PART II. AIRBORNE ORGANISMS

Norton Nelson, Chairman

VIABILITY AND INFECTIVITY OF MICROORGANISMS IN EXPERIMENTAL AIRBORNE INFECTION

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Some appreciation of the implications and meanings of the words *airborne*, *viability*, and *infectivity* is prerequisite to any discussion of airborne infection, although it seems wise to avoid speaking of "definitions." It is difficult to improve on Langmuir's concept (9) of airborne infection as involving the inhalation of *droplet nuclei* (resulting from the evaporation of aerosol droplets) which remain suspended in air for relatively long periods of time. This concept eliminates droplets per se, which are more properly involved in contact infection.

Because the subject of this Conference implies that we are interested in the pathological and medical aspects of airborne infection, our use of the word infectivity will connote pathogenicity since infection per se is an example of parasitism that may or may not include what we refer to as disease. Still, the discussion of viability and infectivity may prove difficult because we are dealing with related but not mutually dependent properties of a cell. Viability generally is considered as the potentiality for multiplication under experimentally defined conditions. Such a process, of course, does seem essential for infection to manifest itself, but certainly all cells that are viable do not infect. For viruses, however, definitions become more complicated because the viruses' so-called viability is measured conventionally in terms of infectivity for the egg, tissue culture, or animal host.

In our laboratories and elsewhere, considerable effort is currently devoted to studies of experimental airborne infection. Many individuals are independently investigating a number of approaches to related problems in aerobiology; the results are sometimes difficult to correlate and interpret. We suspect that there will be times during this Conference when it will be difficult to interpret reported data. The purpose of this paper is to point out, if possible, some of the many factors which must be considered in evaluating and correlating data on this most complicated subject.

The viability and infectivity of airborne organisms are relative processes and must be considered only in relation to the experimental conditions employed. In view of the number of variables that affect the outcome of the host-parasite relationship and the fate of organisms in the aerosolized state, it seems an impossible task to attempt to generalize. Some clarification results, however, if the processes of experimental airborne infection are considered as follows: (i) effects of pre-aerosolization treatment on cells; (ii) effects of aerosolization per se; and (iii) effects of the postaerosolization environment. Even here, as Beveridge (2) points out, there is considerable discrepancy between the behavior of parasites under *natural* versus *laboratory* conditions. For example, the agent of swine fever persists in laboratory culture for long periods of time but dies very rapidly under natural conditions in the pig sty. On the other hand, influenza and foot-and-mouth viruses are quite fragile in the laboratory but survive for some time in nature.

The two principal pre-aerosolization factors that influence the viability and infectivity of cells are strain selection and growth conditions, the latter including such variables as choice of medium, aeration, length of incubation, pH, temperature, and conditions of harvest and storage. Manipulations of these factors usually influence the growth and numbers of cells, the yield of an end product, or the selection of virulent or avirulent cells. For example, we know that bacteria in the logarithmic stage of growth are more susceptible than older cells to such stresses as heat, cold, antibiotics, desiccation, and radiation. Cells in the resting phase or lag phase of growth are more resistant to the stress of aerosolization than are cells in the stage of logarithmic growth (Fig. 1).

Genetic, nutritional, and physiological manipulation may play important roles in selecting cells of increased aerosol stability and infectivity.

