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# Bacteriological Reviews

*A Publication of the American Society for Microbiology*

*It is characteristic of Science and Progress that they continually open new fields to our vision—PASTEUR*

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**ACROSS**

1. Type of bacteria that lives on inorganic matter
7. Antimony
8. Enzyme
10. Discoverer of specific bacillus of soft chancre (1889)
13. Gram-negative stain
14. Discoverer of bacterial agglutination (1896) (init.)
15. Every night
16. Flesh or muscular tissue
19. Lanthanum
20. Pertaining to the ear
22. Toxoid-antitoxin mixture (ab.)
24. Common animal for *in vivo* testing
25. Little stream (L.)
26. ________gony sexual life cycle to plasmodium
27. Spherical bacteria
30. Hydrogen ion concentration
31. Mercury
32. Eminent Japanese bacteriologist (1876-1928) (init.)
33. Type of fats
35. Egg (comb. form)
36. Firm colloid
37. Effective dose (ab.)
38. Type of bacterial movement
42. Type of fermentation tube
43. Toward the head

**DOWN**

1. Bacteria with several spirillae
2. Thrice daily
3. Cold-loving bacteria
4. Hemoglobin (ab.)
5. Type of bacterial pigment
6. Changes $H_2O_2$ to $H_2O$ and $O_2$
9. Selenium
11. Gram-negative bactericide
12. Ribonucleic acid
17. Bacill ry shape
18. Mnemonic for coliform bacteria classification test
21. Indium
23. Axioincisal (ab.)
26. Relating to the spleen (comb. form)
28. Gangosa
29. Tabular information sheet
34. Clear animal liquids
36. Founder of Journal of Pathology and Bacteriology (init.)
38. Bismuth
39. Hydroxyl radical
40. Antitoxic unit
41. Neptunium

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# BACTERIOLOGICAL REVIEWS

*A Publication of the* AMERICAN SOCIETY FOR MICROBIOLOGY

VOLUME 30 — *September 1966* — NUMBER 3


# Second International Conference on Aerobiology (Airborne Infection)

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Consultant Editors


MARK H. LEPPER
University of Illinois
College of Medicine
Chicago, Ill.

ELWOOD K. WOLFE
U. S. Army Biological Laboratories
Fort Detrick
Frederick, Md.



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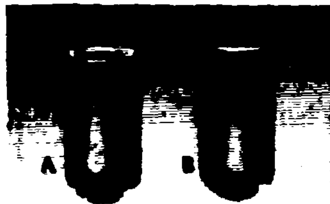


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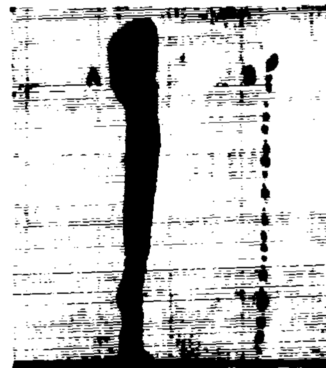


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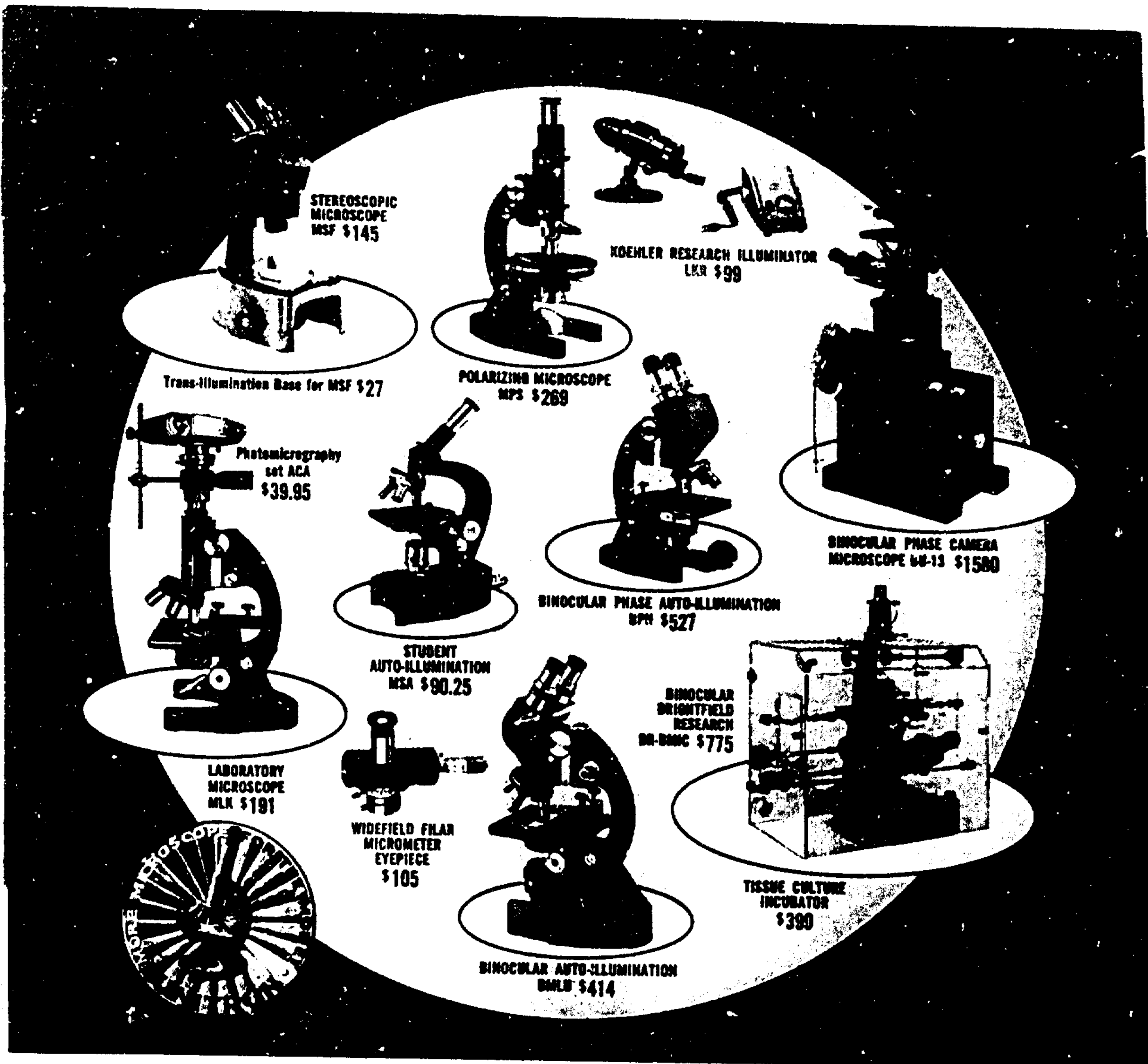


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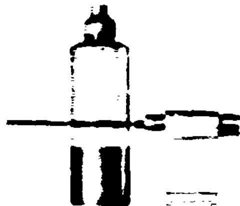

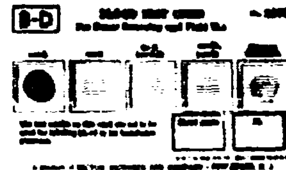

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# Foreword

The meeting termed the First International Conference on Aerobiology was held in October 1963 on the campus of the University of California at Berkeley and was cosponsored by the Naval Biological Laboratory and the University of California. The proceedings were published by the Naval Biological Laboratory as *A Symposium on Aerobiology, 1963*. Prior to this conference, however, the "key" conference which actually originated the series was held in December 1960 at Miami Beach, sponsored by the National Research Council with support from the National Institutes of Health and the U.S. Army Biological Laboratories. The proceedings were published as the September 1961 issue of *Bacteriological Reviews*. The present Second International Conference on Aerobiology continues the series of reports and discussions related to recent investigations in aerobiological fields.

Since the Berkeley conference, research in airborne infection has been stimulated by the problems arising from the increasing concentrations of military recruits in the various training centers. This, in turn, has caused an increase in research in aerobiology in general in order to provide information relating to the basic parameters of biological aerosols and to develop or improve the technology for such investigations. This interest will certainly continue, and conferences of this type can be anticipated at roughly 3-year intervals.

The Program Committee is indebted to the Session Chairmen for their time and competence and to many individuals from the U.S. Army Biological Laboratories and the Illinois Institute of Technology Research Institute for their willing efforts in arranging and conducting the conference.

ELWOOD K. WOLFE
*General Chairman*

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# Airborne Infections—the Past and the Future[1]

HARRY F. DOWLING

*Department of Medicine, University of Illinois College of Medicine, Chicago, Illinois*

Ebb and flow is as characteristic of research as of other activities of man. The transmission of infections through the air is a good example. Interest in airborne infections has had three flood tides in the past four centuries. The first was a long one. We can trace its beginning to 1546, when Fracastorius published his theory of contagion and explained that transmission of infections might occur by simple contact, by indirect contact, or by transmission from a distance. From that time on, the concept of airborne disease captured men's minds. "This most excellent canopy, the air," says Hamlet, "why it appeareth nothing to me but a foul and pestilent congregation of vapors." Hence, in times of epidemics the air was filled with the smoke of sulfur, camphor, tar, or pitch, and people religiously shut out the night air, walked away from noisome smells, or, if they couldn't avoid them, held perfumed kerchiefs to their noses.

The idea that diseases were spread by bad air (i.e., malaria) or by the influence (i.e., influenza) of planets, carried through the air, was too simple and naive an explanation. It was eventually discredited because attempts to apply it failed to stop the spread of infections. By the middle of the 19th century, it had been almost completely discarded by the medical profession (1). Pasteur's demonstration in 1861 that bacteria were consistently present in the unsterilized air and Lister's report in 1867 of the control of suppuration of wounds by measures which included a phenol spray should have stimulated everyone to vigorous attempts to control airborne infections. But this did not happen—for several reasons. For one thing, as I have said, all of the measures that could be conceived for limiting the spread of infection by air had already been tried and had failed. Also, it became evident to those surgeons who studied Lister's techniques carefully (rather than opposing them violently or aping them completely, as most did), that his method owed its success mainly to the suppression of contact infections. This was verified when Macewen about 1880 successfully used instruments and dressings sterilized by boiling and discarded the phenol spray. Finally, other public health measures had been successful and here, as elsewhere, nothing succeeds like success. To understand this, let us try to picture sanitary conditions in our cities around 1850. As Rosenberg (5) so aptly put it:

> "A standing joke maintained that city water was far better than any other, since it served as a purgative as well as for washing and cooking. Most people were sensible enough not to drink it, except when forced by poverty or betrayed through inadvertence."

And Coleridge exclaimed,

> "I counted two-and-seventy stenches
> All well defined, and several stinks
>
> . . . . .
>
> The river Rhine, it is well known,
> Doth wash your city of Cologne;
> But tell me, nymphs! what power divine
> Shall henceforth wash the river Rhine?"

Since filth and putrescence were associated with disease in people's minds, the campaign for clean water gathered momentum. Paralleling this was the concept that fevers resulted from noxious exhalations from putrid animal and vegetable matter. Hence, methods for disposing of waste and sewage were developed gradually during the last half of the 19th century. The results were apparent in the dramatic drop in the mortality and morbidity from water-borne and insect-borne diseases at the turn of the century.

The emphasis, therefore, was on spread by the fecal-oral route and by direct contact. Airborne infections were too hard to understand, and techniques for their control were too complex. So matters stood at the end of World War I. Soon thereafter, a second wave of interest in airborne infections developed.

In 1917, Stillman (6) reported that he had cultured types I and II pneumococci in dust collected from homes of patients with pneumonia caused by the same types. He was able to grow microorganisms from the lungs of mice after exposure to a mist containing pneumococci (7), and he also produced pneumonia with these mists after partially immunizing mice and depressing them with alcohol (8). Branch and Stillman (2) also reported that pneumonia was produced in mice by the inhalation of $\beta$-hemolytic streptococci and *Klebsiella*. These observations were soon overshadowed by other studies in animals of pneumonia

[1] Opening address.

produced by the installation of bacteria, especially the experiments of Robertson and his associates (4) on dogs, which extended over a number of years and added greatly to our knowledge of the pathogenesis of, and immunity from, pneumococcal pneumonia. Valuable as these latter studies were, they diverted interest away from the production of pneumonia by inhalation, since intrabronchial instillation was simpler and surer. Furthermore, the control of pneumonia took a hopeful turn in another direction; good results were obtained from serum therapy, followed by the greater successes of sulfonamides and antibiotics. Thus, the second wave of interest in airborne infections petered out. But the third wave was already forming.

In 1934, Wells introduced the concept of droplet nuclei (9) and followed this with a series of theoretical and applied experiments which broke the logjam and released a new flood of investigations. These investigations were reinforced by the rising interest in the spread of streptococcal infections in army barracks and of staphylococcal infections in hospitals, in the dissemination of viral and fungal infections, and finally in the chemical pollution of the atmosphere. Today we are on the crest of this wave of interest. Let us look at where we are and where we are travelling. To start with the sources of airborne infections, many studies have been made with sick and well persons as sources, but we still are not always sure where the microorganisms originate in the person who disseminates them and how they become dispersed in the air. What is the relative role of the upper and lower respiratory tract and of the skin? Some people spread streptococci and staphylococci more readily than others. We do not aways know why.

Studies have been made on the environment as a source of infection. These have been especially productive in relation to fungi. On the other hand, the principles by which infections are spread in the dust of rooms are only partially comprehended.

Studies have been made on the transmission of infection and the role of droplet nuclei and other small particles in the spread of various diseases, and great progress has been made in this area. I should like to suggest that such studies should not neglect the effect of temperature and humidity on microorganisms while they are in the air. For instance, we still have no idea why the common cold is more prevalent in the colder months. From experiments which my associates and I reported (3), it appears that the explanation does not lie in the chilling of the recipient. Does the virus remain alive longer in cold weather, or in hot dry buildings? Or is crowding of people in the winter months the sole explanation? Finally, with regard to the relationship of microorganisms and chemicals in the air, studies have been made on chemicals which will prevent the spread of bacteria in the air, and some promising results have been obtained with the glycols. On the other hand, we are at present far from knowing whether air pollution augments airborne infection, and, if so, how.

A great deal more attention can be directed toward the recipient. We have a good idea how far microorganisms go into the lung during inhalation and how this is affected by particle size. We are studying the role of the mucus sheath, the cilia, phagocytosis, and local immunity, but we have only scratched the surface so far. Techniques are at hand with which to dig a great deal deeper, and many of them will be reported on in this Conference. The isolation of many of the respiratory viruses and extensive experience with the production of viral infections in volunteers makes it possible, for instance, to study dissemination, spread, and inception of airborne infections in man, something that we could not safely do with bacteria or fungi. Also, the rapid multiplication of antibiotics enables us to try to stop bacteria from growing in air passages even after they have arrived in large numbers through the air.

Finally, there is the control of airborne infections. Because earlier attempts to prevent these infections by ultraviolet light and chemical disinfection were not uniformly successful, the atmosphere in a roomful of investigators of respiratory infections seems itself to be infected with pessimism. Need this be so? The technology of ventilation is advancing all the time, and increasing numbers of people are living the year around in artificial atmospheres which eagerly wait for someone to make them nearly germ-free. Besides, we have failed to capitalize on one strong force, the increasing chemical pollution of the air we breathe. I remember as a boy seeing a performer in a circus dive into a glass tank and eat a banana under water. I was horrified to think that he was eating food that was coated with the bacteria he carried with him every time he dived in. And yet, we are not doing anything different in breathing air contaminated with bacteria. Air in a crowded room may be as contaminated as water in the average farm-pond. But just as our ancestors paid no attention to the water so long as it looked clear, so the public pays no attention to the air when it seems pure. Now that the stench and the irritation from chemical wastes are becoming daily more obnoxious, can we not mobilize interest in cleansing the air? And just as ridding the water of foul tastes was the beginning of purifying it of bacteria, so we may succeed in doing both to the air.

What would happen to our immunity if we breathed in no microorganisms year in and year out? We know the answer from bitter experience with measles in isolated populations or with poliomyelitis in the more advanced countries. To keep up our protection we will have to vaccinate. But how can we immunize against the hundreds of bacteria and viruses, to which people now develop enough immunity to produce a tolerable equilibrium? Perhaps it can be done by isolating the purified antigens and combining them in feasible doses for injection. But it will take a long time to find these antigens, and it is by no means certain that we can prepare all the vaccine necessary or persuade the people to take them if we do. This is where immunization by inhalation comes in. The pioneer studies of vaccination against tetanus and tularemia have shown the way, but to my mind this subject has not received nearly the attention that is its due. The methods of inhalation have been perfected; the bacteria and most of the viruses have been isolated; now the time is ripe to extend the few studies that have been made. One might even visualize, a few years from now, school children and office workers receiving their immunizations without fuss or discomfort while they are sitting at their desks going about their regular work, or, alternatively, inhalo-mobiles that would park outside a school, an office building, or a shopping center, attracting people inside for their inhalations much as X rays for tuberculosis are taken nowadays.

What I have just said may sound grandiose, but I am using this picture deliberately to point up the main theme of my discussion. We have had earlier waves of interest in airborne diseases, but they have died out because other methods of control of infections were more successful and because adequate techniques were not at hand to study and control infections transmitted through the air. As a result, control of contact infections and of food-borne, insect-borne, and water-borne infections is far ahead of control of airborne infection. But the present wave of interest is high, and the techniques are at hand. To paraphrase Brutus,

> "There is a tide in th' pursuit of knowledge,
> Which, taken at the flood, will bring success.
> On such a sea are we now afloat
> And we can take the measures at our hand
> And win our ventures."

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# Mechanisms of Antibacterial Action in the Respiratory System

EDWARD H. KASS, GARETH M. GREEN,[1] AND ELLIOT GOLDSTEIN

*Channing Laboratory, Thorndike Memorial Laboratory, Department of Medical Bacteriology and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and Department of Bacteriology and Immunology and Department of Medicine, Harvard Medical School, Boston, Massachusetts*

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## INTRODUCTION

Perhaps the most striking finding in relation to the antibacterial activity in the respiratory system is the sterility of the bronchopulmonary apparatus from the primary bronchi downward. That these structures are ordinarily sterile, despite the continuous entry of droplet nuclei containing bacteria, has been known for more than 50 years (3). The mechanism by which the bronchopulmonary tree retains its sterility under ordinary circumstances is just beginning to be understood.

Our attention was drawn to the problem by the considerations that if, in fact, the bronchopulmonary tree were normally sterile, and if in chronic bronchitis the lower bronchial secretions were ordinarily heavily populated with bacteria, there might be a basis for investigating the pathogenesis of chronic bronchopulmonary infections from the standpoint of breakdown of mechanism of local antibacterial activity. Initial investigations, therefore, were directed toward the study of the bacteriological flora of the bronchopulmonary secretions, with the use of extreme precautions to avoid contamination of the cultured material by bacteria coming from the upper respiratory passages. These studies (10) confirmed earlier observations (1, 11) that bacteria were rarely found in the bronchopulmonary secretions unless there was manifest inflammation or exudation. By the same token, when such exudation was found, the bacteria tended to be present in large numbers (>$10^6$ colonies per milliliter of secretion), and often the bacteria in the bronchial secretions were not adequately represented in cultures of the sputum.

The present review is not intended as a detailed discussion of the implications of these findings in terms of bacteriological interpretation of cultures arising from the bronchopulmonary apparatus. Instead, attention will be directed toward the implication that the failure to find bacteria could be explained primarily on the basis of continued activity of a potent local antibacterial mechanism. That such a mechanism exists has been indicated by numerous studies in the past. For example, Stillman (16), working with pneumococci that had been instilled into the bronchi of mice, observed that the majority of these organisms were made nonviable by the lung shortly after instillation. Lurie, in his classic experiments on the fate of aerosolized tubercle bacilli, observed that as many as 100 organisms needed to be inhaled to set up one tubercle, even in genetically highly susceptible rabbits, and one or two orders of magnitude more bacteria were required to be inhaled to produce a tubercle in genetically more resistant strains of rabbit (12). It was apparent, then, that the overwhelming majority of the inhaled bacteria were being killed in some manner after inhalation.

## BACTERIAL CLEARANCE IN THE NORMAL LUNG

To study the phenomenon of local killing in the lung, an aerosol apparatus was constructed out of simple and relatively inexpensive materials (8). The apparatus delivered more than 85% of its particles in the form of nuclei between 1 and 3 $\mu$ in diameter, and, in detailed studies of the function of the apparatus, it was found that a set of stand-

[1] Fellow of the American Thoracic Society, National Tuberculosis Association.

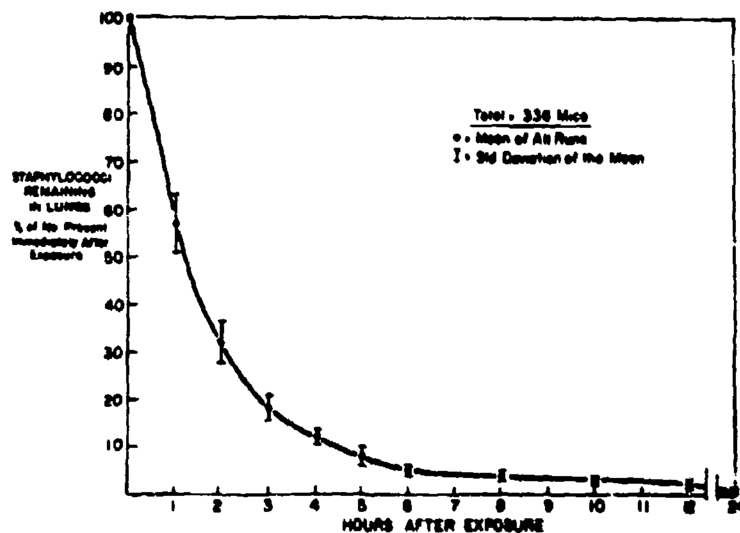


FIG. 1. *Disappearance of Staphylococcus aureus from the lungs of mice after administration by aerosol. The rapid disappearance and small standard deviations are especially noteworthy. (Reprinted from reference 8.)*

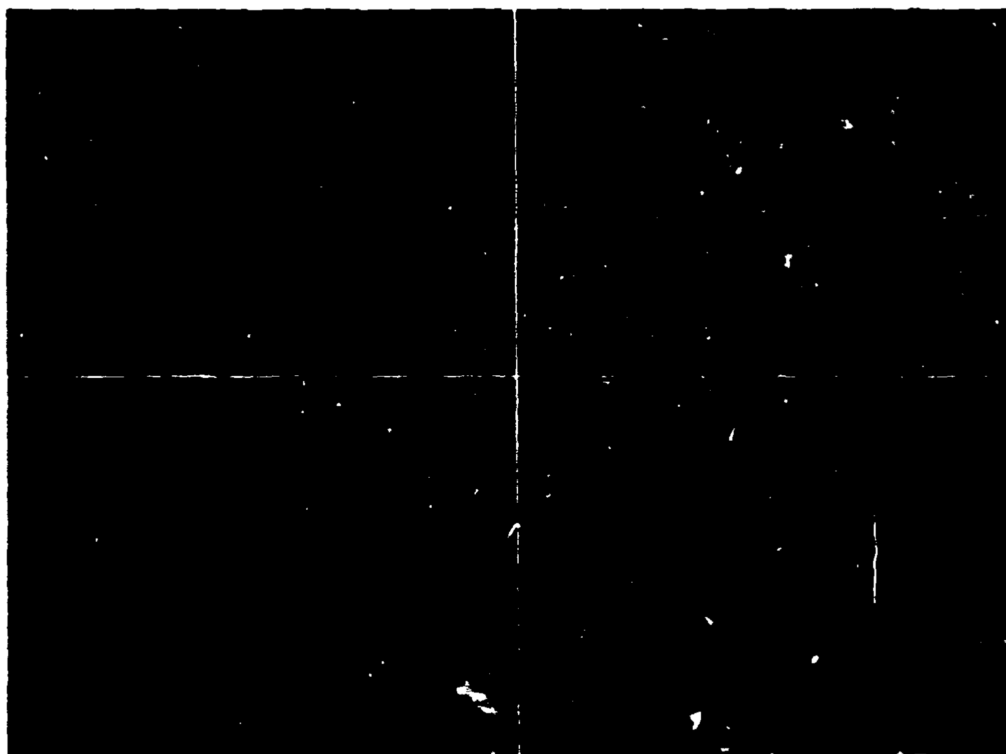

FIG. 2. *Lungs of mice taken 4 hr after exposure to aerosols containing Staphylococcus aureus. The sections have been stained with antibody to the Staphylococcus by use of fluorescein-labeled antibody, in accordance with methods given in reference 6. Note the intense staining of bacterial antigen in some cells lining the alveoli. In a few instances, discrete coccal bodies can also be seen.*

FIG. 3. *Section of mouse lung immediately after 30 min of exposure to aerosol of Staphylococcus aureus stained by Macallum-Goodpasture stain. × 2,500. In the upper photograph, staphylococci appear to have been ingested by an alveolar septal cell. In the lower, staphylococci may be in a mononuclear macrophage in the alveolar septum (see reference 6).*

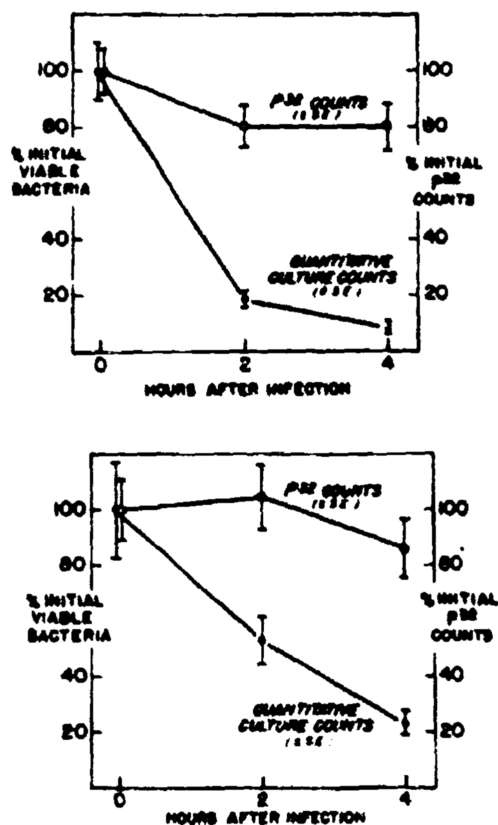


Fig. 4. *Clearance of $P^{32}$-labeled Staphylococcus aureus and Proteus mirabilis from murine lung. The change in number of viable organisms is compared with the change in radioactivity in the lung (6).*

ardized conditions could be obtained whereby a suspension of staphylococci could be delivered into the chamber in sufficient concentration that mice exposed to the aerosol for 30 min and sacrificed immediately thereafter were found to contain in their lungs approximately 50,000 viable units of staphylococci capable of producing colonies. When the lungs were cultured at regular intervals after exposure to the aerosol, the bacteria were found to have become nonviable in exponential fashion, so that within 4 hr approximately 85% of the bacteria could no longer be detected, and within 6 hr all but a few per cent had become nonviable. What was even more striking was the extraordinary reproducibility of the method, as seen by the small standard errors of the mean bacterial counts obtained at various time intervals after infection by aerosol (Fig. 1). The rates of disappearance of the bacterial particles were consistent with the anticipated rate of disappearance of any particles of this size, but as is well known, particles of this size tend not to impinge on the bronchial mucosa, and so the implication was clear that most of the disappearance of the bacteria was likely to be due to cellular systems or other antibacterial systems operating below the tertiary bronchi, beyond the level at which the mucociliary apparatus is active.

