gas washing bottles, the other for permitting removal of air samples to be tested for content of droplet nuclei containing microorganisms. The whole infection chamber is housed in a wooden box which is 28″ wide, 31″ long and 18″ high. As illustrated in Fig. 2, the front of the box has a recess 3″ deep which can be closed by a door hinged at the bottom. Bakelite supports are provided in this recess for 2 air-filters, 2 all-glass flow meters, and the nebulizer venturi apparatus. The plexiglass top is secured to the top of the chamber by means of 12 radially arranged screw clamps visible in Fig. 2.

*The decontamination system* consists of a series of 4 gas washing bottles, each having 350 ml capacity and a very coarse fritted glass filter disc.§ After the air from the infection chamber has been decontaminated (see *Experimental*) by passage through these bottles, it is passed through a dry flask filled with glass beads to remove aqueous droplets before evacuation into the house vacuum system. *The nebulizer-venturi unit,* as illustrated in Fig. 1 and 2, is an all-glass apparatus consisting of a "Vaponephrin" nebulizer annealed to two 25 ml Erlenmeyer flasks.‖ It is so designed that air under pressure from the house air supply is forced into the nebulizer, nebulizes the suspension therein, passes through the tapered exit neck of the nebulizer, is mixed, at a venturi opening at the point where the necks of the two flasks are annealed, with room air coming in through a side arm (½″ i.d.) near the base of the first flask, and, finally, passes through a 1″ internal diameter extension on the base of the second flask into the hose leading to the infection chamber. The suspension to be nebulized is introduced into the nebulizer through the opening provided in the commercially available unit; this can be performed aseptically by use of a syringe and needle. The opening can then be closed with a rubber stopper. *Measurements of airflow* are performed by means of two all-glass manometric type flow meters¶ containing dye-colored water. The compressed air admitted to the nebulizer is adjusted to flow at a rate of 5 liters per minute; this has been found to be adequate to yield 0.08 to 0.09 ml of very fine mist per minute. The main airflow is pushed into the system by atmospheric pressure because the whole interior of the apparatus, where the contaminated air is confined, is kept under negative pressure by the constant vacuum beyond the decontamination system. This main supply is adjusted to flow at 20 liters per minute. By varying the numbers of microorganisms in the suspension introduced into the nebulizer, the proportion of bacteria-bearing nuclei can be varied.

The following theoretical requirements have been fulfilled in the design of this apparatus: 1) The volume of air per unit time moving through the chamber is at least four-fold the volume of air per unit time breathed by 100 mice, the maximum number for which the apparatus was designed. (Total volume of air per minute passing through chamber is 25 liters, and the volume of air per minute breathed by 100 actively moving mice is 5 to 6 liters)(6). 2) The upward movement of air in the chamber is in excess of the settling velocities of all inhalable droplet nuclei. (The volume of the chamber is about 100 liters; the air moves through the chamber at the rate of 25 liters per min; and the height of the chamber is 14″. Therefore, the upward movement of air in the chamber is about 3.5″/min. This is greater than the settling velocity of the largest particles capable of inducing inhalation infection in experimental animals)(7).

*Methods.* The nebulizer fluid used in all experiments to be reported here was a five-fold dilution in distilled water of the Tween-albumin growth medium currently used for mycobacteria in this laboratory(8). The ad-

§ Corning Cat. No. 31750.

‖ Available from E. Machlett & Co., New York City.

¶ Corning Cat. No. 5960, calibrated with a wet-test meter.

dition of bovine serum albumin to suspending media for the airborne infection of rabbits with tubercle bacilli has already been shown to have a stabilizing influence on the viability and infectivity of these organisms(3). And, the five-fold dilution of the Tween-albumin medium gives a solution containing about 0.2% solids, which has been shown to yield aerosols composed of droplet nuclei of size suitable for inhalation(7). The mycobacteria employed in these experiments were cultivated in the above mentioned Tween-albumin liquid medium, for 3 days in the case of the non-pathogenic *M. ranae* and for 7 to 10 days in the case of the pathogenic bovine strain of tubercle bacillus, Vallée. Cultures of the *M. ranae* strain were composed predominantly of well dispersed single cells and were used without further treatment in the standardization experiments described below. Cultures of the Vallée strain contained a low proportion of single cells, being composed mostly of clumps containing 10 to 100 bacilli. Therefore, in one experiment to be reported here, a well dispersed suspension of this strain was prepared by a method like that described by Fenner(6). All experiments described here were 60-minute runs, with an added 10 minutes of airwashing to compensate for the delay in development of the ambient cloud in the infection chamber(4). An additional 20 minutes of airwashing, with the ultraviolet lamp on, was used when experiments involving pathogenic microorganisms were performed.