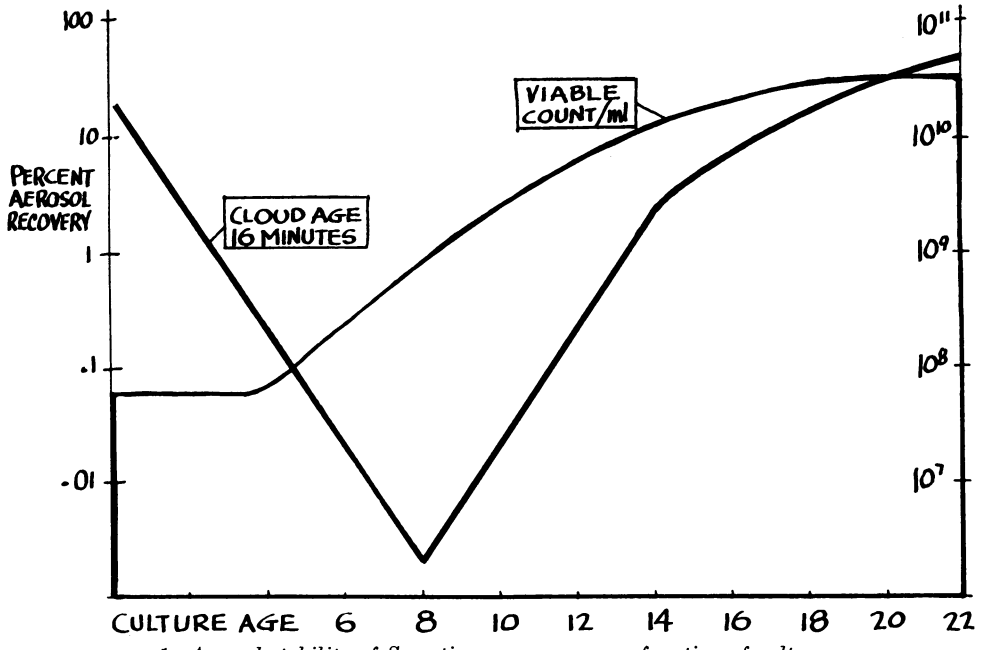

FIG. 1. *Aerosol stability of Serratia marcescens as a function of culture age*

Some obvious approaches such as culturing surviving aerosolized cells and re-aerosolizing them have met with very limited success in selecting aerosol-resistant clones. Braun (3) used irradiated cultures to select cells resistant to drying and antibiotics without success. Similarly, Koh, Morehouse, and Chandler (6) found that cells of *Escherichia coli* that survived 10,000 to 70,000 roentgens of β radiation had all the known properties of the parent cells. However, there is evidence in our laboratories that if we are able to isolate true thermophilic mutants of several pathogens, these mutants will show increased resistance to aerosolization. Furthermore, a salt-tolerant variant studied at Fort Detrick shows increased resistance to aerosolization with retention of infectivity. Delwiche et al. (4) showed that the usual reversion to avirulence when cells of *Pasteurella pestis* were grown in air at 37 C could be overcome by the addition of bicarbonate or aspartic acid to the medium or by growing the cells under nitrogen. Nutritional and physiological studies on some inherently aerosol-stable cells such as staphylococci, mycobacteria, and *Bacillus anthracis* spores have not shown conclusive relationships between properties of these cells and aerosol stability.

The role of storage conditions such as time, temperature, and concentration have been studied extensively in our laboratories. Although generalizations can be misleading, it appears that prolonged storage of cells is accompanied by decreases in viability and infectivity even at relatively low temperatures. Aerosol stability may or may not be affected by storage. The addition of chemical stabilizers will be discussed later. Suffice it to say at this point that the pre-aerosolization treatment of organisms used in studies on airborne infection is of great importance and requires close attention to strain selection, conditions of growth and harvest, and preparations for the act of aerosolization. Studies in our laboratories concerning the effects of aerosolization per se will be discussed by other speakers.

The third phase of our studies, the effect of the postaerosolization environment, is most critical inasmuch as we now are dealing with the host-parasite relationship. We will discuss only a few factors which affect this relationship. Such subjects as host susceptibility and the effects of stress on the experimental host cannot be discussed even though a fascinating literature is accumulating on the role of body irradiation, nutritional deprivations, and the effect of chemicals such as 6-mercaptopurine and endotoxin pretreatment.

Certainly one of the most important parameters involving airborne microorganisms is the size of the particles or droplets comprising the aerosol (7, 8). Many workers have demonstrated that LD_{50} or ID_{50} values of certain airborne pathogens decrease as the aerosol particle size decreases. This has been clearly demonstrated with *B. anthracis*, *Pasteurella tularensis*, *Coxiella burnetti*, *Brucella suis*, and the virus of Venezuelan equine encephalomyelitis. Critical sizes range from approximately 1 to 5 μ. Such particles are retained maximally in the lung and, indeed, must be in this size range to reach the terminal bronchioles and alveoli.