*Clearance by the Alveolar Macrophage System*

To test the implication that bacterial killing occurred primarily in the deeper portions of the lung, two experiments were conducted (6). In the first, fluorescein-labeled antibody was used to detect bacterial antigen in the lungs of mice that had been exposed to the aerosols, and invariably bacterial antigen was found in the epithelial cells lining the alveoli (Fig. 2). Occasionally, relatively intact bacteria could be found in these alveolar-lining cells (Fig. 3). When the bacteria were labeled with radioactive phosphorus and their fate was studied, it was found (Fig. 4) that, when approximately 85% of viability had disappeared, radioactivity had declined by only about 20%. Thus, the decline in viability was not due to transport of the bacteria away from the alveoli, as evidenced by the retention of radioactivity and the observation of bacterial antigen in the alveolar-lining cells. Only a small minority of the bacterial population could have been transported away during the time of maximal killing. The majority of the bacterial cells were destroyed in situ, and the alveolar macrophage system clearly seemed to be the principal agency for such removal.

*Role of Bacterial Species*

That the bacterial species is an important variable in the process of removal was demonstrated by subsequent studies (5) in which the rates of removal of a strain of *Proteus mirabilis*, one of *Staphylococcus aureus*, and one of *S. albus* were studied under comparable conditions (Fig. 5). *S. albus* was removed most rapidly, *S. aureus* somewhat more slowly, and the strain of *Proteus* still more slowly. More recently, observations have been made with a pathogenic strain of *Pasteurella* that frequently produces pneumonia in mice and, as might be expected, clearance of this organism in some, but not all, animals is slower than that of *Proteus*, and occasionally clearance is completely reversed and bacterial proliferation is observed.

The wide variation in clearance of aerosolized bacteria in relation to species suggests that simple mechanical factors, such as the action of the mucociliary apparatus, are an unlikely basis for the antibacterial action, since it seems unlikely

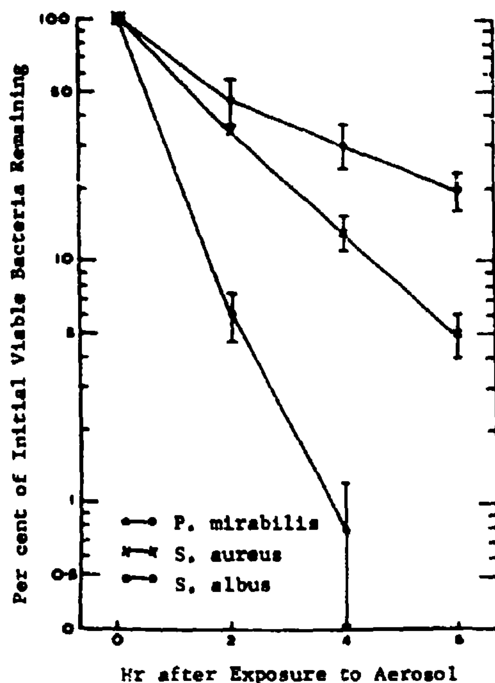


FIG. 5. *Clearance of Proteus mirabilis, Staphylococcus aureus, and S. albus by the normal mouse lung* (5).

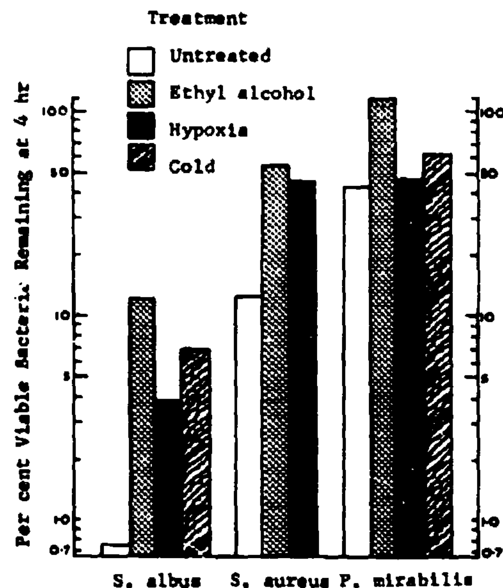


FIG. 6. *Relative effects of ethyl alcohol, hypoxia, and cold on the clearance of Staphylococcus albus, S. aureus, and P. mirabilis by the normal mouse lung. Although in general these circumstances delayed bacterial clearance, the effect of hypoxia in the case of Proteus was negligible. It is also noteworthy that, in the case of Proteus, ethyl alcohol sufficiently depressed bacterial clearance to allow bacterial multiplication to occur* (5).

that a mechanical system would show such a wide range of effectiveness against different species of similar size range. However, such differences in antibacterial activity against different bacterial species are well known in phagocytic systems (2, 14). The observations also indicate that, since the methods of clearance of different bacterial species may be different from one another in a given host, circumstances that alter the rate of clearance may permit one or another species to multiply instead of being cleared.

## BACTERIAL CLEARANCE AND ENVIRONMENTAL AND METABOLIC DISTURBANCES

To investigate the relationship of a variety of metabolic events to the clearing mechanism, experimental animals were exposed to the aerosol of appropriate bacteria and then immediately exposed to a metabolic circumstance that might be expected to alter the rate of bacterial clearance (4). It was found (Fig. 6) that hypoxia equivalent to an altitude of 10,000 ft was sufficient to slow significantly the rate of bacterial clearing, and this effect could also be obtained with diminished oxygen tensions at sea level pressures. Ethyl alcohol inhibited bacterial clearing by the lung and did so in direct relationship to the dose of ethyl alcohol administered. Furthermore, the administration of oxygen to the intoxicated animals did not correct the defect. The latter experiment was performed because of the possibility that ethyl alcohol may have depressed respiration and thereby brought about depression of bacterial clearance. Acute starvation for 24 hr was associated with depressed clearance of bacteria by the lung, and once again the degree of depression of clearance was directly related to the amount of weight lost. In retrospect, however, the latter effect may not be entirely due to the weight loss itself, but may be related to such accompanying metabolic disturbances as acidosis (see below). Cortisol also depressed bacterial clearance significantly.

When the effects of the metabolic agents that inhibited bacterial clearance were tested in animals that had received different microorganisms in the aerosol, it was apparent that not all of the metabolically induced suppression of clearance was uniform regardless of species (5). For example, the clearance of staphylococci was only partly depressed by ethyl alcohol, but the clearance of *P. mirabilis* was completely inhibited and multiplication of the organism occurred. Thus, under conditions of ethyl alcohol intoxication,

with all three species of bacteria present in the lung, it might be expected that *Proteus* would emerge as the most likely organism to produce pulmonary infection. On the other hand, whereas hypoxia markedly inhibited the clearance of staphylococci, there was no depression of clearance of *Proteus* in the hypoxic animals. Presumably an oxygen-dependent system in the alveolar macrophage is operative against certain bacteria such as staphylococci, but not against other organisms, such as *Proteus*. Once again there are indications that specific environmental conditions in the presence of a mixed bacterial flora may favor the emergence of one or another bacterial species from the mixture. In vitro, phagocytosis by alveolar macrophages is depressed when oxygen tension is reduced (13).

### *Bacterial Clearance and Viral Infection*

Another clinical circumstance in which pulmonary bacterial infection has been involved has been the presence of a precedent viral infection. Sellers and co-workers (15) demonstrated that in mice infected with influenza virus the intranasal insufflation of staphylococci was followed by virtually no clearance of the organisms by the lung, whereas in animals not infected with the influenza virus, the staphylococci were readily cleared by the murine lung. Detailed studies of this phenomenon have indicated (Fig. 7) that clearance of inhaled staphylococci is inhibited in the presence of a viral infection. However, quite unexpectedly it was found that the time of maximal inhibition of bacterial clearance by the virus-infected lung was toward the end of the 1st week after the induction of the viral infection. With relatively small inocula of virus, no inhibition of bacterial clearance was observed in the first 5 to 6 days after induction of the viral infection, even though the peak of viral multiplication had been reached by approximately 48 hr. On the other hand, distinct and striking inhibition lasting for 1 to 3 days was observed toward the end of the 1st week at a time when viral titers were falling rapidly. Precisely why this unusual time sequence should occur is a matter for future study. It is noteworthy, however, that, clinically, bacterial infections as superimposed complications of viral infections often appear about 1 week after the initial viral infection.

Previous bacterial infections with the same species seem not to inhibit function of the alveolar clearing mechanism very much, as might be expected from consideration of the relative numbers of bacteria inhaled in relation to the large numbers of alveolar cells available. Thus, when mice were exposed to an aerosol of staphylococci on succeeding days for 1 week, the rate of clearance was not different after seven successive exposures than after the initial exposure.

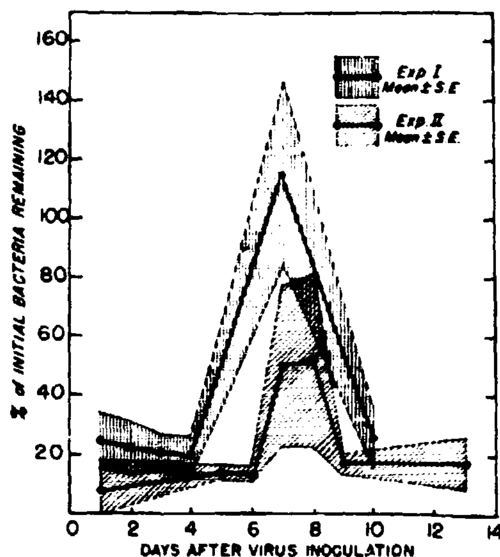


Fig. 7. *Effect of influenza virus infection on clearance of Staphylococcus aureus by the murine lung. The results of two separate experiments are given and plotted as the mean number of bacteria remaining after 4 hr, plotted as the per cent of the initial bacteria. The influenza virus infection was induced with 0.3 $LD_{50}$ dose.*

### *Bacterial Clearance and Pulmonary Injury*

At first glance it might appear that any injury to the lung would be associated with diminished bacterial clearance. However, the widespread nature of the alveolar system might also suggest that focal anatomic lesions would not inactivate a sufficiently large percentage of available cells to inhibit measurably bacterial clearance, except as the anatomic lesions became overwhelmingly severe. The latter of these two points of view seems to be the correct one. Goldstein (*unpublished data*) has produced silicosis experimentally by the intratracheal administration of silica suspensions, and severe coalescent disease was produced in the lungs of the animals. There was remarkably little effect on bacterial clearance, except perhaps in the terminal stages of the silicotic disease, when a variety of other metabolic consequences of severe pulmonary disease also begin to operate.

### *Bacterial Clearance and Tobacco Smoke*

Most recently, an additional effect on pulmonary bacterial clearance by a particle of major

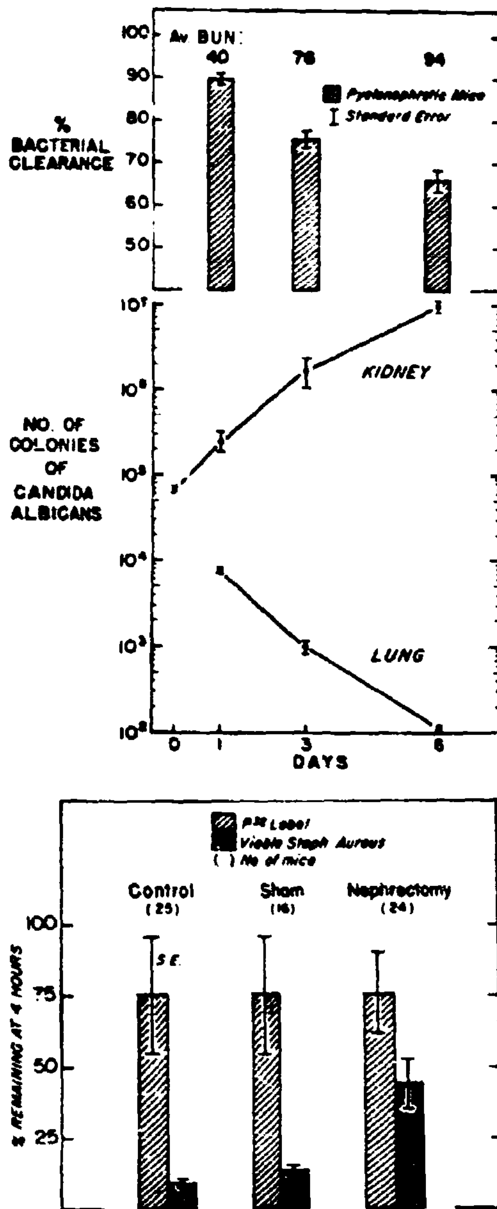


FIG. 8. *Upper portion of the figure indicates the numbers of colonies of Candida albicans in kidneys and lungs of mice after inoculation, and these are related to the levels of blood-urea nitrogen in these animals as well as the per cent clearance of Staphylococcus aureus observed 4 hr after completion of exposure to the aerosol. When numbers of Candida were highest in the lung but blood-urea nitrogen levels were relatively low, there was no significant effect on pulmonary bacterial clearance. As the renal lesion progressed and the numbers of organisms in the lung regressed, the blood-urea nitrogen rose, and there was a corresponding decrease in bacterial clearance. In the lower figure, the effect of nephrectomy on pulmonary bacterial clearance of S. aureus is demonstrated. Although the numbers of bacteria inhaled by each of the three groups is comparable, as evidenced by the comparable levels of $P^{32}$ label in the bacteria, sham surgery slightly depressed bacterial clearance in 4 hr but nephrectomy markedly depressed bacterial clearance.*

public health importance has been observed. It was initially observed by Laurenzi et al. (9) that mice exposed to tobacco smoke suffered inhibition of pulmonary bacterial clearance. More recently, it has been observed by Green and Carolin (*unpublished data*) that the addition of tobacco smoke to cultures of pulmonary macrophages rapidly altered the capacity of the macrophages to cling to the culture flask and greatly diminished the killing power of these cells for added staphylococci. The nature of the agent in tobacco smoke that produces this effect is not yet clear, but the substance is water-soluble and affects macrophage function quantitatively.

A useful methodological innovation that has come from these studies is a consequence of the earlier demonstrations that radio-labeled bacteria may be killed quite rapidly by the lung, but most of the label is readily recovered from the lung after 4 hr, when most of the bacteria are nonviable. In consequence of this observation, it has been possible to study rates of clearance in individual animals rather than in groups of animals, and to do so with considerable precision. It is only necessary to expose animals to radiolabeled bacteria of known specific activity and, after a given period of time, to count the radioactivity and the viability in the homogenates of the lungs. The radioactivity will afford an approximation of the total number of bacteria deposited in the lung, and the direct bacterial counts will indicate residual viability. From these data, the degree of killing can be estimated.

The method has added substantially to the precision of study of the pulmonary antibacterial system, and has made the standard errors of respective points smaller still. Even more important, it has permitted the study of clearance in individual animals and thus has greatly increased the efficiency of the experimental work. Finally, the method offers some hope that it can be adapted to the study of clearance mechanisms in the human being.

### *Bacterial Clearance and Renal Failure and Acidosis*

A recent insight into another major metabolic circumstance that has been clinically associated with apparently increased susceptibility to pulmonary infection has come from the observation that nephrectomized animals or animals whose kidney

function has been reduced in consequence of experimental candidiasis have decreased capacity to clear bacteria from their lungs (Fig. 8). It seems, from the present as yet incomplete analysis of the phenomenon, that the acidosis accompanying the uremic state in these animals is the primary source of the disturbed function of the macrophage system. The implication is clear that pulmonary macrophages harbor enzyme systems that are critical to phagocytosis or bacterial killing, and that are exceedingly sensitive to minute variations in *p*H. The search for such systems should be carried on forthwith.

## SUMMARY

In summary, it is apparent that there is an in situ mechanism for clearing bacteria in the lung. This mechanism, which accounts for most of the antibacterial activity, appears to reside primarily in the pulmonary macrophage, and is relatively independent of the function of the mucociliary apparatus. Parenthetically, it has been observed that later in the course of events pulmonary macrophages laden with bacteria may become free and be carried upward by the mucociliary stream. The pulmonary macrophage system is peculiarly susceptible to a variety of metabolic situations, such as hypoxia, ethyl alcohol, acidosis, cortisol, tobacco smoke, and undoubtedly many others. The macrophage system responds differently to different bacterial species, and the metabolic circumstances that alter bacterial clearance do not affect clearance of each of the bacterial species in the same manner. Thus, a metabolic basis emerges whereby a single organism may emerge from a mixture of organisms as a pathogen under specific environmental circumstances. Viral infections inhibit the clearance of bacteria, but do so, strangely enough, after approximately 1 week of viral infection, and not at the time when viral replication is at its height. Multiple anatomic lesions, such as those accompanying diffuse silicosis, have relatively little effect on bacterial clearance, compared with the effects of the aforementioned metabolic states. Tobacco smoke has a water-soluble substance in it that inhibits the function of pulmonary macrophages.

How these observations relate to the genesis of chronic bacterial infection of the lung is only conjectural at present, but clearly the hypothesis can be stated that a variety of environmental circumstances may conspire to reduce slowly the capacity of the pulmonary macrophage to inhibit bacterial proliferation, and that then a chronic state of bacterial proliferation in the bronchial tree may result. It is conceivable that such a chronic state of bacterial habitation in the lung might be detected by appropriate methods long before manifest clinical pulmonary disease could be found, and in this sense the situation in which asymptomatic infections of the lung might be a precursor to chronic pulmonary infections could be analogous to a comparable situation in the urinary tract (7).

## ACKNOWLEDGMENTS

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## Discussion

VERNON KNIGHT

*Laboratory of Clinical Investigations, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Bethesda, Maryland*

Dr. Kass has reported highly reproducible measurements of the rate of clearance of staphylococci and other bacteria from the lungs of mice after aerosol inoculation. The aerosol particles were 1 to 3 μ in diameter, and the dose, given in a 30-min inhalation, was sufficiently large to permit recovery of at least 50,000 colony-forming units. Studies of lung sections with fluorescein-labeled antibody and by conventional staining methods revealed staphylococcal antigen and some intact bacteria in alveolar lining cells.

With this model, the effect of hypoxia, alcohol, starvation, and other influences was studied. In addition, it was shown that influenza virus infection interfered with the clearance of *Staphylococcus aureus* from the lung.

At this point, it is perhaps of interest to consider briefly the relationship of clearance of staphylococci by alveolar macrophages, referred to by Dr. Kass, with other clearance mechanisms. It is well appreciated at this conference that particles of the size used by Dr. Kass largely escape trapping in the nasopharynx and are carried to the lung. Here a large percentage are deposited, and the remainder are exhaled. Sites available for deposition are the alveoli, the alveolar ducts, respiratory bronchioles, and more proximal airway structures. Although gas exchange occurs quite readily between the tidal air and the alveoli through the layer of residual air in the alveoli, this is effected chiefly by the process of molecular diffusion. In contrast, only 10 to 20% of aerosol in tidal air actually exchanges with residual air with each breath, and molecular diffusion is not a significant factor with particles of the size presently under discussion. It is suggested, therefore, that substantial alveolar penetration will require prolonged periods of breathing of aerosol, probably of the order of that used by Dr. Kass. With a few breaths, particles may be deposited in the lower respiratory tract proximal to the alveoli, and, with further breathing, the site of major deposition will progress peripherally, ultimately to the alveoli, as alveolar wash-in is completed. Parenthetically, I wonder if the slow movement of particles from tidal air to residual air may not be an important means of protection against toxic or infectious particulates in the environmental air.

Once deposited, particles may be removed from alveoli by alveolar macrophages and carried into pulmonary lymphatics. Some macrophages filled with particulates may also be discharged up the airway to the muco-ciliary blanket and then carried up the trachea. In the case of microorganisms which deposit in the respiratory bronchioles, the mode of disposition is not clear. Alveolar macrophages are apparently not available here, and the muco-ciliary blanket begins more proximally. Some studies, however, have described a hyperreactivity of respiratory bronchiolar lining cells which may be a special means of protection in this area. The small volume of lung airway represented by the tracheobronchial tree appears to be the best protected. Inhaled particles which deposit here are carried rapidly up to the posterior pharynx by the muco-ciliary mechanism, where they may be expelled or swallowed.

At present, I know of no studies which adequately describe relative degrees of deposition of small particles in peripheral lung areas in relation to the duration of exposure to small-particle aerosol. I believe the question to be of importance, since, if the foregoing concept is correct, it would be possible to deposit small-particle aerosol in

sites other than the alveoli, and mechanisms other than alveolar macrophages would be called forth to clear them from the lung. Instances of this sort may regularly occur in the natural spread of airborne infection.

Evidence for significant deposition of small particles at sites other than the alveoli is found in the work of McGavran et al. (4), who observed that pulmonary lesions of psittacosis in monkeys, after small-particle aerosol inoculation, developed around foci in respiratory bronchioles and that none were found developing around alveoli. These findings cannot be considered proof, however, since lesions develop a considerable time after inoculation, and a number of factors could influence the site of development of infection during this period.

I was especially interested in Dr. Kass' report that clearance of staphylococci from the lung was impaired in the presence of influenza virus infection, but only after it had progressed for 6 to 8 days. As he suggested, this coincides in time with the occurrence of some human cases of bacterial pneumonia complicating influenza. Harford et al. (2) in 1948 showed a similar result with pneumococci. In their studies, instilled pneumococci multiplied rapidly in mice during the 5th to 6th day of viral influenza, leading to pneumonia and death. Gerone et al. (1) in 1957, in similar experiments, found a rapid increase in pneumococci in the lung and high mortality in mice given bacterial challenge 6 to 9 days after influenza virus PR8 inoculation, but bacterial challenges given before this time were without effect. Although Kass did not report on mortality, it seems likely that an appreciable occurrence of pneumonia and mortality might have resulted in his studies.

Apparently quite distinct from the foregoing was the observation by Janssen, Chappell, and Gerone (3) that guinea pigs given *S. aureus* at the time of inoculation with influenza virus showed a high mortality within 48 hr. Influenza virus or *Staphylococcus* alone in the same doses had no effect. The effect was shown to be dependent on live influenza virus, and it did not occur in animals with influenza antibody produced by prior challenge. On the other hand, killed staphylococci served as well as live cultures in causing death. Animals dying of this synergistic combination howed pulmonary consolidation. However, staphylococci could not be cultured from the lungs (in animals given live cultures), although influenza virus was present in high titer. One is tempted to compare these results with the occasional case of rapidly fatal human influenza in which no evidence of bacterial pneumonia is found. Thus, there may be two forms of interaction of staphylococci and influenza virus in animal infection which have a counterpart in natural human illness, i.e., bacterial superinfection late in the course of influenza (referred to by Kass), and an early, often fatal influenza, apparently unrelated to bacterial infection, but conceivably contributed to by constituents of killed staphylococci [referred to by Janssen et al. (3)] which have remained in the lung.

Dr. Kass' studies of metabolic and other factors which influence lung clearance are of great interest. If the relationship of lung clearance of microorganisms to the pathogenesis of pulmonary infection can be precisely defined, this model could serve an extremely useful purpose in attempts to identify mechanisms of susceptibility and resistance to infection. It would be highly desirable to extend some of these techniques to man, if the safety of the methods could be assured, but the problem is technically an imposing one.