*Experimental.* Of primary import was the effectiveneness of the decontamination system of gas-washing bottles. It is obvious that experiments involving pathogenic microorganisms such as tubercle bacilli could not be suffered until it was demonstrated that microorganisms with similar properties would not be able to pass the decontamination system in significant numbers. Therefore, a flask of sterile medium (oleic acid albumin liquid medium) capable of supporting growth from individual bacterial cells of mycobacteria(8) was introduced at the end of the decontamination system so that air evacuated from the apparatus had to impinge onto the surface of the medium. The 4 gas washing bottles, each containing 300 ml of distilled water and 0.2 ml of tributyl phosphate (as antifoam agent), were autoclaved along with the flask containing glass beads and the flask of culture medium. A suspension of *M. ranae* containing approximately $10^8$ bacterial cells per ml was employed in a routine 60-minute run. The flask of culture medium was then disconnected aseptically and incubated at 38°C. Several runs of this type were made and growth in the test medium never appeared before the fourth day of incubation. Previous studies had shown that at least 100 bacterial cells of this strain had to be inoculated into such a medium for growth to be evident before the fourth day. Thus the great majority of the $5 \times 10^8$ cells nebulized in such experiments was caught mechanically in this decontamination system.

These observations demonstrated that the decontamination system of this apparatus serves as an adequately effective mechanical remover of mycobacteria from contaminated air passing through the apparatus at the rate of 25 liters per min. Nevertheless, as an additional precaution, phenol in a final concentration of 2%, which is bactericidal for tubercle bacilli, was added to the distilled water in each gas washing bottle when these organisms were used in infection experiments.

In order to determine the percentage of fluid sprayed from the nebulizer which passes through the infection chamber as a cloud of droplet nuclei, phenol red was dissolved in a final concentration of 0.02% in the 5-fold dilution of Tween-albumin medium in distilled water, and this solution was used as the nebulizer fluid. The amounts of dye recovered from various parts of the apparatus after a run were determined colorimetrically after the methods of Rosebury(4). The amount of fluid nebulized was determined by weighing the nebulizer-venturi unit before and again after a run. The amount of fluid which impinged and dried on the exit neck of the nebulizer and in the distal flask of the unit was determined by washing out and discarding the nebulizer fluid remaining in the nebulizer after a run and then rinsing the entire nebulizer-venturi unit with dilute alkali. Table I shows the results of three such experiments. It is evident that under the conditions of these experiments approximately

TABLE I. Recovery of Clouds of Phenol Red from Nebulization of this Dye in the Airborne Infection Apparatus after Some Routine 60 Min Runs. Amounts of dye are expressed in milliliter equivalents of the original nebulizer fluid (NF) containing the dye in a final concentration of .02%.

| | Exp. I | Exp. II | Exp. III |
|---|---|---|---|
| Wt of N-V unit + NF, ml: | | | |
| before run | 77.1 | 77.7 | 77.7 |
| after run | 71.7 | 71.9 | 72.3 |
| Fluid lost, ml | 5.4 | 5.8 | 5.4 |
| Impingement loss in N-F unit, ml | .60 | .77 | .64 |
| *A.* Total nebulized, ml | 4.80 | 5.03 | 4.76 |
| Recovery from, ml: | | | |
| tubing from chamber to decontamination system + wash bottle #1 | 1.83 | 1.86 | 1.57 |
| wash bottle #2 | .54 | .67 | .78 |
| wash bottle #3 | .14 | .16 | .20 |
| wash bottle #4 | .05 | .04 | .06 |
| *B.* Total recovered, ml | 2.56 | 2.73 | 2.61 |
| % recovery (B/A × 100%) | 54 | 55 | 55 |

55% of the fluid lost from the N-V unit passes through the infection chamber as a cloud of droplet nuclei. The losses unaccounted for are probably attributable to impingement on the hose leading to the infection chamber and on the walls of the infection chamber itself.