The effects of particle size in experimental airborne tularemia in the guinea pig and the monkey are shown in Table 1. Note the quantitative difference in LD_{50} values between guinea pigs and monkeys exposed to the same aerosol. Obviously this does not reflect a difference in susceptibility to *P. tularensis* because both species demonstrate equal susceptibility to the 1-μ cloud. Reasons postulated for the lower LD_{50} values in monkeys include greater filtration efficiency of the guinea pig lung for larger particulate aerosols and mouth breathing in the monkey. Besides illustrating the importance of the particle size of the aerosol, the data do emphasize the obvious fact that the choice of laboratory animal in experiments of this kind is important.

A major problem in obtaining quantitative data on the response to different particle sizes involves the difficulty in producing homogeneously sized particle clouds. As various disseminating devices produce aerosols of large particles, invariably satellite, small 1-μ particles are produced. We are aware of no atomizing device that will produce a truly homogeneous cloud. Obviously, if enough populated satellites are produced to initiate infection, the number of larger particles inhaled and retained is relatively immaterial. It is important to note also that organisms within particles of a heterogeneous aerosol do not distribute themselves evenly throughout the droplets. The distribution of organisms throughout the available particles of the aerosol is influenced by the concentration of organisms in the material aerosolized. The smaller particles of the aerosol remain unpopulated at low organism concentrations, whereas at higher concentrations the small particles of the aerosol contain organisms. This is an important observation and illustrates the necessity for microscopic examination of collected samples of the aerosol. Use of the proper stain and phase-contrast microscopy makes it possible to visualize bacteria and some rickettsiae within collected aerosol particles, thus enabling the investigator to determine the organism distribution within particles, an important and sometimes critical piece of information.

TABLE 1. *LD_{50} dose in animals exposed to aerosols of Pasteurella tularensis*

Particle size diameter	LD_{50} dose for	
	Guinea pigs	Rhesus monkeys
μ	*no. of cells*	*no. of cells*
1	3	17
7	6,500	240
12	20,000	540
22	170,000	3,000

Figure 2 shows *P. pestis* within an aerosol particle. The slide was prestained with basic fuchsin, the aerosol was allowed to settle on the slide and was examined with the phase-contrast microscope. As mentioned, quantitative studies on the role of particle size in relation to the infectivity of aerosols were difficult because devices that would disseminate homogeneously sized particles were unavailable. Some time ago we employed a vibrating reed device which is capable of producing particles 5 μ and above with a remarkable degree of homogeneity and uniformity, thus eliminating the confusing satellite particles. Particles produced by this device vary in diameter no more than 0.2 to 0.3 μ. Table 2 shows some of the particle size and dose response data obtained using the vibrating reed and exposing restrained, unanesthetized rhesus monkeys. We were surprised by the obviously low LD_{50} value for the 8-μ cloud. If calculated, that value would be expected to be below 60 inhaled cells.

Histopathological observations of monkeys sacrificed at 24 and 48 hr after exposure indicated that *all* primary tularemia lesions in the lung were located in the terminal respiratory bronchioles regardless of 1- or 8-μ particle size. In no case were primary lesions observed at the depth of the alveolus. Animals in both groups showed X-ray evidence of pneumonic lesions.