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# Airborne Disease and the Upper Respiratory Tract

DONALD F. PROCTOR

*Department of Environmental Medicine, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland*

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## INTRODUCTION

Studies of airborne infection have been largely directed at the identification and nature of responsible microorganisms, epidemiology, host immunological defenses, and antibacterial or antiviral drugs. A relatively small effort has involved the possible role of the respiratory mucosa and the nasal passages in the defense against airborne infection. As a result, although a highly sophisticated body of knowledge has accumulated in the former fields, we do not yet know whether the upper respiratory tract constitutes a useful defense against airborne disease, is of no use, or may possibly be a detrimental influence. G. W. Wright pointed out this fact at the first Airborne Infection Conference five years ago (85), in saying, "One might well wonder whether the nose and nasopharynx are more harmful than helpful with regard to infections of the lower air passages."

The long-term objective of the work to be reported in this paper is to discover what part the upper respiratory passages and the respiratory mucosa play in defense against airborne disease and what factors influence their function.

It is conceivable that the nasal passage in man is merely a vestigial remnant of a once highly effective olfactory organ (37, 56) (Fig. 1). Yet, anthropological studies show that evolutionary changes have resulted in nasal dimensions which vary with climate demands (18), thus suggesting that, in man's development, the nose has not entirely regressed to a useless ornament.

On the one hand, available evidence indicates that the nose is less effective in humans than in other mammals as a filter for particles in the inspired air (3, 9, 19, 58), that particles carrying infectious organisms are not only small enough to pass through the nose but also to pass through the tracheobronchial tree into the alveoli (17, 28, 42, 53, 76), and that many patients survive for years while breathing through a tracheotomy (31, 61). Unfortunately, no really adequate study has been done on such patients to determine the effect of tracheotomy breathing on airborne infection.

On the other hand, it is well known that, during the first few days after tracheotomy, pulmonary infections are common and often severe; some evidence from experimental induction of respiratory infection suggests that the nose may serve to protect the lower respiratory tract (13); clinical experience suggests that a diseased nasal passage is seldom found without concomitant lower respiratory symptoms, and, in mucoviscidosis, the abnormal function of mucous membranes seems the most obvious link with the susceptibility of these patients to frequent and severe respiratory infection. At least one study has described a relationship between the effectiveness of the nasal filter and the incidence of silicosis (45).

All of this leads to the conclusion that the role of the upper respiratory tract is still in doubt and requires further investigation.

## ANATOMY OF THE UPPER RESPIRATORY TRACT

It will be helpful if workers in this field will agree upon clear-cut definitions of terms. The upper respiratory tract is that part of the air passages which extends from the larynx to the nostrils and to the lips, including the Eustachian tubes and the paranasal sinuses (Fig. 2).

This may be divided into the nasal passage extending from the mucocutaneous junction at the nostrils to the upper border of the soft palate (including the paranasal sinuses), the nasopharynx from the posterior nasal passage downward to the lower free border of the soft palate (including the Eustachian tubes), the mouth extending from the lips backward to the soft palate,

FIG. 1. *Upper respiratory tract of the Indian barking deer showing the relation between the epiglottis and the palate, assuring nasal air flow even when the mouth is open. This relationship exists in many mammals but not in man. From V. Negus, Comparative Anatomy and Physiology of the Nose and Paranasal Sinuses. E. & S. Livingstone, Ltd., Edinburgh, 1958, with the kind permission of the author and the publishers.*

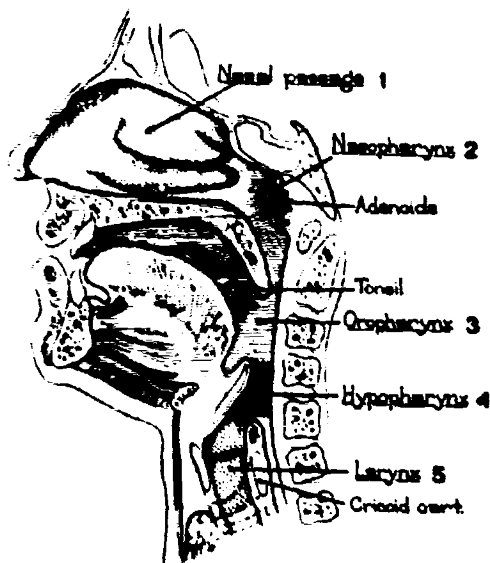


FIG. 2. *Diagram of the upper respiratory tract showing the anatomical divisions suggested in the text. The mouth is included in the oropharynx. The lateral extensions of the hypopharynx downward on either side of the larynx are not shown.*

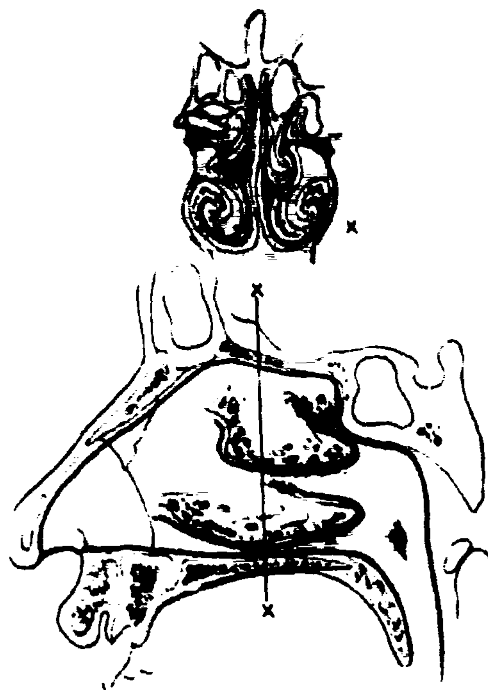

FIG. 3. *Nasal airway. The cartilaginous and bony structures (horizontal hatching) fix the maximal dimensions of this portion of the airway, and the overlying vascular network and mucosa more or less narrow the passage according to their state of congestion. Communication with maxillary antra through middle meati is usually a thinner partition than shown here. Air passage is shown in solid black.*

the oropharynx extending downward from the free border of the soft palate to the epiglottis, the hypopharynx from the tip of the epiglottis downward into the pyriform sinuses laterally and to the aryepiglottic folds medially, and the larynx extending from the aryepiglottic folds down through the cricoiu ring.

The dimensions of the nasal passage are relatively fixed by their cartilaginous and bony framework, but vary in width according to the thickness of the lining mucous membranes (Fig. 3). The nostril hairs placed at the entrance to the nose may play a part in air flow and aerosol distribution in the air stream. From the nostril to posterior nasopharynx in the adult is 8 to 11 cm and from the nasal floor to cribriform plate is 4.5 to 5 cm. The dimensions of the nasopharynx vary with the size of the adenoid mass above and with the position of the soft palate below. The mouth will vary from a broad passage when the tongue and palate are widely separated to a closed space when they are approximated. The oropharynx will be narrow or wide as the tongue is moved backward or forward and will also be affected by the size of the tonsils. The hypopharynx is relatively constant except during deglutition. The laryngeal airway is effected by both deglutition and by the motion of the true and false vocal cords.

The mucous membrane in the respiratory tract is ciliated columnar epithelium from a line just posterior to the anterior ends of the turbinates back into the nasopharynx, except for the olfactory area, but including the lining of the paranasal sinuses. In the nasopharynx there is a transition from ciliated columnar to transitional and then squamous epithelium. Over the adenoid tissue, there are alternating patches of squamous and ciliated columnar, but the crypts are entirely lined with keratinizing squamous. The common belief that adenoid tissue does not exist in the normal adult is untrue. At least small amounts of lymphoid tissue with crypts can be found in man of all ages (2). The Eustachian tubes are also lined with ciliated mucosa. The remainder of the upper respiratory epithelium with the exception of the ciliated posterior wall of the larynx is squamous.

Within the nasal passage, the vascular bed is so rich and subject to such wide changes in dilatation that it is commonly referred to as erectile tissue. There is also a particularly rich vascular supply within the tonsillar and adenoid tissue.

Goblet cells and mucosal glands supply a continuous carpet of mucus which lines the entire upper respiratory tract. This mucus is kept continually on the move by ciliary activity and swallowing from every point in the respiratory tract toward the hypopharynx and thence to the esophagus.

The ciliated mucosa of the trachea extends upward through the posterior commissure of the larynx; but motion of mucus through other portions of the larynx above the cricoid is largely determined by cough or "throat clearing" (Fig. 4).

The paranasal sinuses consist of a group of air spaces in the bones of the face communicating with the nasal air stream through small openings, and, in the case of the frontal sinuses, through long nasofrontal ducts. The direction of the mucociliary stream in all of the sinuses is toward the nasal passage.

The Eustachian tubes are normally closed channels connecting the nasopharynx with the air spaces of the middle ear. Here also ciliary activity moves mucus toward the nasopharynx. These tubes normally open during swallowing and yawning. They are essential for the maintenance of normal pressure within the middle ears and are of importance in respiratory infection as the chief passage through which pathogenic microorganisms gain access to the middle ears.

Whether or not the upper respiratory tract is an important factor in the defense against inhaled materials, it is certainly an important contributor to the adjustment of the temperature and water vapor content of inspired air and probably is important in the maintenance of normal body temperature and water (12, 15, 22, 32, 38, 51, 56–58, 72, 73).

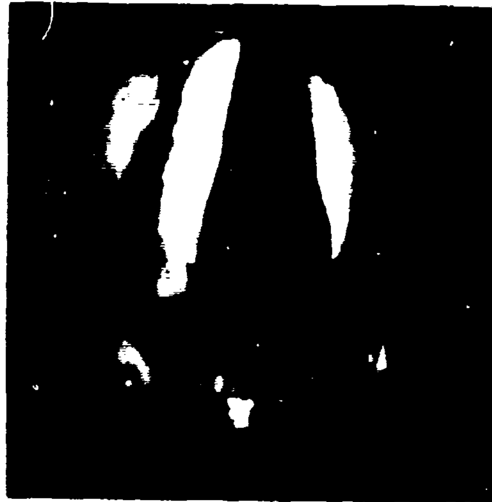

FIG. 4. *Photograph of the human glottis seen from above. Below is the posterior commissure through which the mucociliary stream must pass. The vocal cords are not ciliated. With the kind permission of Paul Holinger, Chicago, Ill.*

Although it is true that these functions can be taken over by the mucosa lining the tracheobronchial tree, observation of patients with tracheotomy (even long term) indicates that the nasal passage is best suited for this purpose. Normally, with nasal breathing, air temperature is close to body temperature, and the air is near to saturation with water vapor by the time it reaches the hypopharynx (63).

## UPPER AIRWAYS, NASAL OR OROPHARYNGEAL

Because the narrow nasal passage is the place of greatest resistance to air flow (24), when the ventilatory demand rises beyond a certain point, one resorts to mouth breathing. Under these circumstances, the tongue is depressed and the palate raised, providing a wide airway with minimal resistance to flow. What work load creates this demand and how much individual variation there is has not been determined.

In like manner, when the nasal airway is sufficiently reduced by physiological alterations in mucosal and submucosal vasculature or by pathological processes, even quiet breathing may occur through the mouth; but in this case the oral airway consists of a narrow slit between tongue and palate. Here there is wide individual variation. Some patients will complain of nasal obstruction

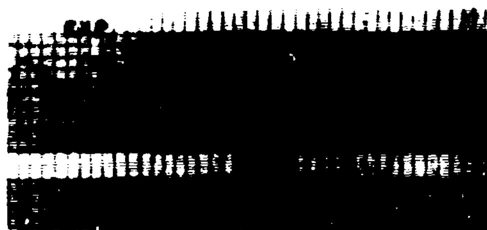

FIG. 5. *Pneumotachogram during nasal breathing (left), followed by talking, with brief inspiration in center. Note air flow during this inspiration is more than twice that in resting breathing. With the permission of the Editor, Inhaled Particles and Vapours II (in press), Pergamon Press, Inc., New York.*

FIG. 7. *Tomograms of the upper airways. To the left, lateral view, and, to the right, anterior view. Compare with Fig. 3 and 8. With the permission of the Editor, Handbook of Physiology, vol., I Respiration, American Physiological Society, Washington, D. C.*

FIG. 6. *Oropharyngeal airway during deep breathing (A) and during inspiration between conversational phrases (B). Lips may be seen to the left. Note narrow airway between tongue and palate in (B). From cine-fluorograph, with thanks to Sue McCarty and Martin Donner, Johns Hopkins Hospital. With the permission of the Editor, Inhaled Particles and Vapours II (in press), Pergamon Press, Inc., New York.*

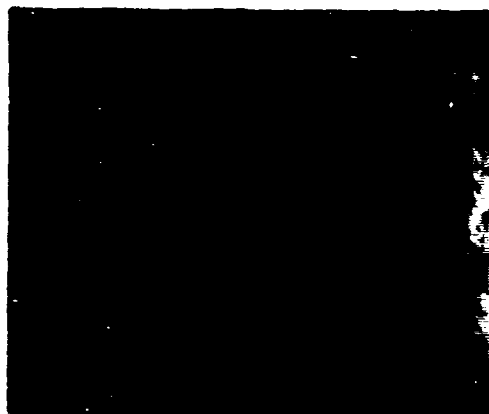

FIG. 8. *(A) Obverse of cast of nasal passage taken at autopsy: (B) Model constructed from same. Nostril is to the left. With the permission of the Editor, Inhaled Particles and Vapours (in press), Pergamon Press, Inc., New York.*

when their measured resistance to air flow is less than that found in others who are unaware of any difficulty in nasal breathing.

Movement of air in and out of the paranasal sinuses and middle ears occurs as a result of respiratory cycle pressure changes when these spaces are in free communication with the moving air stream, and as a result of gas absorption from these spaces when this communication is periodi-

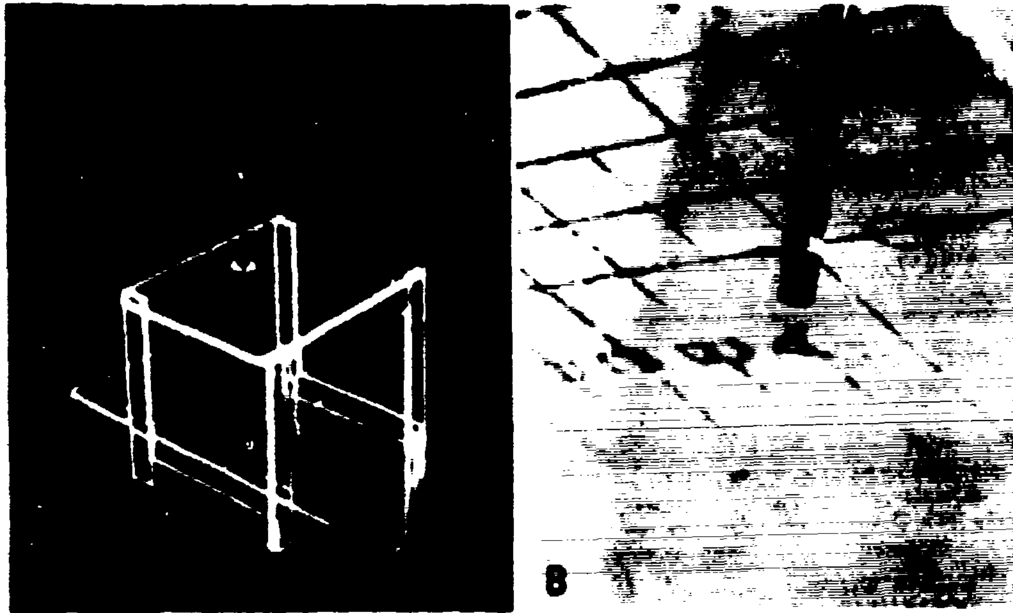


FIG. 9. (*A*) *Angle meter and grid (representing sliding nasal septum) used with models as in Fig. 8B.* (*B*) *Detail of tip of meter, showing three openings projecting into airstream. With the permission of the Editor, Inhaled Particles and Vapours (in press), Pergamon Press, Inc., New York.*

cally interrupted. Under normal circumstances, the gas contained within the sinuses undergoes one full change every few hours (63). Under unusual circumstances, especially those associated with marked atmospheric pressure changes, ventilation of these paranasal spaces may be much greater. Rahn calculated that in the Japanese women divers (Ama) the ventilation of each middle ear approximates 1,800 ml per day (67*a*).

During conversation, inspiration occurs through the mouth, but here again the oral airway is narrow. During singing, when it is necessary to fill the lungs quickly for long phrases, the oral airway is wide as in high ventilatory demands (Fig. 5 and 6).

If resistance to air flow is external, as with respiratory masks, nasal breathing continues until such resistance is extremely severe.

Although it is commonly recognized that nasal congestion sufficient to cause mouth breathing ay be related to a multitude of internal and rnal environmental factors, these relations as emain generally undocumented. Such in- es probably include emotional stimuli, such or sexual conflict, endocrine disturb- ch as hypothyroidism, and sudden inspired air temperature. Especially in motional stress, changes occur in all nbranes but are generally more readily noticed and more pronounced in the nose (35, 49, 54, 60, 75, 84).

The function of the paranasal sinuses in man is open to question. It is clear that these air spaces provide protection for the brain against blows on the face. It seems likely that, in addition, they act as insulators and a source of mucous secretion to supplement the air-conditioning function of the nose (57, 58, 67).

## CHARACTER OF UPPER RESPIRATORY AIR FLOW

The fate of inspired particulate matter depends upon the size and weight of the particle, the character of air flow, and the relationship between the moving air stream and the surfaces over which it passes. Since, within the nose the air stream is narrow (Fig. 7) and moves at a high linear velocity, and since turbulence is more likely to occur there than elsewhere in the respiratory tract, it is of importance that we understand the nature of air flow in the nasal passage and the factors which may significantly alter this flow (20, 38, 59, 66 74, 80).

Because of the difficulty in introducing measuring devices within the nose without interfering with the nasal air stream, models of the nasal cavity have been constructed from casts made at autopsy to permit measurement of simulated nasal air flow at all points (Fig. 8). These measurements

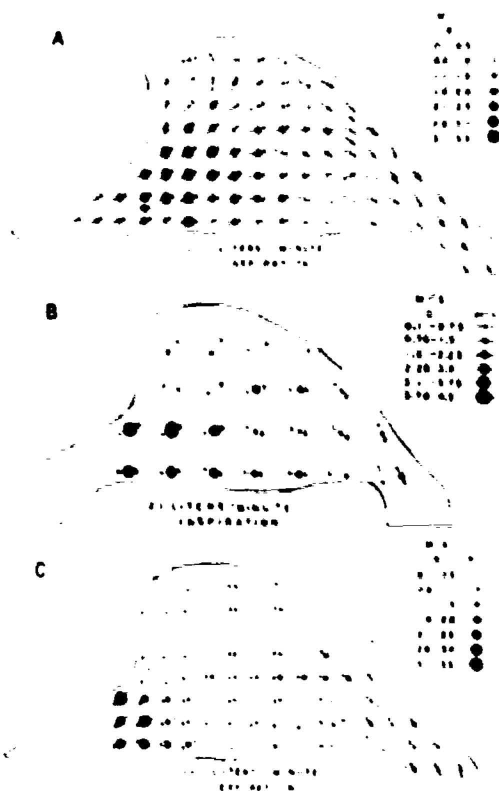


FIG. 10. *Patterns of air flow through one model. Arrows indicate direction of flow and circles indicate linear velocity. (A) Inspiration at 11 liters per min. (B) Inspiration at 21 liters per min. (C) Expiration at 11 liters per min. Note more diffuse distribution of flow in C. With the permission of the Editor, Inhaled Particles and Vapours (in press) Pergamon Press, Inc., New York.*

are made by use of an angle meter introduced into the stream through a simulated nasal septum which can be moved over the surface of the nose model (Fig. 9).

Such studies indicate a concentration of most of the moving air stream during inspiratory flow over a relatively small portion of the nasal surface. This may be useful in shunting the inspired air away from the unciliated olfactory area. It also may be useful when one is breathing noxious air, in that a large surface of neighboring mucosa remains unexposed and able to replenish altered or dried mucus in exposed areas (Fig. 10).

The path of the air stream remains remarkably constant in the face of changing flow from 1 to 100 liters per min. In the models studied thus far, as flow increases there appears to be a greater concentration of the moving air stream in its principal path along the middle meatus. In one model, this change occurred sharply at 16 liters per min, a flow which approximates the peak one might expect through one side of the nose in rapid nasal breathing (Fig. 11). In other models this sharp change has not been so evident, but a similar alteration in flow has been found in all (Fig. 12 and 13).

Changes in the nasal airway resulting from pathological conditions have also been studied in these models. Polyps, septal deviations, alterations at the nostril, enlarged adenoids, and generalized mucosal thickening have all been simulated. Alterations in the main nasal cavity, such as would result from polyps or septal deviations, seem to have the most effect on air flow patterns and might influence particle deposition or concentrate the air stream in small areas, thus producing an undue drying effect upon mucosa.

During expiratory flow, there is a more diffuse spread of the stream through the entire nasal passage including the olfactory area (Fig. 14 and 15). Maximal olfaction occurs just after a sniff when air may rapidly diffuse into the olfactory area.

Landahl has calculated the maximal linear velocity in the respiratory air stream to be about 2 meters per sec, and this occurs in the secondary bronchi (43). In our model studies, it is clear that peaks of at least 5 meters per sec occur briefly in the main stream of flow during quiet nasal breathing. Such velocities in a narrow curving air stream will surely influence the chances of particles contacting the surface.

If inspired particles are hygroscopic, upon entrance into the efficient humidification apparatus of the nose, they will increase in size. Theoretical considerations based on the behavior of particles in tubes or even in the experimental animal may be misleading and cannot substitute for measurements of what actually occurs in the human nose.

The relatively sharp bend of the air stream at the nasopharynx plus the fact that the main air stream at this point travels along the posterior wall increases the chance for impaction of particles on the adenoid tissue.

Studies of oropharyngeal and laryngeal air flow are needed to understand what role these portions of the respiratory tract may play in the fate of inhaled particles.

## FATE OF PARTICLES DEPOSITED ON UPPER RESPIRATORY SURFACES

In collaboration with Henry Wagner, Jr., Betsy Bang, and James Langan, and with the advice of Anna Baetjer, three methods of following muco-

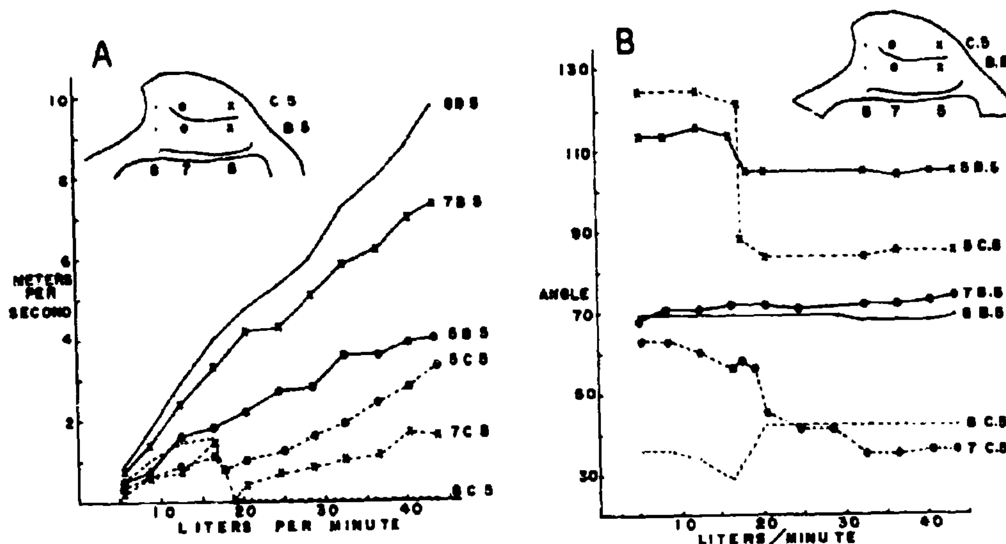


FIG. 11. *Changes in linear velocity (A) and direction of flow (B) with increasing flows through model charted in Fig. 10 at points indicated in inset. Note sharp breaks in both charts at points above main flow paths at 16 liters per min. With the permission of the Editor, Inhaled Particles and Vapours (in press), Pergamon Press, Inc., New York.*

ciliary clearance of particles have been explored one of which was combined with the use of a visible dye. For each technique, 0.02 to 0.1 ml of a saline solution containing 8 to 20 $\mu c$ of radioactivity was injected with a microsyringe on the mucosal surface at the anterior nares. A head mirror and nasal speculum were used to assure placement of the material just behind the anterior end of the inferior turbinate. $I^{131}$ was the isotope most frequently employed. In most studies, the isotope used labeled aggregates of human serum albumin (81), usually from 5 to 100 $\mu$ in diameter, but in one study (Fig. 17) 5 to 15 m$\mu$ (64, 65). In other studies, the isotope was in the form of a solution of sodium iodide, or a solution containing fluorescein mixed with the dye Sky Blue (dimethoxydiphenyl-diazo-bis-8-amino-1-naphthol-5,7-disulfonic acid) [$C_{34}H_{24}N_6O_{16}S_4Na_4$]. Subjects were given Lugol's solution by mouth prior to the study to block entrance of the $I^{131}$ into the thyroid gland.

For the first method, immediately after placement of the isotope, the subject lay prone on a conventional scanning table with the head turned to one side (Fig. 16). A series of scans of the nasal area were then done as quickly as possible until the radioactivity was detected in the nasopharynx (Fig. 17). In most studies, the test was completed in 10 to 30 min, but in one study scanning was continued for 70 min to demonstrate retention in the anterior unciliated area (Fig. 18).