An airborne infection experiment was performed in which 50 three-week-old white mice of the Rockefeller Swiss albino strain were exposed to the virulent bovine strain of tubercle bacillus, Vallée. The mice were equally distributed in the 5 sections of the basket cage. The number of viable tubercle bacilli in the nebulizer suspension was determined as 1.5 x $10^6$/ml by plating out dilutions of this fluid on the oleic acid albumin agar medium(8). 5.8 ml of this suspension was nebulized in the one-hour run. From the experiments with phenol red, it can be estimated that approximately 0.05 ml of this suspension passed through the chamber each minute, diluted in 25 liters of air. Therefore, the concentration of droplet nuclei containing tubercle bacilli was approximately 2.5 x $10^3$ per liter of ambient cloud in the infection chamber during the run. Each mouse is calculated to have inhaled about 4 liters/hour of this air. Therefore, each mouse, on the average, inhaled no more than 10,000 bacilli. The extent and uniformity of pulmonary pathology induced in this airborne infection apparatus was manifest when the mice were killed at 4 weeks after infection. There was no significant difference between the groups of mice infected in the different sections of the basket cage. Fig. 3 illustrates the appearance of the lungs and spleens of 5 unselected mice from one unselected section.

Another experiment was performed in which 77 mice were exposed in this apparatus in the same fashion except that an untreated Tween-albumin culture of the fully grown Vallée strain, diluted with 4 volumes of distilled water, was used as the nebulizer suspension. Suffice it to relate that, although such a suspension contains only a small proportion of well dispensed bacilli, it was possible to induce generalized pulmonary tuberculosis in all the exposed mice. Indeed, all of 10 mice sacrificed at 14 days after infection (2 occupants of each section of the basket cage) showed grossly visible pulmonary lesions, some of barely visible size. And, at 4 weeks after infection, the lungs of all of 15 mice killed at that time manifested widespread pulmonary consolidation, confirming the antemortem observation that all mice surviving at this time had obviously impaired respiratory function.

*Comment.* Two types of apparatus for the airborne infection of experimental animals have been described. In the first kind the animals are exposed in a closed chamber to a mist of aqueous droplets bearing pathogenic agents. It was this type that Glover used(9) when he successfully induced airborne infection of mice with tubercle bacilli. The second type permits quantitatively controllable infection by droplet nuclei, bearing the pathogenic agents, and delivered at a uniform rate to a measured air supply which ventilates the infection chamber at a constant rate. The advantages of the second type over the first have repeatedly been emphasized. It has been demonstrated that in this second type of apparatus it is possible to control the numbers of microorganisms inhaled by experimental animals exposed to droplet nuclei containing the microorganisms as single cells or as very small clumps, provided the number of micro-

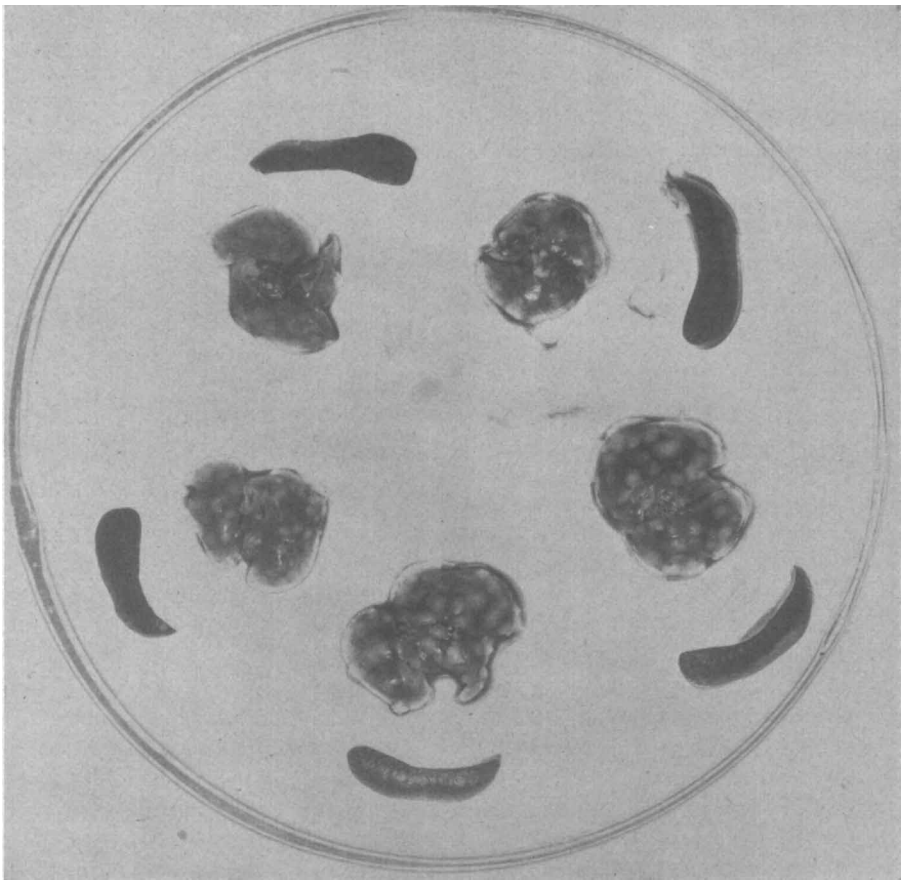

FIG. 3. Photograph of the lungs and spleens of Swiss mice of the Rockefeller Institute strain, exposed four weeks previously to airborne infection with the virulent bovine strain of tubercle bacillus, Vallée. Note numerous tubercles of lungs. Spleens slightly larger than normal. (Photograph by Julian Carlile).