Consequently, in a third series, animals were exposed to *P. tularensis* aerosols containing homogeneous particles 18 μ in diameter. These

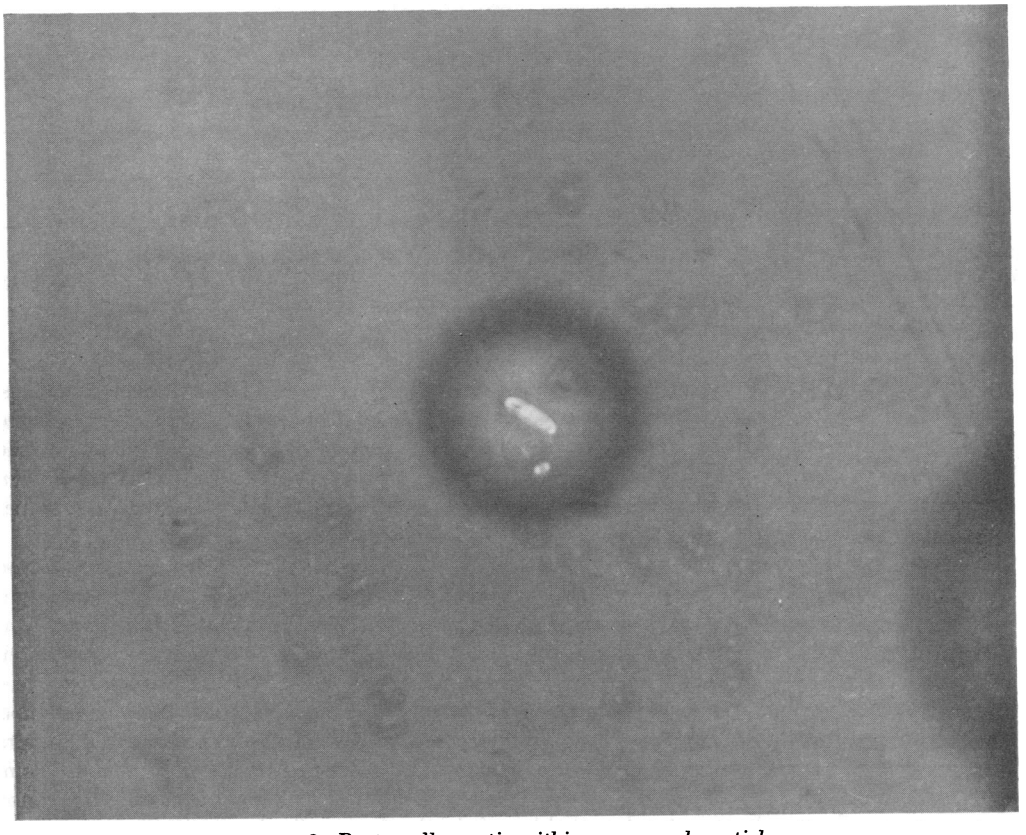

FIG. 2. *Pasteurella pestis within an aerosol particle*

monkeys became ill and died. None showed X-ray evidence of pneumonic disease. Pathological evidence indicated that the distribution of lesions supported a nasopharyngeal portal of entry with spread to the regional lymph nodes, sepsis, dissemination, and death.

In experimental airborne tularemia in the monkey, it is clear that the particle size of the aerosol is of great importance in determining the portal of entry and the nature of the consequent disease. There is evidence that this is likewise true in the case of experimental pneumonic plague.

Problems associated with measuring aerosol decay, both physical and biological, will be discussed by subsequent speakers. With several exceptions, aerosols of microorganisms decay or lose viability very rapidly. The mechanisms of death after aerosolization are very obscure. Webb (11–13) showed that decay of an aerosol occurs in at least two stages, a very rapid death in the first several seconds of cloud age and a slower death rate thereafter, which may result in long periods of time for cloud extinction. These decay rates can be correlated with a mathematical function of both temperature and humidity. Low relative humidity (ca. 20 to 30%) hastens the decay rate for most cells as does very high humidity (ca. 95%); optimal humidity ranges from 40 to 80%. We have done considerable work on the effect of solar radiation on aerosols (1). A special aerosol chamber has been constructed to

TABLE 2. *Dose response in rhesus monkeys exposed to aerosols of Pasteurella tularensis*

Particle size diameter	No. of cells inhaled	No. ill/no. exposed	No. dead/no. exposed
μ			
1	35	8/9	7/9
1	170	10/10	9/10
1	1,360	10/10	10/10
8	60	5/6	5/6
8	230–320	19/20	15/20