Such relatively long scans may provide important information. Both Hilding (33) and Macklin (50) pointed out the possible importance of small areas of poor clearance in the tracheobronchial tree in the role of carcinogenesis. Whether such areas regularly occur in the upper airways or whether they result from specific environmental circumstances is not known.

One study was done on a child with mucoviscoidosis (Fig. 19), but no other patients have been studied as yet.

For the second method, the subject was seated in a chair with a head rest and remained in this position throughout the test (Fig. 20). A double-channel collimated crystal scintillation detector was brought alongside the face and positioned so that the two channels pointe across the nasal passage, one just behind the point of injection, and the other 4 cm farther back. Radioactivity was then recorded at each point until the isotope had been carried backward past the second position (Fig. 21).

The third method (carried out in collaboration with Betsy Bang and James Langan) was similar to the second, except that a single detector was placed in front of the nose, pointed along the line of the nasal passage. Thus, as the isotope was carried backward, detected radioactivity fell in proportion to the square of the distance. In this series of studies, the Sky Blue was mixed with the isotope and looked for in the oropharynx about

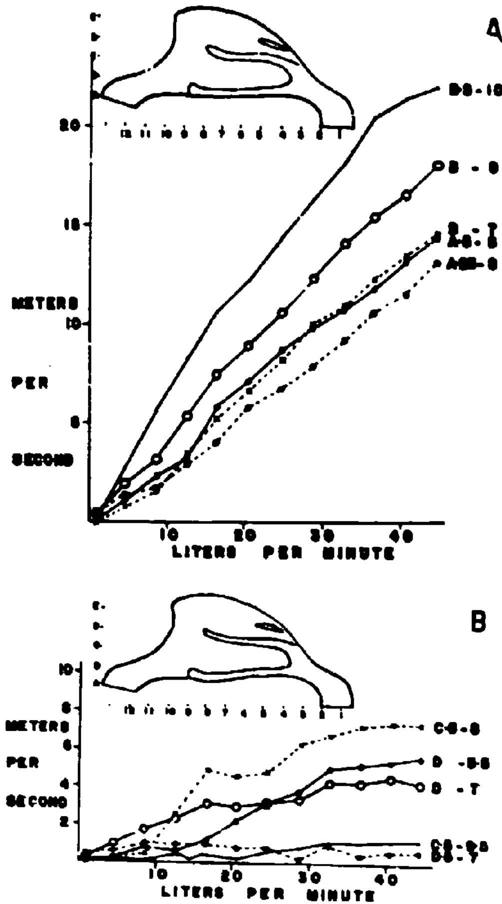


FIG. 12. *Changes in linear velocity with increasing flow in another model, at points in main stream (A) and at points away from main stream (B). Points are indicated on diagram. B·5 refers to the line halfway between (B) and (C), etc.*

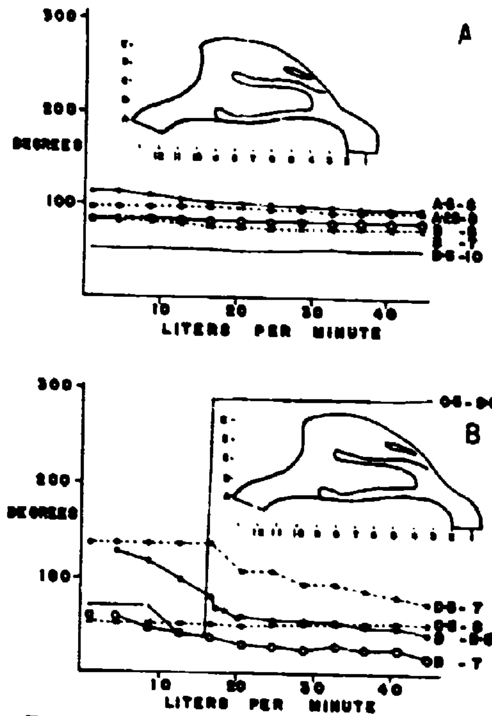


FIG. 13. *Changes in direction of flow with increasing flows, at points in main stream (A) and away from main stream (B) as in Fig. 12: 90° is horizontal to the floor of the nose.*

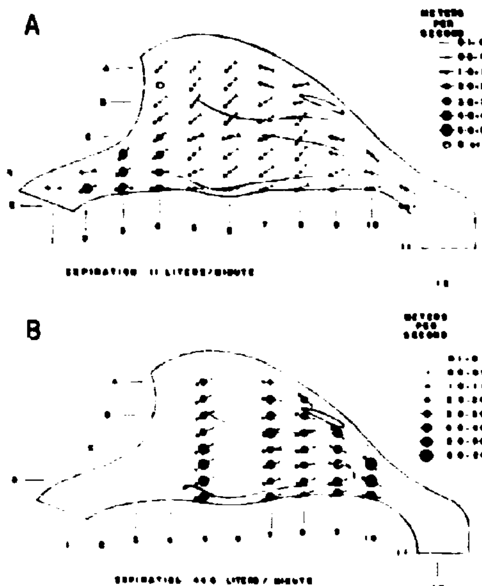


FIG. 14. *Patterns of flow in model charted in Fig. 12 and 13. (A) Expiratory flow at 11 liters per min and (B) at 44.6 liters per min. Note diffuse distribution of flow even in (B).*

once a minute. This dye, which is very clearly visible on mucosal surfaces, produced no unpleasant sensation in the subject, and appeared to have no unfavorable effect upon ciliary activity. In each subject, the appearance of the dye either at the edge of the soft palate or on the posterior pharynx coincided with a fall in radioactivity as detected in front of the nose. In most subjects, the visualization of the dye occurred just before the fall in detected radiation reached a plateau (Fig. 22).

The use of visible materials to study mucociliary activity has the advantage of simplicity (23, 68). Nevertheless, although the isotope technique demands complex equipment, it requires a minimum of cooperation on the part of the subject, gives a much more complete picture of the path of the mucociliary stream, allows one to de-

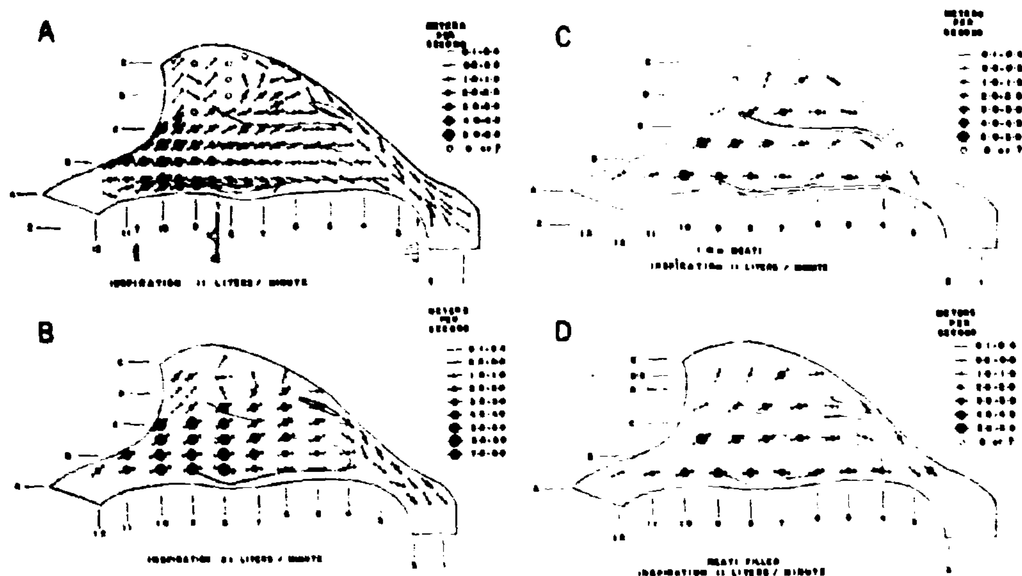


FIG. 15. *Patterns of inspiratory flow in model charted in Fig. 12, 13, and 14. Hatched areas in lower part of A indicate approximate cross section of airway. Letters and numbers refer to the grid locations as shown in charts in Fig. 11, 12, and 13. In C and D, the meati were narrowed (C) and filled (D) to simulate changes in airway to be expected from swollen mucosa.*

tect areas of retention, and permits study of portions of the respiratory tract not readily accessible to visual observation.

Thirty-six subjects have been studied in 64 tests thus far: 27 males and 9 females, ranging in age from 7 to 52 years, only one of whom had gross respiratory disease. This number is insufficient for the establishment of normal values; and studies of normal subjects in varied environmental circumstances and of patients are just beginning. There have been 23 scans, 28 studies with the double detector, and 13 with the single detector.

The mucociliary transport of surface materials in the human nose seems to occur at about the same average speed observed in previous studies of respiratory mucous membranes. It is clear, though, that this is not a uniform speed. Portions of a drop even as small as 0.02 ml may require 2 to 10 min to move 6 to 9 cm backward into the nasopharnyx, whereas other portions of the same drop may require 8 to 15 min to travel the same distance; still other portions (at the anterior unciliated area) undergo no detectable motion.

There is wide individual variation, with some normal subjects showing transport times two to three times faster than others. Not enough studies have been done to discover how much variability there is in a single normal subject from time to time, or how variations may be related to environmental or other influences.

FIG. 16. *Scan superimposed on skull radiogram for orientation. This scan is taken from series to left in Fig. 17 and is from same subject in Fig. 6 and 21 A. With the permission of the Editor, Arch. Environ. Health (64).*

## DISCUSSION

It is believed that microorganisms are airborne in droplet nuclei 2 to 3 $\mu$ in diameter (70, 82). Although there are relatively few studies of nasal particle deposition in man (and in these studies there is not complete agreement), it seems likely that many particles smaller than 5 $\mu$ will penetrate the to lower respiratory tract (29, 30, 34, 45, 53).

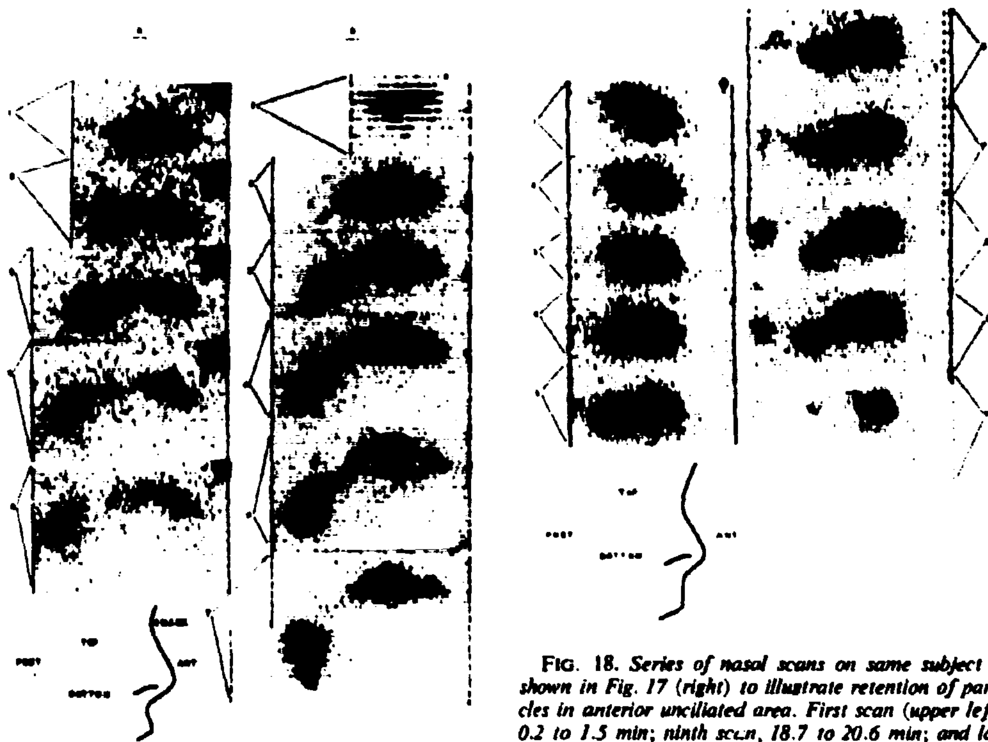

Fig. 17. *Series of nasal scans on 22-year-old normal female in left column and 52-year-old normal male to right. Times after injection reading from above downward, left: (1) 0.9 to 3.5, (2) 3.7 to 6.2, (3) 6.9 to 9.7, (4) shown in Fig. 16, (5) 13.7 to 17.9, (6) 19.7 to 24.9 min. On the right: (1) 1.2 to 2.5, (2) 3.4 to 5.7, (3) 6.3 to 9.7, (4) 9.6 to 13.2, (5) 14.6 to 16.0, (6) poor scan (omitted), (7) 29.5 to 34.7 min. With the permission of the Editor, Arch. Environ. Health (64).*

Fig. 18. *Series of nasal scans on same subject as shown in Fig. 17 (right) to illustrate retention of particles in anterior unciliated area. First scan (upper left), 0.2 to 1.5 min; ninth scan, 18.7 to 20.6 min; and last scan (lower right) 75 to 77 min after injection. With the permission of the Editor, Arch. Environ. Health (64).*

Three factors deserve further investigation in this connection: the possibility that droplet nuclei are hygroscopic and increase in size in the nose, the possibility that coagulation of particles may occur, and the effect of turbulence in the stream.

Since maximal exposure to airborne infection may occur in circumstances where one is indulging in animated conversation, the fate of particles in the oropharynx in these circumstances also deserves further study.

A recent report on the epidemiology of tuberculosis suggests the possibility that the nose may be an important defense. This report, and at least two other studies, indicate the likelihood that cross-infection has occurred between persons singing together, whereas infection did not occur between similar individuals sleeping in neighboring beds, sitting together in crowded classrooms or riding together on crowded buses (7, 36, 33, 78).

Two factors could combine to explain this interesting observation. The passage of air through the vibrating glottis may provide an excellent atomizer for the production of very small mucous particles. At the same time, since inspiration during singing consists of deep breaths through a wide open oropharynx and glottis, maximal opportunity for penetration of airborne particles into the depths of the lungs will result (Fig. 6).

In contrast, inspiration during conversation occurs through a narrow oral slit at relatively high linear velocity, a situation which could result in a filtration of particles similar to that normally found in the nose (Fig. 5 and 6).

If the upper respiratory tract plays a significant role in the removal of particles from the inspired air, the next question involves their fate once deposition has taken place (10, 11). Four possibilities are worthy of investigation: mucociliary clearance with dispatch through swallowing into the stomach, the passage of viable organisms through gastrointestinal mucosa, penetration through the mucous carpet into upper respiratory

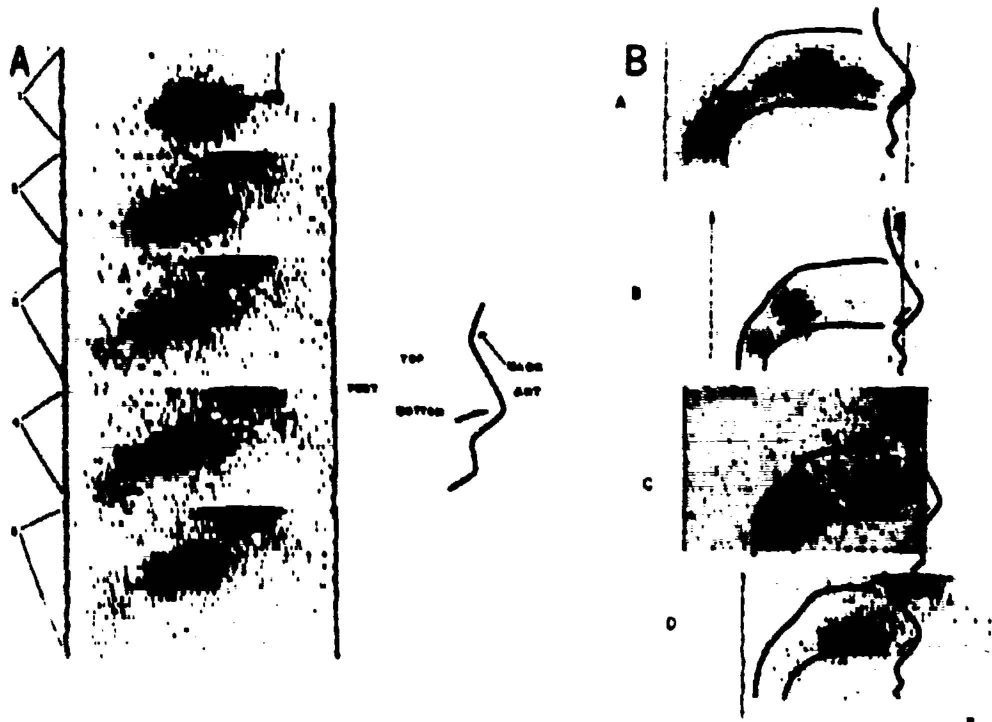


FIG. 19. (*A*) *Series of nasal scans on 7-year-old boy suffering from mucoviscidosis. Times after injection, first scan (top), 0.9 to 2.6; last scan (bottom), 9.3 to 11.6 min. Note relatively poor clearing.* (*B*) *Scans at the 9th min from three normals* (*A, B, and C*) *and the child in A* (*D*). *With the permission of the Editor, Arch. Environ. Health* (64).

mucosal cells, and deposition in the crypts of the adenoids.

Certainly, some organisms are deposited in the adenoids, but we know little about the factors which determine whether active infection ensues or whether the presence of pathogenic microorganisms in lymphoid crypts is a benign infestation leading to an opportunity for the body to thereby develop immunity to them (62).

It is evident that pathogenic bacteria may reside in the upper respiratory tract without producing signs or symptoms of disease (8, 26, 83) What enables such bacteria to institute active infection is not clear. It is conceivable that their deposition and residence, especially in the adenoid crypts, may be entirely innocuous, and that only when mucous membrane defenses are injured in some manner are such bacteria able to invade the body tissues and produce the signs and symptoms of infection.

Information is especially scarce regarding the chemical nature of respiratory-tract mucus Mechanisms by which its water content and viscosity are varied to meet the changing demands of our everyday environment are virtually unexplored. It has been established that antibodies are found in mucus, and it is possible that such antibodies may, on occasion, be more abundant and more effective against airborne infection than those which circulate in the blood stream (4, 14, 27, 46, 65, 79).

The method by which inhaled viruses pass through the moving mucous layer and gain entrance to surface cells remains unclear (79). It does seem that such penetration will be less likely to occur when the particle carrying the virus is kept rapidly on the move in the mucociliary stream. Stasis at any point in the stream would provide the needed opportunity for contact with cells, penetration, and infection. It should be remembered that any influence which slows the stream may lead to stasis. The more slowly the mucous stream moves, the longer it is exposed to the drying effect of the moving air, and the more likely such drying and the consequent rise in viscosity are to lead to the inability of the cilia to maintain mucous motion. Any factor acting directly either to impair ciliary activity or to in-

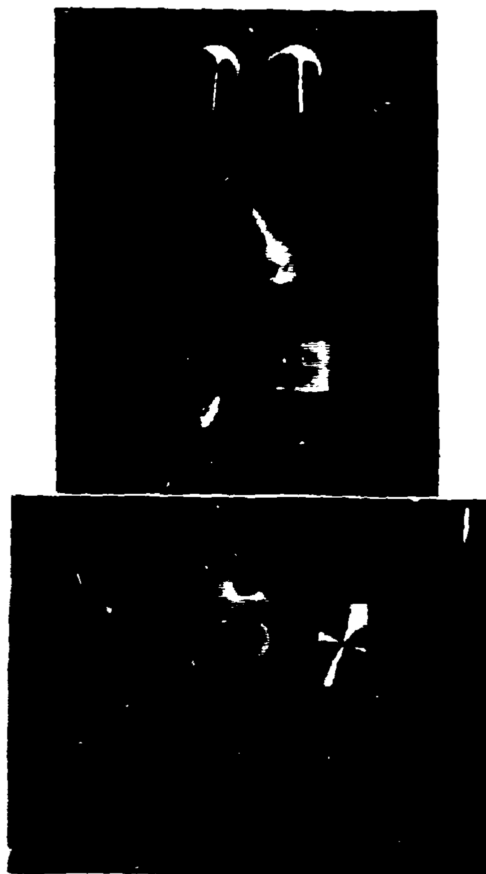

FIG. 20. (*A*) *Double collimated crystal scintillation detector showing slits 4 cm apart.* (*B*) *Detector in place alongside subject's face. With the permission of the Editor, Arch. Environ. Health.*

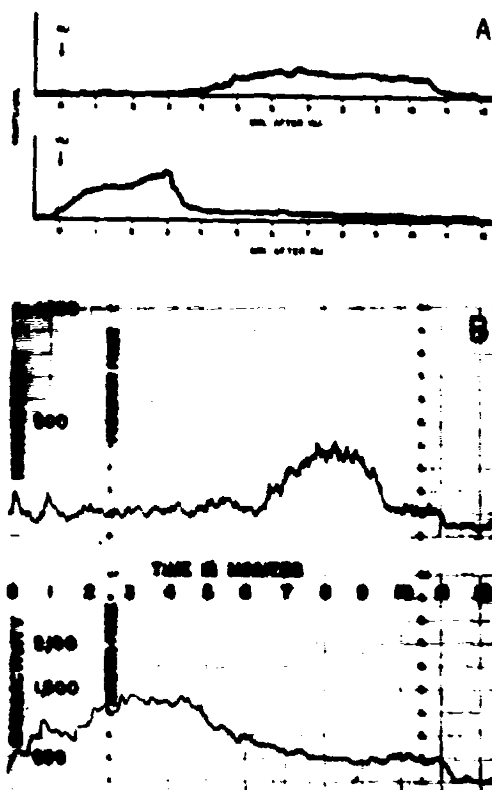


FIG. 21. *Redrawn record from the double detector* (*A*) *and actual record* (*B*). (*A*) *From same subject illustrated in Fig. 6 and 17 (left).* (*B*) *From normal 35-year-old male. With the permission of the Editor, Arch. Environ. Health and Inhaled Particles and Vapours II (in press), Pergamon Press, Inc., New York.*

crease viscosity of mucus may thus reduce the effectiveness of respiratory mucosa, at least as an air conditioner and perhaps as a defense organ.

Although a great many studies have been directed at mucociliary activity and particle clearance, most of this work (owing to the paucity of techniques applicable to the human subject) has been done on in vitro mucosal strips or in the experimental animal (1, 5, 6, 16, 21, 23, 25, 41, 47, 68, 71). The use of radioactive tracer materials and apparatus for their external detection permits the study of mucociliary activity in living man (1, 25, 34, 55, 64, 65).

Although it is possible that many infectious organisms travel in the inspiratory air stream directly to the alveoli, there are broad gaps in our knowledge of particulate behavior in the airways and suggestive areas of disagreement (33, 39, 40,

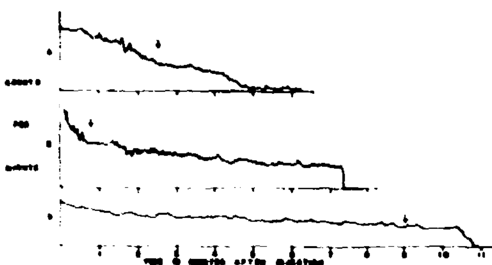

FIG. 22. *Tracings from records obtained with single detector method:* (*A*) *26-year-old normal male;* (*B*) *36-year-old normal male; and* (*C*) *3? year-old normal male. In these records, the fall of radioactivity detected at probe in front of nose results from motion of isotope backward in mucociliary stream. Plateaus in* (*B*) *and* (*C*) *probably represent material retained in anterior unciliated area. Arrows indicate time at which dye became visible in posterior pharynx.*

52, 77). We cannot now state with certainty whether or not the upper respiratory tract is a barrier against airborne disease. We cannot know until we first understand normal human respiratory function (air flow and mucosal function) and what influences disturb it. Only then will it be be possible to search for correlations between such disturbed function and susceptibility to disease.

Many of the conclusions thus far drawn regarding the upper respiratory tract and mucous membrane function come from in vitro studies or the experimental animal. Although some of these conclusions are probably correct, it is time to check them with carefully controlled experiments on the human subject. This is especially true in regard to respiratory air flow and the fate of inhaled particles.

Clinical experience with respiratory infection has long suggested relationships between susceptibility and such factors as change of season, cold weather, allergies, exhaustion, emotional stress, etc. Studies of naturally occurring infections have not produced data leading to clear conclusions, perhaps because they have not included concomitant studies of mucosal function. Studies in the experimental induction of respiratory infection have in general failed to substantiate any of the relationships mentioned, but, instead, indicate that whether or not an individual exhibits signs and symptoms of infection is largely a matter of degree of exposure to, and immunity against, the infectious agent (10, 11, 12, 39, 60, 79).

Now and then, one does find evidence suggesting that susceptibility may vary with other factors, but the most suggestive evidence comes in children with mucoviscidosis. These children seem to have at least average capability for development of antibodies against infectious microorganisms, but, in spite of this, become infected frequently and tend to suffer from unusually severe infections. What is more significant is that their infections are almost exclusively respiratory. Here, the facts strongly indicate either that mucous membrane, when functioning normally, is a potent defense against airborne infection, or that there is some other now unknown factor involved.

To document any possible role of the upper respiratory tract or respiratory mucous membrane in general, we must be able to measure air flow and mucociliary activity in the normal human subject and in the patient before and during respiratory disease. Whether or not naturally occurring respiratory infections are most commonly transmitted through airborne droplets, droplet nuclei, or direct contact has not as yet been clearly established.