bial units per unit volume of nebulized fluid is predictable(3,6). However, without minimizing the importance of the study of the physical aspects of airborne infection, it seems fair to state that preoccupation with this phase of the problem accounted for the complexity and expense of previously described continuous ventilation types of apparatus, thus limiting application of this mode of experimental infection to very few laboratories. The model described here should permit wider use of airborne infection methods with mice as the experimental animals.

Tubercle bacilli, cultivated in Tween-albumin liquid medium, can be used to prepare well dispersed and stable suspensions consisting predominantly of single bacilli, and the numbers of living bacterial cells in such suspensions can be accurately predicted(8). Thus, it is possible with this apparatus and with suspensions of tubercle bacilli prepared in this fashion to induce simultaneously a uniform type of pulmonary tuberculosis in large numbers of mice. It is of some interest that the extent of pulmonary disease induced by this method is much greater than that caused by the same number of tubercle bacilli injected into mice of the same strain and age by the intravenous route(10).

*Summary.* A safe and relatively inexpensive laboratory apparatus for the experimental airborne infection of as many as 100 mice at a time has been described. It has been used for the production of murine pulmonary tuber-

culosis with tubercle bacilli cultivated in Tween-albumin liquid medium.

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## Effect of Filter Paper, *para*-hydroxybenzoic Acid, and Fixed Tissue on Phagocytosis. (19539)

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Wood *et al.* have described "surface phagocytosis" at length under the following conditions(1-4): "The material with surface to be tested was spread flat in the bottom of a Petri dish lined with filter paper previously soaked in Locke's solution. The leukocyte-pneumococcus mixture was distributed over the test surface, and the dish was sealed and placed in the incubator (37°C) for 1 to 3 hours. In this way the preparations were kept moist. When the Petri dishes were opened, impression smears were made from the test surfaces and were stained with methylene blue." Substances such as glass, paraffin, and cellophane were reported as inactive, while a variety of materials including fixed tissue sections, fiber glass, and filter paper alone promoted phagocytosis. The common denominator in the cited experiments was wet filter paper, which might have been responsible for phagocytosis. The inactive materials were those impermeable to liquid.

This report deals with the influence of filter paper on phagocytosis of *E. coli*. During this work, an active principle in filter paper, *para*-hydroxybenzoic acid, was reported by Davis(5,6) as a new bacterial vitamin and antagonist for *para*-aminobenzoic acid. The effects of *para*-hydroxybenzoic acid and of fixed tissues were also examined.

*Methods. E. coli suspensions.* Cultures were grown for 18-20 hours in tryptose broth containing 1% dextrose, 3.7 μg Fe/ml, and 0.1 μg thiamin HCl/ml. The bacteria were harvested and washed in saline, standardized turbidimetrically to 4 x $10^{10}$ cells/ml, and checked by dilution plate counts. The bacteria were killed by treatment with 1% $CH_2O$ for 24 hours at room temperature, washed 3 times in saline, and sterility was checked by plating. The number of bacteria employed in each set of comparative experiments was the same but varied among the different sets.

*Filter paper.* Strips measuring 1 x 4 or 1 x 5 cm of Whatman No. 1 or No. 42 paper were rolled and placed in the bottom of 12 ml tapered centrifuge tubes. Bacterial suspensions containing from 4 x $10^9$ to 2.4 x $10^{10}$ organisms in 0.4 to 0.6 ml were pipetted into these tubes and allowed to remain in contact for 30 to 60 minutes, after which the filter paper was squeezed out and removed. Chamber counts in several instances showed no significant loss of bacteria after this treatment. *Para-hydroxybenzoic acid.* Eastman C.P. *para*-hydroxybenzoic acid, either free or as the Na-salt, was used in $H_2O$ solutions containing from 0.00001 to 1 mg/ml. One to 3 ml of these concentrations of *para*-hydroxybenzoic acid were added to 2.5 x $10^9$ to 6 x $10^9$ cells for 30 to 60 minutes, centrifuged, and the cells resuspended in saline with or without extra washing in saline or Krebs-gelatin. *Tissue.* Washed frozen sections 5-25 μ thick of for-