TABLE 3. *Effect of aerosol age on respiratory virulence of Pasteurella tularensis for guinea pigs*

Experimental parameters	Average aerosol age				
	UCTL atomizer		Collision atomizer		
	1.37 min	330 min	1.37 min	330 min	24 hr
Respiratory LD_{50}	18 cells	199 cells	11 cells	156 cells	540 cells
95% confidence limit*	(16–23)	(158–250)	(9–17)	(108–307)	(426–704)

* The majority of these results are an average of 11 tests.

TABLE 4. *Effect of aerosol age on respiratory virulence of Pasteurella pestis for guinea pigs*

Expt no.	Aerosol age	No. animals inoculated	Inhaled dose	Per cent mortality
	min			
1	5	10	9.2×10^4	100
	45	10	1.7×10^5	20
2	5	10	6.8×10^4	100
	45	10	7.0×10^4	10
3	5	10	1.2×10^5	90
	45	10	1.2×10^5	30

permit exposure of aerosols to natural sunlight under controlled conditions of temperature and humidity. Preliminary studies indicate that (i) large-particulate clouds are more resistant than small-particulate clouds to the lethal affect of solar radiation, (ii) dry disseminated aerosols (dust) were more resistant to solar radiation than wet disseminated aerosols, and (iii) in wet aerosols atmospheric moisture (relative humidity above 70%) afforded significant protection against the lethal affect of solar radiation.

Data supporting the various hypotheses relating to viability and infectivity of airborne cells are painfully meager and probably reflect our inadequate techniques for recognizing and measuring the various phenomena in operation. Scott (10), Ferry, Brown, and Damon (5), and Webb (11) all confirm the thesis that the one common factor affecting airborne viability is the relationship between cellular proteins and water. Webb suggests that death results from the movement of water molecules in and out of the cell in an equilibrium system, resulting in the collapse of the natural structure of cell protein. Treatment of aerosol-stable cells with ribonuclease or lysozyme rendered them aerosol sensitive. Cells of *E. coli* which are freely permeable to ions, small molecules, sugars, and peptides are air sensitive, whereas cells of *Staphylococcus citreus* and *Staphylococcus albus* are not so permeable and are more aerosol stable.

Webb (12) and others have shown that aerosol stability can be improved by adding a variety of compounds to the spray material. These compounds include amino acids, long chain protein derivatives, some sugars, and polyhydroxycyclohexanes. In our laboratories, the roles of nonmetabolized carbohydrates, metabolic inhibitors, and many other classes of compounds are being studied. If we are able to determine the causes of death of aerosolized organisms, we are confident that the geneticist, the nutritionist, and physiologist will be better able to develop strains or mutants which meet our requirements.

In addition to loss of viability of cells suspended in the aerosolized state for prolonged periods of time, it has become apparent that in the cases of *P. tularensis* and *P. pestis*, at least, there are demonstrable losses of virulence or infectivity (Tables 3 and 4). The mechanisms involved are quite obscure and apparently are somewhat independent of losses in viability. This is illustrated by the difference in mortality caused by essentially equal doses of *P. pestis*. It is not known whether the "less" virulent cells in the 45-min-old cloud would regain their original virulence if regrown in a growth medium and reaerosolized for 5 min.

In summary, investigators concerned with experimental airborne infection should pay close attention to:

1) Specific strain of organism chosen for study.

2) Conditions of growth and harvest of the organism.

3) Age of culture at time of aerosolization.

4) Suspending medium of the organism to be aerosolized.

5) Technique of aerosolization, including disseminating device, temperature, and relative humidity.

6) Techniques of aerosol sampling.

7) Particle size distribution of the aerosol.

8) Distribution of organisms (if possible) within the aerosol particles.

9) The age of the aerosol at time of animal exposure.

10) The decay rate, both physical and biological, of the aerosol.

11) Choice of experimental animal

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