The techniques reported here represent a beginning toward the development of methods applicable to human studies aimed at the eventual answer to these questions. When applied in circumstances where environmental conditions are carefully controlled, and, especially when the isotopes are delivered in airborne suspensions comparable to naturally occurring aerosols, our knowledge should be improved. Now it should be possible to determine ranges of normal function in man of all ages, variations in normal function associated with environmental change, and association between such variations and airborne disease.

It is too early to say which of the three isotope techniques thus far explored is most useful or whether some other method may prove to be more effective. At the present time, it appears that the serial scans are most helpful in picturing the path of flow and, especially, in detecting areas of poor clearance. The double collimated detector seems most useful in quantitating speed of mucociliary flow between any two points. The single detector is the simplest mechanically.

These same techniques are also applicable to the study of areas of deposition of the isotope-labeled airborne particulates, as well as the determination of the eventual fate of those deposited.

At present, more questions regarding the upper respiratory tract and mucous membranes have been raised than answered. Among these are the following.

How much of the variability already noted is really "normal," and to what degree might the extremes of these "normal" variations be related to infection if they coincide with exposure? What pathological conditions in the upper respiratory tract increase susceptibility to infection? Are significant variations attributable to alterations in ciliary activity, respiratory tract mucus, or both? How are these functions affected by variations in environmental temperature or humidity, emotional stress, endocrine activity, pharmacological agents, etc.?

When these questions are clearly answered, research in the field of airborne disease may be more logically directed toward, or away from, the upper respiratory tract or the respiratory mucosa, or both.

## SUMMARY AND CONCLUSIONS

Certain questions regarding the role of the upper respiratory tract in airborne disease remain unanswered. Among the most urgent are the following. What airborne materials are likely to be deposited upon the respiratory mucosa? What factors influence their removal from the air stream and their point of deposition? What is the fate of materials once deposited upon mucosal surfaces?

What factors influence their clearance, not only from the respiratory tract, but from the body?

Techniques are herein reported for the study of the respiratory air stream, and, through the external detection of radioisotopes, for the study of the deposition of airborne particles and their motion in the respiratory mucociliary stream. It is hoped that pursuit of these studies may cast some light on the transmission and pathogenesis of airborne disease.

ACKNOWLEDGMENT

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# Measurement of Different Mechanisms for Elimination of Bacteria from the Lung

RAGNAR RYLANDER

*Department of General Hygiene, National Institute of Public Health, and Institute of Hygiene, Karolinska Institutet, Stockholm, Sweden, and Institute of Hygiene, Umeå University, Umeå, Sweden*

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## INTRODUCTION

Several clinical and experimental studies have shown that the normal defense of the lung against bacteria might be affected by environmental agents, such as toxic gases (2), alcohol (7), and decreased temperature (3).

Although experimental studies of the disappearance rate of viable bacteria or of changes in mortality among bacteria-exposed animals are of great interest, a complete understanding of the recorded effects requires the study of the different elimination mechanisms separately. In turn, certain of the agents used to produce changes are able to affect one or more of these mechanisms.

This report presents a brief description of experimental methods used for the elucidation of the function of the different elimination mechanisms, and suggests an experimental set-up whereby the efficiency of the various mechanisms can be measured when the short-term elimination of bacteria from the lungs is being studied. In view of the bacteriological findings in chronic bronchitis (5), the present work has been performed with nonpathogenic bacteria, which were originally cultured from the mouths of the animals.

## METHODS AND RESULTS

Guinea pigs were exposed for 10 min to a flow of radioactive bacteria in a monodisperse aerosol in a stainless-steel exposure chamber. The number of bacteria present at various sites in the lung was determined by use of bacteriological and autoradiographic (1) techniques. The number of bacteria at various times after the exposure was expressed as the percentage of the initial number remaining.

The number of viable bacteria in the whole lung was determined by use of a grinding technique similar to that described earlier (6). However, to ensure complete lysis of phagocytes, saponin was added to the grinding fluid in the present experiments. The number of viable bacteria was found to decrease fairly rapidly from these whole-lung preparations (Fig. 1). The results are generally in accordance with earlier reports. As the disappearance rate from the whole-lung preparations is a determinant of all the elimination mechanisms, the following experiments were performed to elucidate the individual functioning of the different mechanisms.

The airways were washed with sterile saline by use of a modification of the technique of LaBelle and Brieger (4). The lungs were flushed three times via the trachea with 10 ml of saline under aseptic conditions; then the number of bacteria in the fluid was determined. When *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, which all were present in the normal mouth flora, were used, it was found that the rate of decrease of viable bacteria in the fluid was more rapid than the decrease from whole-lung preparations (Fig. 1). If saponin was added to the wash-out fluid, a larger number of viable bacteria was found. If the autoradiographic technique was used, even more bacteria (*E. coli*) could be detected in the fluid (Fig. 2).

When the insides of isolated pieces of trachea, standardized with respect to length, were flushed with saline, the number of viable bacteria was found to be rather high during the first few hours after exposure but decreased later on. When saponin was added to the tracheal wash-out, a significant increase in the number of bacteria was only occasionally found (Table 1).

## COMMENTS

The above findings suggest that, during the duration of these experiments, most of the *E. coli* that deposit on the mucus are carried upwards out of the lung without prior phagocytosis. The very rapid decrease in number of bacteria found

in the wash-out from the airways is apparently dependent upon the mucus transport. As viable bacteria and autoradiographically detectable bacteria could be expected to be removed by mucus at identical rates, the difference (Fig. 2) indicates that other mechanisms may be involved. Although the wash-out fluid cannot be expected to remove all bacteria from the minor airways and alveoli, the presence of phagocytes in the airway wash-out fluid and the almost complete absence of phagocytes in the trachea wash-out fluid indicate that at least a certain number of the peripheral airways are subject to flushing with removal of free or loosely attached phagocytes. The difference in disappearance rates of viable bacteria and autoradiographically detect-

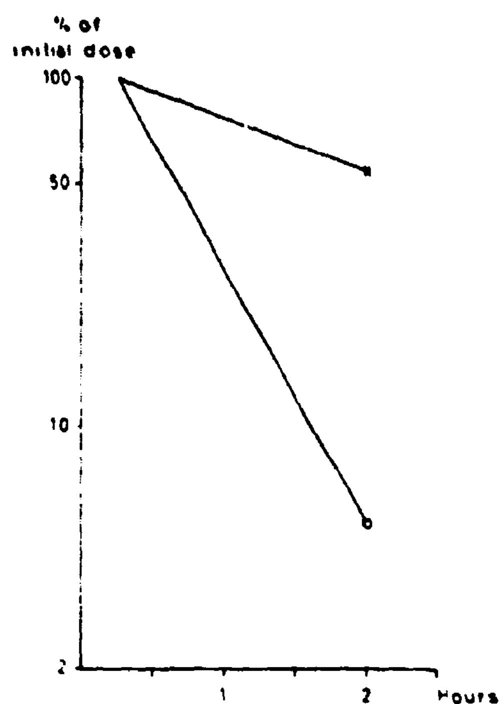


FIG. 1. *Decrease in viable bacteria (Escherichia coli) in whole lung (X) and in airway wash-out fluid (O)*

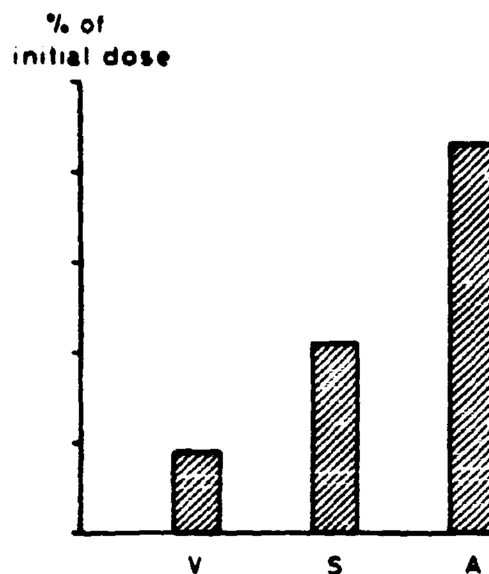


FIG. 2. *Number of Escherichia coli present in airway wash-out fluid 2 hr after exposure. V = viable; S = viable after saponin treatment; A = autoradiographically counted.*

TABLE 1. *Number of viable Escherichia coli cells in trachea wash-out fluid before and after saponin treatment*

| Time after exposure | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 hr | | 1 hr | | 2 hr | | 3 hr | | 4 hr | |
| V* | S* | V | S | V | S | V | S | V | S |
| 117 | 130 | 55 | 56 | 44 | 30 | 4 | 0 | 6 | 4 |
| 81 | 42 | 271 | 382 | 6 | 8 | 0 | 2 | 20 | 22 |
| 153 | 160 | 15 | 12 | 0 | 0 | 329 | 440 | 9 | 12 |
| 139 | 144 | 106 | 108 | 14 | 22 | 60 | 46 | 166 | 160 |
| 25 | 18 | 6 | 4 | 21 | 8 | 0 | 0 | 1 | 0 |
| 10 | 40 | [illegible] | 34 | 10 | 0 | 1 | 10 | 0 | 2 |
| 118 | 150 | 19 | 24 | 11 | 16 | 170 | 310 | 0 | 0 |
| 123 | 108 | 14 | 82 | 1 | 0 | 7 | 24 | [illegible] | 0 |
| 330 | 1,228 | 11 | 6 | 9 | 14 | 0 | 4 | 0 | 2 |
| 108 | 206 | 62 | 208 | 42 | 42 | 0 | 0 | 1 | [illegible] |

* V = viable *E. coli* cells before treatment; S = viable *E. coli* cells after saponin treatment.

able bacteria from the airway wash-out fluids could then be due to an uptake of bacteria by phagocytes, whereby the bacteria cannot be cultured but are autoradiographically detectable. This theory is supported by the observed difference in removal rates of viable bacteria with or without treatment of the wash-out fluid with saponin. The increased yield of bacteria when saponin is added to the fluid indicates that some bacteria initially retain their viability when within or adherent to the phagocytes.

The reduction in the number of bacteria in airway wash-out fluids is thus, apart from being a determinant of mucus transport, also due to the removal of bacteria from the airways by *uptake in the phagocytes*.

The decrease in number of viable bacteria in the whole lung preparations is obviously the combined effect of mucus removal and of the bacteriocidal effect of the phagocytes.

Another elimination mechanism may be considered, namely, the removal of phagocytized bacteria out of the lung via an interstitial route. Cultures from blood and autoradiographic preparations from liver, kidney, and spleen did not reveal the presence of any bacteria, thus excluding the possibility that direct elimination via the blood plays a major role in the present experiments and with the types of bacteria studied here.

To be able to test the elimination mechanism discussed here, one would have to use two different organisms, one of which is subject to only one of the elimination mechanisms. Spores seem to be suitable for this purpose.

The efficiency of the mucus removal, of the phagocytic removal from the airways, and of the bactericidal effect of the phagocytes in the lung can thus be tested with the following model

The animals are exposed to an aerosol containing radioactive spores and nonradioactive bacteria, e.g., *E. coli*. The reduction in the number of spores in whole lung grinding will then give an estimate of the capacity of the mucus removal. The reduction in the number of viable bacteria in wash-out fluids from the airways will give an estimate of the capacity of the phagocytic and mucus removal, and the reduction of viable bacteria in whole-lung grindings will provide an index of the bactericidal activity of the phagocytes in the lung and of mucus removal. Effects on any of the above functions can then be evaluated, provided that the three functions are tested simultaneously and that the effects are expressed as deviations from the normal.

Preliminary results from experiments performed with the above model indicate that the mucus removal during the first 2 hr after exposure accounts for about 15% of the total removal under the experimental conditions used. This rate agrees with results from experiments where the elimination of monodisperse plastic aerosols of various size ranges has been followed (Holma, *personal communication*). Experiments are in progress wherein the possible effects on any of the three elimination mechanisms discussed above of continuous exposure to low doses of $SO_2$ and to dust are being evaluated.

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# Effect of Route of Inoculation on Experimental Respiratory Viral Disease in Volunteers and Evidence for Airborne Transmission

ROBERT B. COUCH,[1] THOMAS R. CATE,[2] R. GORDON DOUGLAS, JR.,[1] PETER J. GERONE, AND VERNON KNIGHT[1]

*Laboratory of Clinical Investigations, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Bethesda, Maryland, and U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland*

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## INTRODUCTION

Initiation of respiratory viral infection, with some possible exceptions, appears to depend upon deposition of infectious virus at some point on the respiratory tract. There appear to be two possible mechanisms of transmission, contact or airborne. The former term is meant to refer to transfer of virus by physical contact between an infected and a susceptible subject, or indirectly through personal articles or fomites. Transmission by this route would result in deposition of virus predominantly in the nasopharynx.

Airborne transmission is intended to mean transfer of infection by means of small-particle aerosols (11, 16). These particles are the evaporated residues of infected respiratory secretions which are of such small size (mostly less than 5 μ in diameter) that they will remain airborne for long periods of time. As a function of their small size, such droplets, when inhaled, deposit predominantly in the lower respiratory tract. Particles between 5 and 15 μ to 20 μ in diameter represent an intermediate stage, and most particles in this size range will be trapped in the nose, although some will penetrate to below the larynx. (Lower respiratory tract will refer to that portion of the respiratory tract below the larynx.) Still larger particles may be produced by coughing and sneezing, etc., but since, because of their large size, they do not produce stable aerosols, transmission will ordinarily occur only by direct impaction on the nasopharynx of persons in the immediate vicinity of an infected case. Such transmission would be difficult to distinguish from that resulting from contact, and is best considered under this category.

This report will describe studies of the transmission of respiratory viral diseases which were a joint undertaking of the U.S. Army Biological Laboratories, Fort Detrick, Md., and the Laboratory of Clinical Investigations, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

[1] Present address: Baylor University College of Medicine, Houston, Tex.

[2] Present address: Washington University College of Medicine, St. Louis, Mo.

The first part of the report will describe an investigation of the infectiousness of respiratory viruses given by methods which attempt to simulate natural contact and airborne transmission, namely, nasal drops and aerosols containing virus. Coxsackievirus A type 21, a strain of rhinovirus, and adenovirus type 4 were used in these studies.

The second part will describe recovery of virus from natural aerosols produced by coughing and sneezing and from air of rooms contaminated by such discharges. In addition, preliminary results of an experimental attempt to transmit respiratory viral infection in volunteers by the airborne route will be presented.

## MATERIALS AND METHODS

### *Volunteers*

Subjects were healthy adult male inmates from several federal correctional institutions and were selected on the basis of serum antibody determinations, willingness to participate, and demonstration of good health. For studies performed at the Clinical Center, National Institutes of Health, volunteers were isolated two or three to a room for 3 to 4 days prior to inoculation and 10 to 14 days after inoculation. Examinations were performed daily by physicians having no knowledge of which of several respiratory agents was administered to a particular volunteer.

An experimental transmission experiment was performed at the Federal Prison Camp, Eglin Air Force Base. Fla. Volunteers were housed in a converted barracks building, and were evaluated before inoculation and twice daily after inoculation by physicians who knew which volunteer was inoculated and which was an exposed susceptible. Complete separation of the two groups, as described in the text, was carefully maintained; however, only partial separation from the remaining camp population was maintained.

### *Inocula*

Virus strains used in these studies were obtained from Marines with acute respiratory disease at Parris Island, S.C., or Camp Lejeune, N.C. (through the courtesy of K. M. Johnson, H. H. Bloom, and R. M. Chanock). Each inoculum had been passaged once or twice (see Results) in either human embryonic kidney (HEK) or human embryonic fibroblast (HEF, strain WI-26) tissue cultures (17). The harvests in each case were frozen and thawed, pooled, centrifuged at 1,000 × *g* for 20 min, and filtered through 800-mμ membrane filters (Millipore). The filtrates were stored at −60 C until used. Each inoculum was safety-tested for adventitious agents in a manner previously described (19). In addition to the above described procedures, the coxsackievirus A type 21 strain 48654 $HEF_2$ was submitted to vacuum concentration and trifluortrichlorethane (Gelman) treatment. Further details of these procedures have been described (6, 8, 9).

### *Inoculation Procedures*

Volunteers received aerosol inoculation by means of a molded rubber face mask attached to a cylindrical chamber containing a continuous flow of aerosol generated by a Collison atomizer. Virus was approximately 10 sec old at the time of inoculation. This equipment and other necessary auxiliary components were contained in a mobile truck and semitrailer and have been previously described (15). Each man inhaled 10 liters (±5%) through the nose, and exhaled by mouth into a discharge bag. Each inoculation required 30 to 60 sec and usually followed a training period on a previous day with use of the same equipment. The size of particles in the aerosol ranged from 0.2 to 3.0 μ in diameter. Particles 1 to 2 μ in diameter comprised 54% of the total particle volume and contained 68% of recoverable virus. Further details of the aerosol will be described in a subsequent report in this symposium (14). Aerosol inoculations with particles 15 μ in diameter were performed with the same equipment, except that the vibrating reed method of Wolf was used to generate the aerosol (25). Volunteer doses for both aerosols were calculated from virus assays in simultaneously collected Shipe impinger samples of the aerosol.

Nasopharyngeal inoculations were performed by the instillation of 0.25 ml of virus inoculum into each nostril of the volunteer while he was prone. This inoculation was accompanied by a sensation of liquid in the nose but not by a desire to expectorate or swallow. In addition, some volunteers received 0.5 ml of inoculum into each nostril as well as 0.5 ml sprayed into each nostril by a no. 127 DeVilbiss (12) hand atomizer. Studies on the aerosol produced by this atomizer have shown that 99.95% of the inoculum is contained in particles greater than 5 μ in diameter and most would be deposited in the nasopharynx (*unpublished data*).

### *Collection of Cough, Sneeze, Talking, and Room Air Samples*

Particles produced in selected expiratory events were collected for size analyses and virus

assay. In addition, room air samples were collected in a large-volume air sampler. Description and analysis of the methods used will also be described in a subsequent report in this symposium (14).

*Virus Isolations and Identification Procedures*

Specimens obtained varied with the virus being studied but included nose, throat, and anal swabs, nasal washes, and expectoration specimens. Specimens were collected prior to and subsequent to inoculation. Expectoration specimens were stored in that form until tested. Nasal washes were performed with 10 ml of Veal Infusion Broth (Difco) containing 0.5% bovine albumin with antibiotics; swabs were agitated in 2 ml of this medium and then discarded. All specimens were stored at −20 C until tested. Testing for virus was performed by inoculating 0.4 ml of specimen fluid into one HEK and HEF tissue culture tube that contained 1.5 ml of equal parts of medium 199 and Eagle's MEM, 2% inactivated calf or chicken serum, and antibiotics. The cultures were incubated in a roller drum turning at 12 rev/min at 33 to 34 C and were observed for cytopathic effect (CPE). This observation period was 14 days for coxsackievirus A type 21 and rhinovirus NIH 1734, but 60 days for adenovirus type 4. All the latter studies were performed in HEK cultures. Tissue culture fluid and cells were harvested when CPE involved 75 to 100% of the cell sheet. For coxsackievirus A type 21 and adenovirus type 4, the first and last isolates, as well as intervening isolates, when indicated, were identified by hemagglutination-inhibition (HI) with 20 antibody units of specific hyperimmune guinea pig serum or rabbit serum. HEF cultures were used for identification of comparable specimens of rhinovirus NIH 1734 by neutralization of 32 to 100 $TCID_{50}$ of virus with specific hyperimmune guinea pig serum. Further details of these procedures have been reported (6, 8, 9).

*Serological Tests*

Serial fourfold dilutions of inactivated serum were tested for neutralizing antibody for each virus by mixing equal volumes of the serum dilution with a test dose of virus, incubating at room temperature, inoculating each of two tissue culture tubes with 0.2 ml of the mixture, and observing thereafter for CPE.

All neutralizing antibody titers, calculated by the method of Karber, are expressed as the initial dilution of serum completely inhibiting CPE of 32 to 100 $TCID_{50}$ of coxsackievirus A type 21 and adenovirus type 4, but 10 to 16 $TCID_{50}$ of rhinovirus NIH 1734. Further details of the procedures have been reported (6, 8, 9, 13).

## RESULTS

*Response to Inoculation with Aerosol and Nasal Drops*

*Coxsackievirus A type 21: 50% human infectious doses* ($HID_{50}$). Volunteers free of detectable antibody were inoculated with a range of doses of coxsackievirus A type 21 by small-particle aerosol (diameter of particles, 0.3 to 2.5 μ), large-particle aerosol (diameter of particles, 15 μ), and nasal drops (0.25 ml in each nostril). An example of the type of response obtained is shown in Table 1. Twenty-eight volunteers received strain 49889 $HEK_1$ in a small-particle aerosol, and 18 became infected. The doses, number of volunteers who received each dose, and the number who became infected, as determined by virus isolation and antibody rise, are shown. Based on these findings, the $HID_{50}$ for this inoculum administered in this way corresponds to 28 $TCID_{50}$ (Spearman-Karber method; 13). Only two of the infected volunteers failed to develop illness, indicating that the 50% infectious dose and 50% illness dose are nearly the same.

In this experiment, three volunteers developed unexplained mild cases of rhinitis. Experience with over 300 volunteer inoculations indicates that such an illness is recorded in about 15% of uninfected individuals. The phenomenon occurs even though virus is inactivated with specific hyperimmune serum, in men with all levels of serum antibody, and irrespective of virus type or materials and methods used for inoculum preparation (8). Attempts to isolate a causative agent in HEK, HEF, and rhesus monkey kidney tissue cultures have been unsuccessful.

The $HID_{50}$ for strain 49889 $HEK_1$ and another inoculum (strain 48654 $HEF_2$) of coxsackievirus

TABLE 1. *Response of antibody-free volunteers inoculated with 0.3 to 2.5-μ particle aerosol of coxsackievirus A type 21 (strain 49889 $HEK_1$)*[a]

| Inhaled dose ($TCID_{50}$) | No. of volunteers | No. infected | No. ill |
|---|---|---|---|
| 832 | 1 | 1 | 1 |
| 676 | 3 | 3 | 2 |
| 316 | 3 | 3 | 3 |
| 83 | 2 | 2 | 2 |
| 71 | 5 | 5 | 4 |
| 47 | 4 | 3 | 3 |
| 18 | 4 | 1 | 2[b] |
| 6 | 6 | 0 | 2[b] |

[a] $HID_{50}$ = 28 $TCID_{50}$.
[b] Three cases of afebrile URI without infection.

TABLE 2. *$HID_{50}$ for coxsackievirus A type 21*

| Inoculum | Inoculation method | No. of volunteers | No. infected | $HID_{50}$ | 95% Confidence limits |
|---|---|---|---|---|---|
| Strain 49889 $HEK_1$[a] | Aerosol, 0.3 to 2.5-μ particles | 28 | 18 | 28 $TCID_{50}$ | 15–49 |
| Strain 48654 $HEF_2$[b] | Aerosol, 0.3 to 2.5-μ particles | 14 | 8 | 34 $TCID_{50}$ | 22–52 |
| | Aerosol, 15-μ particles | 29 | 12 | 32 $TCID_{50}$ | 13–78 |
| | Nose drops | 14 | 7 | 6 $TCID_{50}$ | 3–13 |

[a] One passage in human embryonic kidney tissue cultures.
[b] Two passages in human embryonic fibroblast tissue cultures.

TABLE 3. *Clinical response of antibody-free volunteers to coxsackievirus A type 21*

| Inoculum | Inoculation method | No. of volunteers | No. infected | No. ill | Predominant illness: Afebrile URI[a] | Predominant illness: Febrile URI | Predominant illness: Febrile LRI[b] |
|---|---|---|---|---|---|---|---|
| Strain 49889 $HEK_1$[c] | Aerosol, 0.3 to 2.5-μ particles | 28 | 18 | 16 | 1 | 3 | 12 |
| | Coarse spray and nose drops | 13 | 13 | 8 | 2 | 6 | |
| Strain 48654 $HEF_2$[d] | Aerosol, 0.3 to 2.5-μ particles | 14 | 8 | 8 | 1 | 7 | |
| | Aerosol, 15-μ particles | 29 | 12 | 11 | 1 | 8 | 2 |
| | Nose drops | 14 | 7 | 5 | 2 | 3 | |

[a] Upper respiratory tract illness.
[b] Lower respiratory tract illness.
[c] One passage in human embryonic kidney tissue cultures.
[d] Two passages in human embryonic fibroblast tissue cultures.

A type 21 administered by each of the described methods in shown in Table 2. As can be seen, the $HID_{50}$ is virtually identical for the three aerosol titrations; however, for virus administered by nasal drops, it is about fivefold less. Natural virus (virus recovered from naturally infected individuals, but not cultivated in vitro) administered by small-particle aerosol (not shown) produced infection in one of two volunteers at a dose of 28 $TCID_{50}$, and in none of six who received 7 $TCID_{50}$, suggesting a similar degree of infectivity (8).

The $HID_{50}$ for each aerosol inoculum is based on inhaled virus. Available information indicates that only 50 to 75% of particles of the size range in the small-particle aerosol would be retained and that the majority of these would deposit in the lower respiratory tract (11, 16). This indicates that the true $HID_{50}$ for the inocula administered in this way is appreciably less than that indicated in Table 2. All of the nasal drop inoculation was retained, and therefore the $HID_{50}$ for this method corresponds to the $HID_{50}$ given in the table. Since virtually all 15-μ particles would be retained, and the majority would be trapped in the nose, one would expect the $HID_{50}$ by this route of inoculation to be similar to that obtained by nasal drops. No explanation is presently available for the observed difference.

The clinical responses that correspond to the strains and inoculation methods in Table 2 are shown in Table 3. In addition, the responses to 3,000 $TCID_{50}$ of strain 49889 $HEK_1$ administered to the nasopharynx by coarse spray and drops are included (22). The frequencies of occurrence of illness in each of the five categories were not significantly different. As can be seen, the predominant clinical response to strain 49889 inoculated by small-particle aerosol was febrile lower respiratory tract illness. All 12 volunteers with this response were clinically diagnosed as having acute tracheobronchitis. The pertinent data obtained on a volunteer from a more recent experiment, but typical of the syndrome, are shown in Fig. 1. Characteristic of this syndrome was the occurrence of pain in the neck (tracheal) and chest, the latter usually being substernal. Cough, often paroxysmal, was usually non-

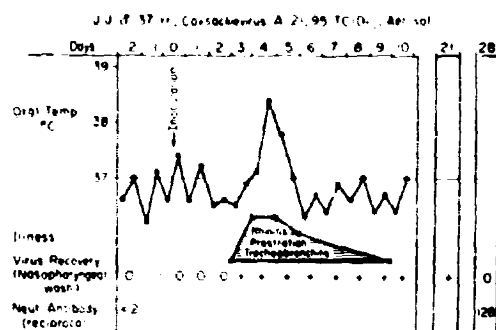


FIG. 1. *Case report of an antibody-free volunteer inoculated with coxsackievirus A type 21 by small-particle aerosol.*

productive, although auscultation of the chest occasionally revealed scattered rhonchi, and, in two cases, there was X-ray evidence of pneumonia. These lower respiratory tract symptoms were accompanied by malaise, myalgias, chilly sensations, sweats, headache, and anorexia. Illness was not limited to the lower respiratory tract, however, since 9 of the 12 volunteers with tracheobronchitis also had upper respiratory tract illness that was characterized by rhinorrhea and nasal obstruction. Four of the remaining six infected volunteers had upper respiratory tract illness only, and the other two had infection without apparent illness.

In contrast to the small-particle aerosol response, 8 of 13 volunteers who received nasopharyngeal inoculation developed upper respiratory tract (nasopharyngeal) illness only. The fact that virus was deposited in the nasopharynx in this case and predominantly in the lower respiratory tract in the former suggested that virus deposition site accounted for this difference and that it might be the factor that determines the clinical response. However, when strain 48654 was administered by small-particle aerosol, the lower respiratory tract illness, which was characteristic of strain 49889 given in this way, was not seen. The predominant clinical response to strain 48654 in a small-particle aerosol was febrile upper respiratory tract illness (Table 3). Thus, virus deposition site and inoculum differences both appeared as important factors in determining the type of clinical response.

Febrile upper respiratory tract illness was also the predominant clinical response for strain 48654 administered by 15-$\mu$ particle aerosol and by nasal drops (Table 3). Not shown are clinical responses to natural virus and to still another strain of virus administered by small-particle aerosol (8, 20) For these inocula, febrile upper respiratory tract illness also predominated. This combined experience suggests that virus deposition site may be an important factor in determining the type of clinical response that occurs. However, for coxsackievirus A type 21, most strains appear to lack the capability of producing lower respiratory tract illness when presented such an opportunity by virus deposition at this site.

In all other aspects, the clinical responses were similar for each inoculum and inoculation method. The incubation period was 2 to 5 days, illness usually lasted 2 to 3 days, fever rarely exceeded 38.5 C, and fever usually persisted less than 1 day.

The effect of pre-existing serum neutralizing antibody on the responses following inoculation of volunteers with coxsackievirus A type 21 (strain 49889) has not been completely evaluated, but the data available are shown in Table 4. As can be seen, all individuals with intermediate titers of antibody were infected after nasopharyngeal inoculation, but infection occurred in only 5 out of 11 with high titers. A similar suggestion of reduction in infection also occurred in the small-particle aerosol groups.

*Rhinovirus NIH 1734:* $HID_{50}$. Volunteers free of detectable antibody to this virus were inoculated with a range of doses of rhinovirus NIH 1734 by small-particle aerosol and by nasal drops. The $HID_{50}$ for each inoculation method is shown in Table 5. Nasal drop doses of 1 $TCID_{50}$ and less were extrapolated values based on dilutions of a pool with known virus concentration, and aerosol doses of 2 and less were extrapolated from measured concentrations of virus in aerosols produced, during the inoculation period, by higher concentrations of virus. Repeated tests of several dilutions of virus run in sequence have been shown to produce proportionate changes in aerosol virus concentration. Assays for virus were performed in HEF (WI-38) tissue cultures, in a

TABLE 4. *Response of volunteers with pre-existing antibody to inoculation with coxsackievirus A type 21*

| Level of antibody | Nasopharyngeal inoculation | | | Aerosol, 0.3 to 2.5 $\mu$ particles | | |
|---|---|---|---|---|---|---|
| | No. of volunteers | No. infected | No. ill | No. of volunteers | No. infected | No. ill |
| Intermediate (1:4-1:128) | 6 | 6 | 4 | 5 | 3 | 2 |
| High (1:256 or greater) | 11 | 5 | 0 | 3 | 0 | 3* |

* Each illness was mild rhinitis.

TABLE 5. $HID_{50}$ for rhinovirus NIH 1734

| Inoculation method | No. of volunteers | No. infected | $HID_{50}$ | 95% Confidence limits |
|---|---|---|---|---|
| Nasal drops | 17 | 11 | 0.032 $TCID_{50}$ | 0[a] |
| Aerosol, 0.3 to 2.5 μ particles | 26 | 20 | 0.68 $TCID_{50}$ | 0.2–2.0 |

[a] Indicates no intermediate response between 100 and 0% infection.

TABLE 6. Clinical response of volunteers to inoculation with rhinovirus NIH 1734

| Inoculation method | No. of infected volunteers | No. ill | Illness: URI | Illness: URI-LRI[a] | Illness: LRI |
|---|---|---|---|---|---|
| Coarse spray and nose drops | 48 | 43 | 41 | 2 | 0 |
| Aerosol, 0.3 to 2.5 μ particles | 41 | 33 | 23 | 5 | 5 |

[a] Upper and lower respiratory tract illness.

manner described previously (6). Other types of tissue culture [HEK and HEF (WI-26)] and tissue culture assay [HEF (WI-38) plaque assay] were tested and found to be equal to or less sensitive than the cultures and methods used.

As can be seen in Table 5, the $HID_{50}$ for both inoculation methods was below the practical limits of detection. Failure to infect all volunteers with small-particle aerosol inoculation first occurred at an inhaled dose of 2 $TCID_{50}$, and none of three who inhaled 0.06 $TCID_{50}$ became infected. The $HID_{50}$ for this inoculation method was 0.68 $TCID_{50}$ (Spearman-Karber; 13). In contrast, all volunteers who received 0.1 $TCID_{50}$ by nasal drops became infected, although none became infected at two lower doses. The $HID_{50}$ for this method corresponded to 0.032 $TCID_{50}$. These results indicate an approximately 20-fold disparity between infectivity for the virus given by the two methods. The disparity could be accounted for by assuming that the 10 to 20% of small-particle aerosol particles that deposit in the nasopharynx are responsible for all infection in volunteers inoculated in this way. However, the fact that this is not the case is suggested by the occurrence of lower respiratory tract illness in some of these volunteers. In any event, the data suggest that the nasal mucosa is somewhat more susceptible to rhinovirus NIH 1734 than is the lower respiratory tract. Although the difference was less for coxsackievirus A type 21, it was similar in direction.

The clinical responses of all volunteers who have received either nasal or small-particle aerosol inoculation with rhinovirus NIH 1734 are shown in Table 6. As can be seen, the characteristic response to either method of inoculation is an upper respiratory tract illness which in all respects is a common cold. The pertinent data obtained from one of the volunteers inoculated by nasal drops are shown in Fig. 2. His response consisted of a common cold syndrome characterized by nasal obstruction and discharge, and was accompanied by throat irritation and systemic symptoms. The extent of the rhinorrhea is shown in the figure. Fever was absent in this volunteer and occurred in less than 10% of the volunteers, regardless of method of inoculation.

As can be seen in Table 6, lower respiratory tract illness (acute tracheobronchitis) was predominant in five volunteers who received small-particle aerosol inoculation, and diffuse respiratory tract disease without a predominant localization was seen in five others. Predominant lower respiratory tract illness was not seen in men inoculated by nasal drops, although two volunteers exhibited a combination of upper and lower respiratory tract illness. These findings suggest that aerosol inoculation may produce lower respiratory tract involvement, but the characteristic response to infection produced by either method is an upper respiratory tract illness.

The incubation period of the illnesses produced by both inoculation methods was 2 to 4 days, the illness usually lasted 2 to 3 days, and fever, when it occurred, was usually 1 day in duration.

The effect of pre-existing serum neutralizing antibody on responses to inoculation with rhinovirus NIH 1734 is shown in Table 7. As can be seen, no significant reduction in frequency of infection occurred unless high levels of serum antibody were present. This reduction in frequency of infections occurred for both methods of inoculation and was accompanied by a similar reduction in illnesses. [Data are grouped for convenience. Individual values were tested in Spearman's rank correlation or Yates mean score tests (13). Reduction in infection and illness with increasing serum antibody was statistically significant ($P < 0.05$) for both inoculation methods.]

*Adenovirus type 4: $HID_{50}$.* Nine volunteers free of detectable antibody to adenovirus type 4 received small doses of this virus by small-particle aerosol. Six volunteers received the virus by 15 μ particle aerosol. The results of these

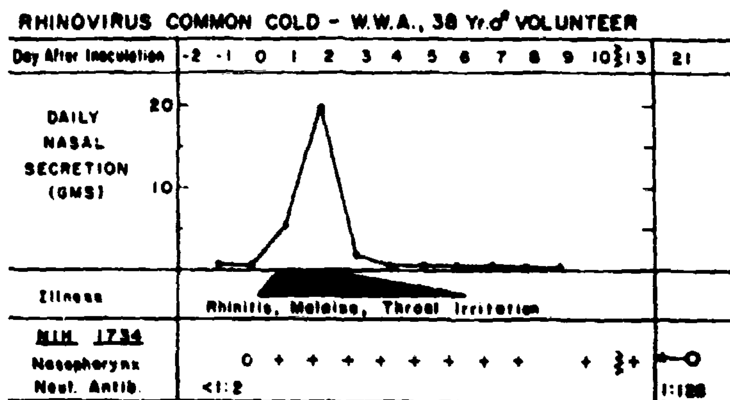


FIG. 2. *Case report of an antibody-free volunteer inoculated with rhinovirus NIH 1734 by course spray and nasal drops. (Reproduced with the permission of the Journal of Clinical Investigation.)*

studies are shown in Table 8. As can be seen, all volunteers who received doses of 11 and 5 $TCID_{50}$ by small-particle aerosol became infected, but only one out of three became infected at a dose of 1 $TCID_{50}$. Other volunteers were inoculated in this way, and, although the data are incomplete, the studies indicate that the $HID_{50}$ for small-particle aerosol inoculation is about 1 $TCID_{50}$. It should be stated that these virus assays were performed in HEK tissue cultures, the most sensitive tissue available for adenovirus, and the cultures were observed for 60 days for CPE with subpassage as needed. This time period was shown to provide maximal detection of adenovirus (9).

Only one dose level of adenovirus type 4 has been administered by 15-$\mu$ particle aerosol, and this was 1,000 $TCID_{50}$. All six volunteers who received this dose became infected. Preliminary results on inoculation of volunteers by nasal drops indicate that the $HID_{50}$ by this method is about 20 $TCID_{50}$. This combined experience with adenovirus type 4 suggests that a greater dose of this virus is required to initiate infection in the nasopharynx than in the lower respiratory tract.

Also shown in Table 8 are the clinical responses seen in the volunteers inoculated by aerosol. As can be seen, all volunteers infected by means of small-particle aerosol inoculation became ill, and the illness was usually febrile. Three volunteers had predominantly upper respiratory tract illness, and, in three others, illness was predominantly in the lower respiratory tract. The latter included one instance of mild pneumonia. Only three of the six volunteers infected by 15-$\mu$ particle aerosol inoculation became ill, two with febrile upper respiratory tract illness and one with pneumonia. The incubation period for these

TABLE 7. *Response of volunteers with pre-existing antibody to inoculation with rhinovirus NIH 1734*

| Level of antibody | Nasopharyngeal inoculation | | | Aerosol, 0.3 to 2.5 $\mu$ particles | | |
|---|---|---|---|---|---|---|
| | No. of volunteers | No. infected | No. ill | No. of volunteers | No. infected | No. ill |
| Low (1:2-1:8) | 3 | 3 | 2 | 5 | 4 | 4 |
| Intermediate (1:16-1:64) | 9 | 8 | 7 | 8 | 6 | 4 |
| High (1:128 or greater) | 13 | 8 | 4 | 4 | 1 | 1 |

illnesses varied between 6 and 13 days, duration of illness varied between 2 and 10 days, and fever between 1 and 8 days. In addition, the severity of illness, as manifested in respiratory tract involvement and constitutional symptoms, also was quite variable. Upper respiratory tract findings occurred in all men in the 15-$\mu$ particle aerosol group, whereas this finding was variable in the small-particle aerosol group. The pertinent data obtained on one of the volunteers who exhibited the syndrome described as acute respiratory disease (ARD) of military recruits are shown in Fig. 3. Bacteriological cultures were negative for pathogens, and spontaneous recovery occurred without antibiotic therapy.

It is notable that the syndromes of febrile respiratory tract illness that occurred after aerosol inoculation resemble the naturally occurring type 4 adenovirus diseases of military recruits (5, 7, 21). Previous studies by others, in which volunteers were inoculated in the nasopharynx, usually resulted in asymptomatic infection or mild afebrile upper respiratory illness (1). Inocula-

TABLE 8. *Response of antibody-free volunteers to adenovirus type 4*

| Inoculation method | Dose[a] | No. of volunteers | No. infected | No. ill | Illness: Afebrile URI[b] | Illness: Febrile URI | Illness: Febrile LRI[c] |
|---|---|---|---|---|---|---|---|
| Aerosol, 0.3–2.5-μ particles | 11 | 3 | 3 | 3 | 1 | 1 | 1 |
| | 5 | 3 | 3 | 3 | | 1 | 2 |
| | 1 | 3 | 1 | 1 | | 1 | |
| Aerosol, 15-μ particles | 1,000 | 6 | 6 | 3 | | 2 | 1 |

[a] Expressed as $TCID_{50}$.
[b] Upper respiratory tract illness.
[c] Lower respiratory tract illness.

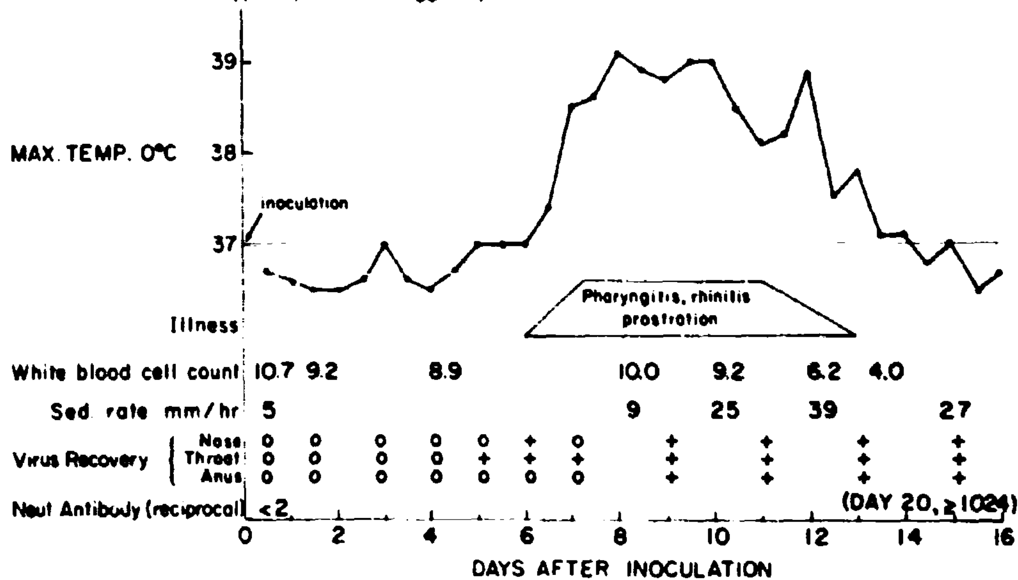


FIG. 3. *Case report of an antibody-free volunteer inoculated with adenovirus type 4 by 15-μ particle aerosol. White blood cell counts are times $10^3$ per cm. (Reproduced with the permission of the American Review of Respiratory Diseases.)*

tions into the conjunctival sac resulted in occurrence of conjunctivitis only or pharyngoconjunctival fever, illnesses which rarely occur naturally in type 4 infection, and which were not seen in the present studies (1). These findings suggest that the unique feature of the present inoculations, deposition of virus in the lower respiratory tract, was the major factor accounting for the recruit type illnesses. This is supported by the fact that small doses of virus given by small-particle aerosol produced illness in all volunteers infected by this method of inoculation, whereas the large dose given by 15-μ particle aerosol caused illness in only three of six infected men. Evidence indicates that most of the 15-μ particles were deposited in the upper respiratory tract, but the possibility exists of deposition in the lower respiratory tract either by direct inhalation or as a result of particle fragmentation (16). It is suggested that in three men this occurred and caused febrile illness.

Three volunteers with pre-existing antibody titers of 1:32 to 1:64 received 6 $TCID_{50}$ by small-particle aerosol, and none became infected or ill.

inoculations of volunteers with pre-existing antibody into the nasopharynx or conjunctival sac by others have also demonstrated the protective effect of serum antibody (1).

### *Evidence for Airborne Transmission*

*Detection of virus in particles produced by coughing, sneezing, and normal expiration.* By use of methods (14) for recovery of virus from particles produced by coughs and sneezes, virus titration was carried out on 61 cough collections and 58 sneeze collections from volunteers infected with coxsackievirus A type 21 (Table 9). The collection method involved coughing or sneezing into a collapsed weather balloon through a tight-fitting face mask. The air in the balloon was evacuated through a Shipe impinger to remove airborne particles, and material impacted on the wall of the balloon was collected by rinsing with sterile tissue culture fluid. When the results of both samples were combined, 39% of cough specimens and 50% of sneeze specimens were positive for virus. Thirty per cent of air samples were positive for both events, and the mean quantity present was 30 $TCID_{50}$ and 60 $TCID_{50}$ for cough and sneeze samples, respectively. This close similarity in results is of interest in view of the approximately 20-fold greater number of particles and particle volumes produced by sneezing (14). This finding suggests that the concentration of virus in secretions released in small particles produced by coughing is greater than that produced by sneezing.

Analysis of balloon wall samples revealed a disparity between the two events. Wall samples of sneezes were more frequently positive than the wall samples of coughs, but the mean quantity present was only twofold greater. However, the mean quantity of virus present in the wall samples from sneezes does not include four sneezes in which gross contamination with large quantities of nasal secretion occurred. The wall samples of these sneezes contained 30,000 to 500,000 $TCID_{50}$ of virus. The reasons for the disparity in frequency of detection of virus on the balloon wall for the two events are not known at the present time, since studies have revealed similar particle size distributions for both events (12, 14).

TABLE 9. *Virus recovery from particles in coughs and sneezes produced by volunteers infected with coxsackievirus A type 21*

| Phenomenon | No. tested | Per cent positive | Source | Per cent positive | Mean quantity ($TCID_{50}$) |
|---|---|---|---|---|---|
| Sneeze | 58 | 52 | Air[a] | 30 | 60 |
| | | | Wall[b] | 45 | 100 |
| Cough | 61 | 39 | Air[a] | 30 | 30 |
| | | | Wall[b] | 20 | 50 |

[a] Assay of Shipe impinger collection of particles suspended in air in balloon.

[b] Assay of 10-ml liquid rinse of balloon wall.

Breathing samples were tested by collecting the entire amount of expired air in Shipe impingers through a closed system for 30-min periods. This testing constituted sampling of air expired for 2 hr per day from four infected volunteers during the period that included occurrence of illness and maximal virus shedding. A volume equivalent to 12 hr of expired air was tested in this way, and all samples were negative for virus.

A number of factors were evaluated to determine the cause for virus release in the process of coughing and sneezing. These evaluations suggested that the presence of nasal obstruction and discharge was the most important determinant for release of virus when infected persons sneeze [with nasal obstruction and discharge, 19 of 24 sneeze samples were positive; without nasal obstruction and discharge, 11 of 34 samples were positive ($P < 0.001$)]. In contrast, positive cough specimens bore a relationship only to the quantity of virus present in respiratory secretions, and this relationship occurred for air samples only [combined nasal and oral secretions, Yates mean score, test, $P = 0.05$ (13)]. Since cough particles would presumably be derived from pharyngeal and lower respiratory secretions, it is suggested that the concentration of virus in these secretions varied proportionately with the secretions tested.

*Virus in room air.* The contribution to room air contamination by coughing, sneezing, and possibly by other expiratory phenomena of man involves frequency and occurrence of the phenomenon, inactivation of virus, and physical loss of aerosol particles, in addition to quantity of virus released. The significance of these factors in determining environmental contamination was tested by collecting particles present in the air of rooms occupied by volunteers infected with coxsackievirus A type 21 and then assaying the collections for virus.

The large-volume air sampler was used to collect particles from approximately 70% of room air after a period of 2 hr with no ventilation. Shown in Table 10 are the results of testing 30 such samples collected during the acute phase of illness and maximal virus shedding. Of the 30 samples, 14 were positive, and, as can be seen, the frequency of positive samples increased with increasing quantity of virus present in respiratory

TABLE 10. *Relation of virus quantity in respiratory secretions to virus in room air samples*

| Mean (3 vol.) virus quantity in secretions | Air sample | | |
|---|---|---|---|
| | No. of tests | No. positive | Mean virus quantity |
| 10-30[a] | 5 | 1 | 5[b] |
| 30-100 | 11 | 2 | 160 |
| 100-300 | 5 | 4 | 250 |
| 300-1,000 | 6 | 4 | 50 |
| 1,000->1,000 | 3 | 3 | 500 |

[a] Expressed as $TCID_{50}$ per milliliter of secretion.
[b] Expressed as $TCID_{50}$.

secretions [Smirnoff test, $P < 0.01$ (13)]. The mean virus quantity in positive samples is shown in the last column and was sufficiently variable so that no quantitative relationship to virus in respiratory secretions was detected, although it is of interest that the largest quantity present in room air, 28,000 $TCID_{50}$, was in the room with the highest virus concentration in secretions.

Since both positive cough and room air samples were related to quantity of virus in respiratory secretions of infected volunteers, it was suggested that coughing was responsible for contamination of room air with virus. When the results were analyzed by room, it was found that the presence of virus in cough air samples from volunteers occupying a room was significantly related to the recovery of virus from the air of that same room on the same day. [Positive room air samples, 10 of 11 rooms with positive cough air samples; negative room air samples, 2 of 7 rooms with positive cough air sample ($P = 0.03$)]. This further suggested that cough is the important intermediary between virus in secretions and virus in room air. No such relationship was detected for sneezing. These findings are not surprising, since cough as a symptom was recorded as being frequently present in these same volunteers at this time, whereas sneezing was not.

*Preliminary report on a transmission experiment.* An experiment designed to test whether the occurrence of air contamination with virus is sufficient to produce airborne transmission has been performed (*unpublished data*). Nineteen placebo-inoculated volunteers were exposed to air surrounding infected volunteers by housing the two groups in a converted barracks and separating them with a double-wire barrier. Even distribution of air on both sides of the test building was accomplished by means of large floor fans and was proved by generating an aerosol containing a fluorescein dye on one side and then collecting and analyzing air samples from different locations throughout the building. Coxsackievirus A type 21 infection was produced in 10 volunteers with aerosol inoculation, and all exposed individuals became infected with this virus during the course of the study. A specific separation of results in terms of contact and airborne-acquired infection is not completed, but it is possible to state that airborne transmission unquestionably occurred.

## DISCUSSION

The theory that respiratory viruses are transmitted by the airborne route has been popular in the past, primarily because it seemed reasonable to assume that coughing and sneezing, common symptoms of viral respiratory disease, produce aerosols that would accomplish such transmission. Despite this assumption, proof that man produces aerosols that contain virus and that sufficient viral contamination of air occurs to result in this type of transmission, both essential requirements for airborne transmission, has not been obtained (24). The results presented in this report provide this important information. It was shown that individuals infected with respiratory viruses, in this case coxsackievirus A type 21, produce airborne virus in quantities sufficient to infect susceptible individuals. The capacity to produce viral aerosols was tested for three expiratory events. Breathing samples were uniformly negative for virus, whereas cough and sneeze samples were frequently positive. Thus, whereas in man the former event is probably insignificant in producing transmission of the respiratory viruses, it seems likely that it is important in the mouse-influenza system of Shulman and Kilbourne (23) in which airborne transmission has also been conclusively demonstrated. For man, coughing and sneezing appear to be the significant events for producing viral aerosols.

Studies in which virus released by coughing and sneezing was collected in a balloon and separated into an air phase and a wall phase provided quantitative results that correspond roughly to virus involved in airborne transmission and contact transmission, respectively. Virus was recovered more often from the air sample from coughs than from the wall samples, although the wall samples of sneezes were more commonly positive than the air samples. This would suggest that sneezing may be of some significance for that form of transmission involving direct impaction of large particles in the nasopharynx, whereas cough contributes primarily to small particle

aerosol transmission. Despite the differences in frequency of recovery, the difference in mean quantity of virus in each phase was small and quite similar for each event. These findings are in contrast to the findings of Buckland et al. (5), in which the vast majority of virus released in sneezing was found on the sides of a large sampling bag. However, the different collection methods involved may account for this disparity.

Despite a larger number of particles in sneezes than in coughs, the quantity of virus expelled in the two events was remarkably similar, suggesting that, in these volunteers, the concentration of virus in secretions atomized in coughing was relatively greater than that in secretions atomized in sneezing (12, 14). Inoculation of these volunteers was performed by small-particle aerosol, and, although lower respiratory tract secretions were not quantitated, virus is known to have been deposited at this site and probably induced infection there. Thus, it is possible that the method by which infection was induced may have contributed to the virus recovery results from coughing.

The fact that infected persons are capable of producing airborne virus does not necessarily indicate that virus can be transmitted in this way. Viral aerosols produced by infected persons are subject to dilution in room air, biological decay, and sedimentation. Nevertheless, assuming normal breathing by susceptible volunteers and an infectious dose of about 6 to 30 $TCID_{50}$, assay of air samples from rooms occupied by infected volunteers indicated that transmission would be accomplished in from 5 min to 24 hr. Furthermore, in view of the observed efficiency (11') of the air-sampling equipment, larger than measured doses of virus were actually available for inhalation (14). In addition, the present data suggest that cough is a most important event in producing viral contamination of air.

The findings described above stimulated the performance of an experiment to test the assumption that airborne transmission is possible, and preliminary results revealed that airborne transmission occurred from infected cases to susceptibles across a wire barrier.

Airborne and contact transmisison was simulated in volunteers by aerosol and nose drop inoculation, respectively. Studies with three different strains of coxsackievirus A type 21 indicated a similar $HID_{50}$ of about 30 $TCID_{50}$ for this virus, when predominant deposition was in the lower respiratory tract (small-particle aerosol), and a lower value when nasal drops were used. Since the latter inoculation method provided deposition only in the nasopharynx, it is suggested that the nasal mucosa exhibited a greater susceptibility to infection with this virus than did the lower respiratory tract. Another picornavirus, rhinovirus NIH 1734, exhibited an even greater difference between the $HID_{50}$ for nasal drop inoculation and for small-particle aerosol inoculation. Thus, the data suggest that, for both of these viruses, the nasal mucosa is the preferred site for infection. Although definitive comparisons are incomplete, present evidence suggests a disparity in infectivity in the opposite direction for adenovirus type 4. This virus exhibits a high degree of infectivity for the lower respiratory tract, but the nasopharynx appears to lack this degree of susceptibility.

The most common illness response to each virus that followed inoculation by nasal drops and small-particle aerosol is shown in Table 11. For comparative purposes, the most common naturally occurring illness response to each virus is also listed. As can be seen for coxsackievirus A type 21, regardless of method of inoculation as well as dose, febrile upper respiratory illness usually results in volunteers, whereas naturally occurring illness is reported to be usually afebrile (2, 18). This disparity may well be explained by the fact that fever in volunteers is usually so brief in duration that, without 24-hr observation, the majority of volunteers would have been designated afebrile. The predominant lower respiratory tract illness that was seen with one

TABLE 11. *Characteristic natural and experimentally induced clinical responses to respiratory viruses*

| Virus | Experimental inoculation | | Natural inoculation |
|---|---|---|---|
| | Nasopharyngeal | Aerosol, 0.3 to 2.5 μ particles | |
| Coxsackievirus A type 21 | Febrile URI[a] | Febrile URI | Afebrile URI |
| Rhinovirus NIH 1734 | Afebrile URI | Afebrile URI | Afebrile URI |
| Adenovirus type 4 | Afebrile URI | Febrile URI or LRI, or both | Febrile URI or LRI, or both |

[a] Upper respiratory tract illness

inoculum administered by small-particle aerosol appears to have been relatively unique, and was due to properties of the virus in that inoculum that are not usually exhibited by strains of this virus.

For rhinovirus NIH 1734, afebrile upper respiratory tract illness occurs in volunteers regardless of inoculation method and is also the characteristic natural clinical response to this and other rhinoviruses (4, 10). Data thus far available indicate that naturally occurring adenovirus type 4 disease can regularly be reproduced in volunteers only by aerosol inoculation. Nasal inoculation, throat swabbing, and conjunctival inoculation have all failed to reproduce naturally occurring type 4 adenovirus disease (1).

It is therefore suggested that adenovirus type 4 disease is transmitted in natural circumstances primarily by the airborne route. The information available on coxsackievirus A type 21 and rhinovirus NIH 1734 indicates that either airborne or contact transmission would result in the upper respiratory tract illness characteristic of naturally occurring illness. However, the small-particle aerosol inoculation results suggest that airborne transmission would produce a more varied response and account for the lower respiratory tract illness which is sometimes associated with naturally occurring upper respiratory tract disease (4, 10, 18).

Thus, the data presented on production of airborne virus, environmental air contamination with virus, and the demonstration of airborne transmission summarized in the present report indicate that airborne transmission probably occurs naturally. Present information, however, does not indicate whether airborne transmission is the predominant mechanism of natural transmission. At the present time, it seems most reasonable to suggest that both contact and airborne transmission occur in natural circumstances, and that the predominant method of transmission varies with the virus and the opportunity presented in a particular situation. For those viruses and situations in which airborne transmission predominates, it may be possible to devise suitable methods of control of respiratory viral infection.

## SUMMARY

Volunteers were inoculated with respiratory viruses by means of nasal instillations and inhalation of aerosols. The former method was used to simulate contact transmission, and the latter to simulate airborne transmission. The $HID_{50}$ for coxsackievirus A type 21 was about 30 $TCID_{50}$ by aerosol and 6 $TCID_{50}$ by nose drops. Similar determinations for rhinovirus NIH 1734 revealed $HID_{50}$ of 0.68 $TCID_{50}$ by aerosol and 0.032 $TCID_{50}$ by nasal drops. The clinical response was characteristically an upper respiratory tract illness for both viruses by both inoculation methods, although coxsackievirus A type 21 illness was usually febrile, and rhinovirus illness usually was not. Incomplete infectivity studies with adenovirus type 4 suggest a disparity in the opposite direction for this infection. Aerosol inoculation revealed an $HID_{50}$ of about 1 $TCID_{50}$ and thus far is the only inoculation method which regularly reproduced naturally occurring ARD.

The suggestion that airborne transmission accounted for some naturally occurring acute respiratory disease was further evaluated by studying the production of airborne virus by coughs and sneezes and the contamination of room air with virus. Coughing and sneezing regularly produced quantities of virus sufficient to infect, whereas breathing did not. Room air samples revealed contamination probably sufficient to infect susceptibles. In addition, preliminary results of a transmission experiment with coxsackievirus A type 21 indicate that airborne transmission unquestionably occurred. It was concluded that both contact and airborne transmission of the respiratory viruses probably occur in natural circumstances, and that the predominant method of transmission may vary with the virus and with the particular environmental situation.

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# Discussion

EDWIN D. KILBOURNE
*Cornell University Medical College, New York, New York*

The investigator of experimental infection has advantages—and, indeed, he *makes* these advantages—that the student of natural infection cannot have. He can operate with deliberation, he can pick his time and place, he can pick his subject, and his infecting agent (indeed, also its dose), he can control the environment and the route of inoculation, and he can observe the event we call infection both in prospect and in retrospect. Yet he lacks, and always will lack, the cardinal advantage possessed by the student of natural disease—for, unlike his colleague, he is not studying natural disease. This must never be forgotten. Dr. Couch and his associates are well aware of this point. Their interesting and provocative paper is a measured and careful analysis of basic investigations of an incredibly neglected aspect of medicine: the transmission of human respiratory infection.

There is much to discuss in this paper, as the authors themselves have discovered, and I should like to focus on the aspects of the study that seem to have broad implications for the pathogenesis and transmission of infection, rather than on the technical problems of air sampling and calculation of virus dose, although, to some extent, these matters are inseparable.

First, I would emphazise that the principal and best-documented part of this study concerns virus *input* and its effects rather than virus *output* and transmission. Commendably, the authors have recognized implicitly the inhomogeneity of "respiratory viruses" by their selection of three prototypes for study, and, further, have studied (with differing results) two strains of one prototype, coxsackievirus A21. This leads me to my first question. In Table 1, in which the data for determination of human infectious dose of coxsackievirus A21 are presented, we note that in contrast to the common phenomenon of infection without disease, we have disease without infection (after doses of 18 and 6 $TCID_{50}$)! This observation may be an important one and is not adequately explained by the notation that 15% of volunteers may have unexplained mild rhinitis, particularly since no control group is included in the experiment cited. Is this a nonspecific reaction, or is it rather a subtler measure of low-grade infection than the conventional indices of virus recovery and antibody response? A similar observation concerning experimental A21 injection has also been made by Buckland and associates. If so, it is implied that the human and tissue culture infective doses may be close to unity. I do not wish to belabor an apparently trivial point, but we note that the virus used in this titration is the A21 strain, 49889, which is more virulent than the other A21 strain used, in its capacity to produce lower respiratory tract disease. Why this greater capacity to induce disease? Because of intrinsic difference in virulence of the virus in nature, because of the vagaries of virus selection during its isolation and passage, or because the human infectious dose has been miscalculated, with the result that a larger dose of this strain has actually been given? This type of problem will continue to beset us as we attempt to distinguish qualitative from quantitative factors in experimental infection. Incidentally, is the febrile course of experimental coxsackievirus A21 infection in contrast to the afebrile course observed in the field a reflection of selection of subjects with no antibody, or does it reflect differences in the conditions of infection?

But the striking fact that emerges from the studies of coxsackievirus A21 infection is the importance of the nature of the viral inoculum in determining the localization and severity of infection. The implication is clear that in the perhaps special case of the 49889 strain, lower respiratory tract disease requires initial implantation of virus at that site. It is also clear that one may have presumed implantation of virus in the lower respiratory tract without evidence of disease at that site, but with evidence of disease above. What a fine demonstration of the importance of host determination of clinical response in a precisely controlled situation. Restudy of such volunteers with an antigenically heterologous virus might confirm our strong suspicion that certain individuals are unduly subject to lower tract involvement in the course of upper respiratory tract infections. Now the other part of the question is: how does an aerosol of small particles produce upper respiratory tract disease? Probably not by extension from below as judged by the similar incubation periods of aerosol and nasal inoculation disease, but perhaps by the fraction of small particles that are retained in the upper tract. It must be kept in mind that there is a range of particle size with either method of inoculation.

The experiments with rhinovirus infection are also of interest and suggest again that a *sine qua*

*nism* for respiratory virus infection of the lower tract is initiation of infection by aerosol. The observation that a true common cold virus can indeed produce generalized respiratory disease is an important one. Incidentally, I disagree with the authors when they impute an all-or-none effect of pre-existing antibody in ameliorating disease. The number of cases is far too small (a chronic problem, incidentally, in volunteer studies) and the data suggest a graded response in terms of infection.

Turning to adenovirus infection, it is especially interesting that simulation of the natural disease is apparently dependent on infection by aerosol, despite the fact that its principal manifestations are in the nasopharynx. Influenced by our studies of influenza, Jerome Schulman and I have long contended that the site of predominant symptomatology is not necessarily an indication of the site of primary viral invasion. It is true, however, that natural infection with either influenza or adenoviruses may be associated with primary virus pneumonia, so the potential for that clinical manifestation is probably omnipresent.

In conclusion, we can note with great interest the brief mention by Dr. Couch and his associates of transmission of infection from man to man under conditions that exclude direct or indirect contact and that point to true airborne inoculation. The double wire screen used by Dr. Schulman with influenza in mice has now found application with man, coxsackievirus, and Quonset huts. We await further exploitation of this system by Dr. Couch and his colleagues with the imagination and thoroughness they have shown in the past.

The proper study of mankind may be the mouse. In an experimental mouse influenza virus model developed in our laboratory by Dr. Schulman, we have found that virtually all transmission of infection can be attributed to small-particle aerosols. This conclusion depends essentially on two types of observation: (i) that physical separation of contacts from transmitters exerted no effect on the transmission rate, and (ii) that increasing the rate of ventilation through the chamber where contact occurred decreased proportionately the rate of transmission. Both observations are inconsistent with transmission by larger droplets. Furthermore, in very recent experiments, we have recovered influenza virus from the air in proximity to transmitter mice in quantities that are virtually identical to the calculated output required to account for the observed transmission rate (a calculated 2.3 infectious doses per infector per 24 hr).

It is clear that experimental infection with a number of viruses may be initiated by any of several routes by either small or large particle inocula. It is now time to determine for each virus what, in fact, is its principal natural mechanism of transmission as it journeys from man to man. This can be done with the techniques now available, and, indeed, indirectly by such environmental controls as ultraviolet irradiation, employed 30 years ago by Wells.

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# Aerogenic Immunization of Man with Live Tularemia Vaccine

RICHARD B. HORNICK AND HENRY T. EIGELSBACH

*The University of Maryland School of Medicine, Baltimore, Maryland, and U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland*

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## INTRODUCTION

Vaccines are generally administered by the subcutaneous or intramuscular route. However, the immune response produced after parenteral administration is inadequate in many instances to ensure optimal host resistance. Many infectious diseases are acquired via the respiratory tree; possibly, the immunizing antigen would be more effective in inducing high-grade host defense if the route of administration were identical to the route of acquisition of the disease. Active immunization against airborne infection by inhalation of living, attenuated microorganisms has been proved with experimental animals and, in some instances, has become routine (1-4, 11-14, 16, 20, 22). The potential for immunization of man by aerogenic vaccination with single or combined live vaccines has been recognized in the Soviet Union (1-3) and in the United States (5, 8, 9). In the Soviet Union, vaccination of man with aerosols of dried, viable tularemia vaccine, singly or in combination with living vaccines of other microorganisms, has received considerable attention. Systemic reactions were reported by Alexsandrov et al. (2) in 2 of 138 volunteers inhaling an estimated 750,000 organisms contained in an aerosol of dried tularemia vaccine. Kerostovtsev, Onikiyenko, and Khokhlov (17) noted similar complaints in three of eight persons inhaling 7,500,000 cells of a comparable product. Immunity has been measured primarily by serological procedures and by reaction to skin test preparations, but has not been proved by increase in resistance of the vaccinee to challenge with fully virulent organisms. Tigertt (23) has reviewed selected Soviet articles on viable tularemia vaccines, and Lebidinsky (18) has reviewed the published American literature on this subject.

In the United States, live tularemia vaccine prepared from *Francisella tularensis* strain LVS (live vaccine strain) (7) and administered percutaneously has been proved immunogenic and superior to killed vaccines in studies with volunteers by Saslaw et al. (21) and by McCrumb (19). Studies by the latter investigator revealed that, although immunized volunteers were protected against challenge by the respiratory route with 200 to 2,000 virulent organisms, resistance could be overcome in about 50% of men when the challenge dose was increased approximately 10-fold. (The genus *Francisella*, honoring the late Edward Francis of the U.S. Public Health Service and providing better taxonomy, will appear in the next edition of *Bergey's Manual*.)

In an effort to enhance the immunity provided by LVS, aerogenic vaccination was studied by Eigelsbach et al. (10, 11) and White et al. (24). It was demonstrated that this route of vaccination was not associated with untoward reactions, and only a mild, nongranulomatous response was observed in the respiratory bronchioles of monkeys that received aerosolized liquid vaccine. Animals so vaccinated evidenced excellent protection when challenged with virulent organisms.

In a recent unpublished study (H. T. Eigelsbach and J. J. Tulis) designed to determine the effect of aerosolized vaccine dose on reactivity and

TABLE 1. *Effect of dose and route of inoculation on the immunogenicity of live tularemia vaccine for the monkey*

| Vaccination | Vaccine dose | No. of animals | Survival at 120 days after aerogenic challenge* |
|---|---|---|---|
| | | | % |
| Respiratory | $10^7$ | 16 | 94 |
| Respiratory | $10^5$ | 15 | 60 |
| Respiratory | $10^3$ | 17 | 47 |
| Dermal | Acupuncture | 16 | 81 |
| Control | None | 12 | 0 |

* With $10^4$ cells of strain SCHU S4.

immunogenicity for monkey, groups of 15 to 17 *Macaca mulatta* inhaled $10^3$, $10^5$, or $10^7$ cells of live tularemia vaccine strain LVS. Another group of 16 animals received LVS percutaneously by acupuncture; in this case, the actual number of cells introduced is unknown, because a substantial portion of the inoculum remains on the surface of the skin. Vaccination by either procedure proved innocuous, and resulted in comparable peak mean titers except in the aerosol group receiving the lowest dilution of organisms. Of the 17 animals that inhaled $10^3$ organisms, 9 failed to develop agglutinins. The mean titer of the intradermal vaccinees rose earlier and faster than did titers of the aerogenic vaccinees. At 60 days after vaccination, these animals, as well as nonvaccinated controls, were challenged aerogenically with $10^4$ cells of strain SCHU S4 (Table 1). All controls died within 30 days: 120 days after challenge, the per cent survival in the $10^7$, $10^5$, and $10^3$ groups vaccinated aerogenically and in the group vaccinated dermally was 94, 60, 47, and 81, respectively. Because monkeys are less resistant to tularemia than is man, their benign response to aerosolized liquid LVS tularemia vaccine indicated that this vaccine might also be safe for man when administered aerogenically. Initial studies in volunteers indicated (9) that respiratory doses ranging from 200 to 30,000 organisms were innocuous and that approximately 1,500 inhaled cells were required to induce serological conversion consistently. These studies were expanded at the University of Maryland Research Ward at Jessup, Maryland House of Correction, and are the subject of the present report.

## AEROGENIC VACCINATION OF VOLUNTEERS

### *Materials and Methods*

*F. tularensis* LVS and highly virulent challenge strain SCHU S4 (6) were cultivated in a modified casein partial-hydrolysate liquid medium (R. C. Mills et al., Bacteriol. Proc., p. 37, 1949). Cultures, harvested after 16 hr of incubation with continuous shaking at 37 C, contained 35 × $10^9$ to 40 × $10^9$ viable organisms per milliliter. For aerogenic immunization with strain LVS or challenge with strain SCHU S4, aerosols were generated with a nebulizer that produced particles primarily in the range of 1 to 5 $\mu$ diameter. Methodology was comparable to that previously described by Griffith (15).

Prior to aerosolization of LVS for use as a vaccine in man, all available information pertaining to its safety was evaluated. Extensive experience gained in volunteers and laboratory workers at Fort Detrick by the acupuncture route (8) attested to the attenuation of this strain. Serious reactions, such as secondary pneumonitis or bubo formation, were not seen. Immunogenicity was evident from the excellent protection noted in vaccinated volunteers exposed to aerosol or intracutaneous challenge. The aforementioned thorough animal evaluations suggested that no untoward reactions were likely to occur in man after inhalation of LVS.

### *Clinical Reactions*

Five groups totaling 253 volunteers free from tularemia agglutinins were exposed to aerosolized LVS. The dose ranged from $10^4$ to $10^8$ organisms. Reaction rates correlated directly with size of inoculum. After inhaling a dose of $10^4$ LVS cells, about 30% of 42 volunteers had minor systemic complaints. The majority noted minimal upper respiratory symptoms, such as sore throat or slight cough. Practically all had pea-sized cervical nodes after exposure to aerogenic LVS. None had fever or roentgen evidence of pneumonic infiltration. The signs and symptoms were quite insignificant and would have been overlooked with casual examination or questioning.

A more severe reaction was associated with inhalation of a $10^8$ inoculum. As a result of this massive dose, 90% of the volunteers were symptomatic with headache, coryza, chest pain, and malaise. Actually, they had mild typhoidal tularemia. In 80%, there were temperature elevations of >100 F which occurred on the average at 3 days and lasted an average of 2.5 days. Of 42 men receiving this huge dose, 3 were treated with streptomycin, and several others were put to bed for periods of 2 to 3 days. Chest X rays revealed transient infiltrations in a few of the vaccinees. In general, the reaction just described can be likened to a "flu-like" syndrome. This condition did not incapacitate the majority of volunteers; they were able to con-

tinue their prison routine. Similar, but milder reactions, were seen in 79% of volunteers inhaling $10^6$ cells of LVS.

### Serological Response

Results of serological studies have shown the correlation between size of inhaled dose (antigenic mass) and acquisition of serum agglutinins. Volunteers receiving the largest number of organisms developed demonstrable serum antibodies in a surprisingly short time. By the second week postvaccination, 92% of the volunteers had agglutinins, and at 3 weeks a 97% incidence was recorded. This rapid acquisition of serum antibodies following the large inhaled antigenic mass was more rapid than the rate following LVS administered by acupuncture (65% at 2 and 82% at 3 weeks). The preliminary studies with smaller aerogenic inocula revealed a delayed response when compared with the intracutaneous route of vaccination. The results of these series of investigations suggested that large groups of nonimmune people can be immunized more rapidly by the respiratory than the intracutaneous route; however, a high incidence of systemic reactions would result from exposure to large-dose vaccine aerosols. Although there is more rapid seroconversion noted with the latter method (large-dose aerosol), the geometric mean titers were no different after 8 weeks whether vaccination was accomplished by acupuncture or with smaller-dose aerosols.

Conversion rates were reduced and geometric mean agglutinin titers were delayed as the inhaled dose was lowered. After the $10^4$ log dose of LVS, geometric mean titers did not begin to rise significantly until the 3rd week postvaccination, and antibody levels comparable to those associated with acupuncture vaccination occurred between the 4th and 5th weeks (Fig. 1). Both geometric mean titers lagged behind those of the two largest aerogenic vaccine doses. Similarly,

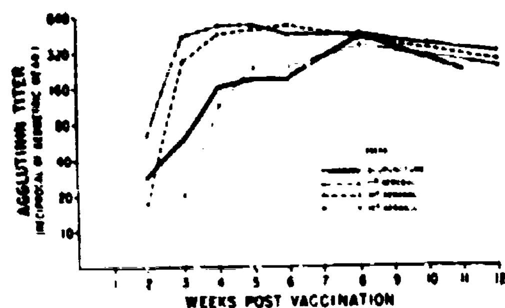


FIG. 1. *Agglutinin response to LVS vaccine administered in varying doses by the aerogenic route compared with response after intradermal inoculation.*

TABLE 2. *Response of volunteers to large-dose respiratory challenge after aerogenic vaccination*

| Interval between vaccination and challenge | No. challenged[a] | No. with fever [illegible] | No. requiring therapy[b] | Per cent protection |
|---|---|---|---|---|
| *months* | | | | |
| 2 | 22 | 15 | 6 | 73 |
| 4 | 30 | 18 | 0 | 100 |
| 6 | 16 | 10 | 0 | 100 |
| 14 | 32 | 26 | 16 | 50 |
| 18 | 2 | 2 | 1 | 50 |
| Summation, 2-18 | 102 | 71 (70%) | 23 (23%) | 77 |
| Controls | 47 | 44 (94%) | 42 (89%) | |

[a] With $2.5 \times 10^4$ organisms, strain SCHU S4.
[b] Criterion for treatment was 103 F or greater for over 24 hr.
[c] Uncorrected with respect to control data.

seroconversion rates peaked at the 90% level 5 weeks postvaccination, compared with 3 weeks. Nevertheless, geometric mean titers eventually reached antibody levels achieved with larger aerosol doses.

## CHALLENGE OF AEROGENIC VACCINEES WITH VIRULENT *F. TULARENSIS*

### Aerogenic Challenge

The presence of circulating tularemia agglutinins is not tantamount to resistance to the disease. It remained, therefore, to evaluate the degree of protection of the volunteers to challenge with virulent *F. tularensis*. Table 2 outlines the results of aerogenic challenge with $2.5 \times 10^4$ organisms. This challenge represents approximately 2,500 times the minimum infective dose for man, which has been estimated to be 10 to 50 organisms (21). This was a severe challenge and probably far exceeds the number encountered during natural exposures. In this experiment, the interval between vaccination and time of challenge did not appear to be a determining factor in the extent of protection. At 2 months 73% of 22 and at 14 months 50% of 32 volunteers exposed developed disease and were treated with antibiotics. (The difference was not significant by the chi square test.) These two groups received the same dose of aerosolized vaccine. Those men challenged at 4 and 6 months received the two highest doses of LVS ($10^8$ and $10^9$), and the subsequent mild vaccine infection may have contributed to the excellent overall resistance of the groups.

Table 3 illustrates the significance of the method of vaccination of these volunteers in relation to

TABLE 3. *Relationship of route and dose of LVS vaccine to resistance to tularemia aerosol challenge*

| Dose | Route | No. challenged | No. (%) with fever (≥100 F)[a] | Per cent requiring treatment[b] | Per cent protection[b] |
|---|---|---|---|---|---|
| $10^8$ | Respiratory | 30 | 18 (60) | 0 | 100 |
| $10^6$ | Respiratory | 16 | 10 (62) | 0 | 100 |
| $10^4$ | Respiratory | 56 | 43 (77) | 41 | 59 |
| | Dermal[c] | 46 | 29 (63) | 46 | 54 |
| | None | 47 | 44 (94) | 89 | |

[a] Mean incubation period: volunteers vaccinated aerogenically or by acupuncture, approximately 4 days; controls, approximately 3 days.
[b] Uncorrected with respect to control data.
[c] Acupuncture technique.

TABLE 4. *Response of volunteers to large dose respiratory challenge after acupuncture vaccination*

| Interval between vaccination and challenge | No. challenged[a] | No. with fever (≥100 F) | No. requiring therapy[b] | Per cent protection[c] |
|---|---|---|---|---|
| *months* | | | | |
| 2 | 5 | 1 | 0 | 100 |
| 4 | 13 | 10 | 4 | 69 |
| 11 | 8 | 6 | 6 | 25 |
| 14 | 19 | 11 | 10 | 47 |
| 36 | 1 | 1 | 1 | |
| Summation | | | | |
| 2-36 | 46 | 29 (63%) | 21 | 54 |
| Controls | 47 | 44 (94%) | 42 (89%) | |

[a] With $2.5 \times 10^4$ organisms, strain SCHU S4.
[b] Criterion for treatment, 103 F per os or greater for over 24 hr.
[c] Uncorrected with respect to control data.

resistance to respiratory challenge. High-grade protection was acquired by the men inhaling $10^8$ or $10^6$ doses of LVS. A somewhat lower grade protection was observed in man immunized aerogenically with $10^4$ LVS or by acupuncture; similar protection resulted in both groups. The incidence of *infection* in all four groups of vaccinees was equivalent (60 to 77%, had fever of 100 F or greater), but the incidence of the disease was quite different. The 28 infected men in the two groups who had received large doses of LVS by the aerogenic route reacted to the initial infectious process developing from the severe respiratory challenge, but the acquired resistance prevented progression to overt disease requiring specific treatment.

The average incubation period for the control subjects was less by 1 day than that of the vaccinees. The shorter incubation period in the controls plus equal incubation time for all vaccinees, acupuncture as well as aerogenic, suggests that respiratory exposure to LVS did not sensitize the lung parenchyma. If a hypersensitivity reaction had occurred in men vaccinated aerogenically, immediate febrile or systemic reactions might have been expected. No evidence of such reaction was observed.

Table 4 presents data accumulated from additional experiments wherein volunteers, immunized by the acupuncture technique, were challenged aerogenically at varying intervals postvaccination. Although the numbers of subjects were small, results were similar to those observed after small-dose aerosol LVS. Immunity waned at about 1 year to the same extent.

Unvaccinated volunteers without demonstrable tularemia agglutinins served as controls in these aerosol challenges. Five of 47 men failed to develop disease. Actually, four men represent these five failures; two were rechallenged and developed pneumonic tularemia, a third was reexposed on two additional occasions before disease was induced, and the fourth has not been rechallenged. Each appeared to be a complete "miss" at time of exposure without subsequent subclinical infection, because antibodies were not demonstrable. Mechanical difficulties, i.e., loose-fitting masks, were implicated as the significant reasons for failure to produce disease and not natural host resistance, because of susceptibility to rechallenge. Similar incidents may also have occurred in the exposed vaccinees. The low frequency of "misses" and presumed equal distribution would not invalidate the per cent protection observed in the challenged vaccinees.

### *Intradermal Challenge*

Small numbers of volunteers receiving vaccine by the respiratory route have been challenged by the intradermal inoculation of 1,000 to 10,000 infectious doses per man of SCHU S4 strain (Table 5). Protection was excellent. Not only was there no evidence of lessened immunity after 6 months, but also resistance to massive challenges was uniform. The disease rates were comparable to those following challenge of volunteers vaccinated by acupuncture. The clinical appearance of inoculation sites was strikingly different from the lesions in controls. The skin lesion resembled a delayed hypersensitivity reaction in the immune individual; control

TABLE 5. *Response of vaccinated volunteers to intradermal challenge*

| Type of vaccination | Challenge dose (organisms) | Interval between vaccination and challenge | No. challenged | No. requiring therapy[a] | Number protected/total | Controls requiring therapy/total |
|---|---|---|---|---|---|---|
| | | *months* | | | | |
| Acupunture | $3 \times 10^{[illegible]}$ | 6 | 10 | 1 | 9/10 | 10/10 |
| | $8 \times 10^{3}$ | 6 | 8 | 1 | 7/8 | 1/1 |
| | $1 \times 10^{4}$ | 2 | 3 | 1 | 2/3 | 2/2 |
| | $1 \times 10^{6}$ | 2 | 3 | 1 | 2/3 | |
| Total | $3 \times 10^{[illegible]}$–$1 \times 10^{[illegible]}$ | 2–6 | 24 | 4 | 20/24 (83%) | |
| Aerogenic | $1 \times 10^{[illegible]}$ | 2 | 7 | 0 | 7/7 | – |
| | $1 \times 10^{[illegible]}$ | 6 | 4 | 0 | 4/4 | 4/4 |
| | $1 \times 10^{[illegible]}$ | 2 | 3 | 2 | 1/3 | – |
| Total | $1 \times 10^{[illegible]}$–$1 \times 10^{[illegible]}$ | 2–6 | 14 | 2 | 12/14 (86%) | |

[a] Criterion for treatment, development of a typical ulceroglandular infection similar to that in controls.

subjects showed progressively developing ulcers. Based on this small experience, it appears that aerosolized LVS produces effective immunity to ulceroglandular tularemia.

### *Relationship of Antibody Titers to Immunity*

Analysis of agglutinating antibody titers in the vaccinees suggests that higher levels were associated with less severe illness, and groups of inmates requiring treatment had baseline geometric mean titers one-half the value of those groups not treated. On the other hand, absence of disease in the respiratory challenge group which received the massive dose of aerogenic LVS cannot be explained solely on the basis of elevated titers. Geometric mean titers in this group were equivalent to those of the other aerogenically vaccinated groups. Challenge results were quite different; 6 of 22 men had disease when exposed 2 months after small-dose aerosol vaccination, but none of 30 men had disease following challenge at 4 months after large-dose vaccine aerosol. Thus, although absolute level of agglutinins cannot be correlated with immunity, presence of these antibodies in the sera of volunteers exposed to virulent challenge suggests that members of the group will resist infection to a greater degree than unvaccinated controls.

## DISCUSSION

Immunization of man against tularemia can be accomplished safely by employing aerosolized living attenuated vaccine. The dose necessary to ensure development of serum antibodies in at least 90% of volunteers is $10^4$ organisms. Systemic subjective reactions at this dose were not significant, and close clinical observation was necessary to reveal subtle objective findings, i.e., appearance of pea-sized cervical lymph nodes.

The inhaled dose can be increased without undue risk if more rapid induction of antibodies is desired. As many as $10^8$ organisms have been delivered to volunteers as an immunizing dose. Low-grade febrile disease occurred in more than 90% of the volunteers with this dose. However, the reaction was mild and self-limiting, and did not interfere with the daily routine of most inmates. After this vaccination, a high-grade immunity was observed against a severe aerogenic challenge conducted 4 months after vaccination. Assurance is provided, thereby, that, even if unlikely dilution errors would create such concentrated aerosols, exposed healthy young adults would experience only mild discomfort. Acceptability of this aerosolized antigen is questionable in people with chronic lung disease, congestive failure, or other diseases affecting the integrity of respiratory defense mechanisms. Perhaps small doses of aerosolized LVS could be tolerated in such patients. Sufficient evidence bearing on this point is unavailable.

These studies validate the respiratory route as a means of introducing an attenuated bacterium into the human host. It remains to be determined whether this route is more advantageous than the conventional dermal site. Aerosolized vaccine does lead to an immune state. The incidence of disease after challenge of volunteers vaccinated by this method was less than that recorded in the men challenged after immunization by acupuncture. This difference occurred primarily in groups that received the larger doses of aerosolized LVS. These men had mild tularemia after vaccination, and the virulent challenge can

almost be considered a rechallenge. This type of immunization provoked more resistance to infection. Circulating antibodies alone are not sufficient to explain differences in protection; the geometric mean titers were identical for the aerogenic vaccine groups challenged at 2 and 4 months, respectively; yet, disease rate was greater in the former. Perhaps the lower disease rate results from the ability of lung tissue previously exposed to LVS to confine better the inhaled pathogens through better phagocytosis, tissue antibody effect, or other local defense mechanisms. Thus, it seems reasonable to expect that in respiratory-acquired infectious diseases prior vaccination with sufficient antigen given by the aerogenic route will produce increased host protection. Present evidence is insufficient to allow conclusions regarding the protection afforded aerogenically vaccinated individuals against the ulceroglandular form of tularemia. Following the reasoning above, the acupuncture method should be the best way to prevent this disease. The differences in distribution of vaccine by the two routes into the two organs initiates dissimilar reactions for developing local tissue defense. Therefore, analogous reasoning cannot be applied to the skin.

One disadvantage of the aerogenic vaccination technique is the lack of a "marker" indicating vaccine reaction. The scar from the acupuncture route is obvious for weeks. Nevertheless, the need for visible evidence of reaction to vaccine is lessened when over 90% of an exposed population are immunized by simple inhalation of LVS. In addition, serological proof of vaccination is easily obtained.

The elaborate exposure equipment used in these studies allowed for precision in uniformity of particle size and quantitation of the inhaled dose. The application of aerosolized vaccines on a mass basis will require simple, less complicated apparatus. Efforts to create such instruments should be encouraged. Soviet literature contains reference to mass aerogenic vaccination of troops exposed in tents (6). Vaccination by the respiratory route for tularemia is effective, and this fact should serve as an impetus for future experimental studies with viral and bacterial vaccines.

## SUMMARY

Live, attenuated LVS tularemia vaccine has been administered via the respiratory route in doses ranging from $10^4$ to $10^8$ organisms. Mild self-limiting typhoidal tularemia was induced by doses of $10^6$ to $10^8$ vaccine organisms. Rapidity of induction of agglutinin titers in the human host varies directly with size of inhaled inoculum. Immunity to aerogenic virulent *F. tularensis* challenge appeared to be greater than that produced by the conventional acupuncture method of vaccine administration. Protection against ulceroglandular tularemia was also demonstrated. The pulmonary tree in man can be safely and successfully utilized for application of *F. tularensis* strain LVS and possibly for other microorganisms.

## ACKNOWLEDGMENTS

Volunteers for these studies were well informed and willing inmates at the Maryland House of Correction. Their patience and courage, so generously displayed throughout these studies, is gratefully acknowledged. Appreciation is expressed to W. R. Griffith of Fort Detrick who supervised the aerosol exposures.

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# Respiratory Antibody to *Francisella tularensis* in Man

E. L. BUESCHER AND J. A. BELLANTI

*Department of Virus Diseases, Walter Reed Army Institute of Research, and Department of Pediatrics, Georgetown University School of Medicine, Washington, D.C.*

For several years my colleagues, J. Bellanti and M. Artenstein, and I have studied the occurrence of specific antibodies in secretions of the respiratory tract, and have attempted to evaluate their biological significance (2, 3, 3a). This interest stemmed from the need for a simple biological marker to identify persons most likely to resist overt respiratory infection with any of several viruses. As with respiratory tularemia, the presence or titer of *humoral* antibodies to respiratory viruses is *not* synonymous with resistance to clinical disease upon infection. When these investigations were begun, it was our purpose to identify such markers. We chose to reinvestigate the occurrence of local antibody in respiratory secretions by use of more modern virological and immunological methods. Such antibodies were indeed found in nasal secretions of normal individuals. Not every individual possesses antibody to each respiratory virus; rather, detectable antibodies occur in patterns which varied from person to person (2, 3). Although there is no doubt that local antibody exists in the respiratory tract, little is known of its influence upon the pathogenesis of respiratory infections. Recently, we studied respiratory antibody to *Francisella tularensis* in man; these observations are pertinent to the questions raised by Drs. Hornick and Eigelsbach concerning effectiveness of aerosol immunization against respiratory tularemia.

Last year, with H. Dangerfield and D. Crozier of the Medical Unit, Fort Detrick, Frederick, Md., we studied respiratory antibody in 14 volunteers before and after aerosol infection with virulent *F. tularensis* (SCHU-S4 strain); this investigation will be reported in detail elsewhere. Eight volunteers were immunized percutaneously 3 months previously with LVS (tularemia vaccine, live attenuated) vaccine containing approximately $10^8$ viable LVS cells per 0.1 ml. Six served as susceptible controls. One-half of each group was challenged by aerosol containing approximately 2,500 organisms; the other, with 25,000 cells. Nasal secretions were collected from these individuals by previously described methods (2) twice daily for 3 days before and for 5 days after challenge, and at weekly intervals thereafter for 6 weeks. Daily collections of nasal washings from each volunteer were pooled, concentrated approximately 10-fold by lyophilization after dialysis against distilled water, and studied for hemagglutinating antibody to polysaccharide prepared from the SCHU-S4 strain. Antibody determinations were made by the method of Alexander (1) modified for microtiter technique. Hemagglutinating antibody was measured because it is more readily detected in higher titers than are cell agglutinins (4).

Nasal antibody was indeed detected 3 months after percutaneous immunization, prior to challenge by aerosol infection (Table 1). Titers of nasal antibody ranged from 1:2 to 1:32 per 0.05 ml of concentrated nasal washing, and, for the most part, were significantly lower than those observed simultaneously in serum. There was no clear correlation between titers of antibody in serum with those found in secretions (Table 2), although too few individuals were studied to make absolute comparisons. However meager, the

TABLE 1. *Occurrence of serum and nasal antibody in eight persons to Francisella tularensis 3 months after percutaneous immunization*

| Determination | Antibody[a] titer | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 or > |
| Nasal washings | 3[b] | 1 | 2 | 1 | 1 | | | | |
| Serum | | | 1 | | | | | 2 | 5 |

[a] Reciprocal per 0.05 ml.
[b] Results expressed as number of persons with indicated titer.

TABLE 2. *Correlation between serum and nasal antibody titers 3 months after percutaneous immunization*

| Nasal antibody[a] | Serum antibody[a] | | | | |
|---|---|---|---|---|---|
| | 8 | 256 | 512 | 1,024 | 2,048 |
| 2 | • | • | • | | |
| 4 | | | | • | |
| 8 | | | • | | • |
| 16 | | | | • | |
| 32 | | • | | | |

[a] Hemagglutinin per 0.05 ml. At serum antibody dilution of 1:16 through 1:128, no hemagglutination occurred.

TABLE 3. *Development of nasal and serum antibody after aerosol infection with Francisella tularensis*

| Challenge dose | Patient no. | Determination | Titer[a] preexposure | Titer[a] at indicated day postexposure: 1 | 3 | 7 | 14 | 21 | 42 |
|---|---|---|---|---|---|---|---|---|---|
| ±2,500 cells | 1 | Serum | <2 | — | — | <2 | 256 | 1,024 | — |
| | | NW[b] | <2 | <2 | <2 | 2 | 16 | 32 | 32 |
| | 2 | Serum | <2 | — | — | 2 | 32 | 4,096 | 16,384 |
| | | NW | <2 | <2 | <2 | <2 | 2 | 128 | — |
| ±25,000 cells | 3 | Serum | <2 | — | — | <2 | 64 | 128 | 256 |
| | | NW | <2 | <2 | <2 | <2 | 8 | 8 | 16 |
| | 4 | Serum | <2 | — | — | <2 | 64 | 512 | 512 |
| | | NW | <2 | <2 | <2 | <2 | 4 | 16 | 8 |

[a] Per 0.05 ml of serum or nasal washing.
[b] NW = nasal washing.

data suggest that detectable nasal antibody occurred in persons with serum antibody titers of 1:256 or greater. The quantitative relationships between titers of nasal and serum antibodies remain to be determined.

Susceptible volunteers, when exposed to aerosols containing either 2,500 or 25,000 living cells, similarly developed nasal antibody (Table 3). Antibody was detected as early as 7 days after exposure (patient no. 1), was regularly present at 14 days, and increased in titer to levels essentially similar to those observed in percutaneously immunized personnel 3 months after vaccination (1:8 to 1:32), except for patient no. 2, whose nasal antibody titered 1:128 on the 21st day after infection. Again, titers were significantly lower than those observed simultaneously in serum. Each of the four individuals experienced respiratory tularemia, and was treated with antibiotics in the conventional fashion (6). Thus, it is clear that, irrespective of the method for infection, human beings develop nasal antibody to *F. tularensis*.

This hemagglutinating antibody of nasal secretions was found to be associated primarily with γA immunoglobulin components. Antibody-bearing secretions from each of two individuals, either the result of immunization or infection, were absorbed with goat antisera against human γA and γM immunoglobulins (Table 4). Absorption with antihuman γA immunoglobulin removed all hemagglutinin from each secretion; in contrast, absorption with antihuman γM immunoglobulin failed to remove significant amounts of antibody. Further, nasal antibody appeared to be significantly different from that of serum in the same individuals (Table 5). When high titered postimmunization or postinfection sera and nasal washings were subjected to gel filtration (Sephadex G-200), patterns of eluted

TABLE 4. *Removal of hemagglutinin from nasal secretions by specific absorption*

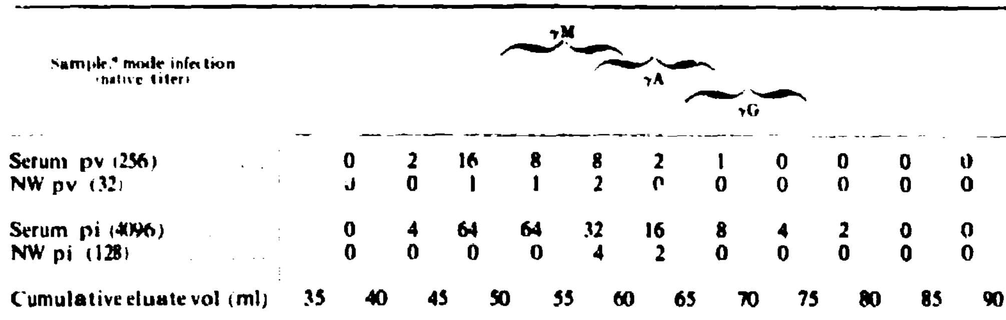
| Antibody induced by | Subject | Antibody titers after absorption with: Nothing | γM | γA |
|---|---|---|---|---|
| Immunization | 1 | 8 | 4 | <2 |
| | 2 | 8 | 8 | <2 |
| Infection | 1 | 8 | 4 | <2 |
| | 2 | 8 | 2 | <2 |

hemagglutinin differed between serum and secretions. The majority of antibody activity in serum was associated primarily with the γM immunoglobulins, whereas nasal antibody was found primarily in eluates containing γA immunoglobulins, and this pattern was the same after either percutaneous or aerosol infection.

These observations show that there is no significant difference in the nature of local or humoral distribution of hemagglutinin to *F. tularensis* between persons infected percutaneously or by the respiratory route. If this antibody in any way reflects resistance to overt infection (and there certainly are reasons to question this assumption), it may be properly concluded that such differences as might be effected by varying the route of vaccine administration would be only chronological. Hornick and Eigelsbach showed that the humoral antibody response following aerosol immunization is more rapid than the response to percutaneous vaccination (5). Whether local antibody appears in the respiratory tract less rapidly after percutaneous immunization is, of course, unknown, but is readily subjected to test in percutaneously immunized volunteers.

Even if respiratory antibody appears more

TABLE 5. *Partition of nasal and serum antibodies to Francisella tularensis by Sephadex gel filtration*




| Sample,ᵃ mode infection (native titer) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum pv (256) | 0 | 2 | 16 | 8 | 8 | 2 | 1 | 0 | 0 | 0 | 0 |
| NW pv (32) | 0 | 0 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Serum pi (4096) | 0 | 4 | 64 | 64 | 32 | 16 | 8 | 4 | 2 | 0 | 0 |
| NW pi (128) | 0 | 0 | 0 | 0 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |
| Cumulative eluate vol (ml) | 35 / 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 |

ᵃ NW = nasal washing. The serum samples both contained immunoglobulin components γM, γA, and γG; the NW samples contained γA and γG.

promptly after aerosol immunization, there are few circumstances which demand this extraordinarily prompt immune response. Further, it is clear that, despite the presence of respiratory antibody, the immunity induced by any method of immunization can be overwhelmed by challenge with more than 10,000 virulent cells. Finally, Hornick's experience shows that administration of LVS vaccine by aerosol is not without risk of reaction (5). Indeed, to obtain optimal protection for up to 6 months, it appears necessary to administer over $10^8$ to $10^9$ viable vaccine cells. Approximately 80% of those receiving these doses of vaccine had, as a reaction, overt but mild respiratory tularemia. This appears to be a greater price for an additional short interval of immunity than we would be willing to pay.

Finally, it is clear that this experimentation is seriously limited by the lack of a good reproducible marker for immunity (resistance to overt infection). It is not now possible to evaluate local antibody as a marker for immunity to *F. tularensis*, although in one other respiratory infection there appears to be a good correlation between presence of respiratory antibody and resistance to infection. Experiments with parainfluenza virus type 1 in man show that persons with nasal neutralizing antibody are more resistant to challenge infection than are those without, irrespective of their humoral antibody status (Smith et al., New Engl. J. Med. *in press*). Thus, recent experimental evidence strongly suggests that detailed analysis of respiratory secretions may well provide better markers for immunity to respiratory infections. This experimental approach is not technically difficult today, and should be extended further into the problem at hand.

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# Antibiotic Prophylaxis and Therapy of Airborne Tularemia

WILLIAM D. SAWYER,[1] HARRY G. DANGERFIELD, ARTHUR L. HOGGE, AND DAN CROZIER

*U. S. Army Medical Unit, Fort Detrick, Frederick, Maryland*

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## INTRODUCTION

Streptomycin was the first effective antibiotic for the therapy of tularemia, and remains the drug of choice (1, 14, 21). Alternatives are needed, however, because of (i) the possibility of infection by streptomycin-resistant *Francisella tularensis* (11), (ii) the need for injection of streptomycin with the attendant inconvenience and discomfort, and (iii) the toxicity of streptomycin. Of the many other antibiotics active against *F. tularensis*, the best evaluated and most frequently used are the tetracyclines and chloramphenicol (2, 9, 11, 12, 20). (Because chloramphenicol offers no advantages over the tetracyclines in the treatment of tularemia and has significant toxicity, only the tetracyclines will be considered hereafter except in reviewing earlier work.) Patients with acute tularemia respond well to therapy with either streptomycin or tetracycline; symptoms rapidly remit, and defervescence is prompt (Fig. 1). The late consequences of treatment with the two antibiotics differ, however. Relapses rarely follow exhibition of reasonable doses of streptomycin but occur frequently after therapy with conventional regimens of tetracycline (2, 11). Such relapses result from the persistence of bacteria in the tissues, not the emergence of tetracycline-resistant organisms; retreatment with tetracycline is effective (Fig. 1).

In addition to their use in the management of tularemia, antibiotics may be employed for prophylaxis, used here to mean treatment instituted during the incubation period to prevent illness. Results have been similar to those achieved in the therapy of acute disease: streptomycin prevents illness, but broad-spectrum drugs merely delay disease. McCrumb et al. (9), for example, consistently protected volunteers by administration of streptomycin for 5 days after intradermal inoculation with *F. tularensis*, whereas only two of five volunteers were protected from tularemia by 5 days of prophylactic treatment with chloramphenicol.

Comparison of their actions against *F. tularensis* in vitro may help to explain the difference in effectiveness of streptomycin and tetracycline in both the prophylaxis and therapy of tularemia. Streptomycin is bactericidal in vitro, and may eradicate the organisms without the intervention of host mechanisms. Tetracycline, even in high concentration, merely suppresses multiplication; organisms persist in the tissues until destroyed by host defenses. *F. tularensis*, like other intracellular pathogens (5,17), is cleared from the cells slowly even when multiplication is prevented, e.g., by a bacteriostatic antibiotic. The relative inefficiency of host defense against *F. tularensis* is a crucial factor in determining the effectiveness of prophylaxis and therapy of tularemia with bacteriostatic agents. In the Conference on Airborne Infection held in 1960, McCrumb cited the imperfect results achieved with bacteriostatic drugs and suggested that either prolonged or intermittent treatment might be required if they were to be completely effective (10). The success of such regimens in other intracellular infections, e.g., scrub typhus (8, 16) and Q fever (18, 19), prompted the present studies of tetracycline prophylaxis and therapy of experimental airborne tularemia in *Macaca mulatta* and man.

[1] Present address: Department of Microbiology, The Johns Hopkins University School of Medicine, Baltimore, Md.

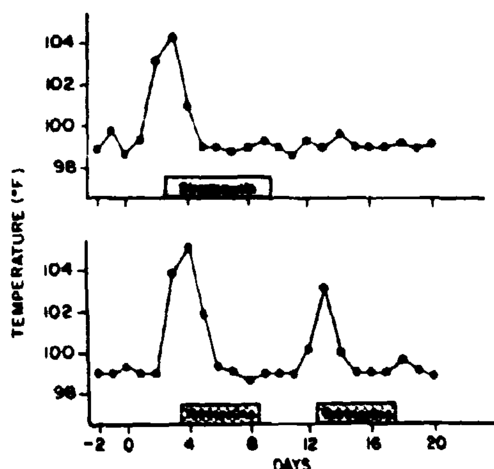


FIG. 1. *Therapy of experimental human airborne tularemia with streptomycin and with tetracycline. Doses were: streptomycin, 1 g twice daily; and tetracycline, 0.5 g four times daily.*

## TETRACYCLINE PROPHYLAXIS

### *Simian Tularemia*

Monkeys were exposed to aerosols of *F. tularensis* SCHU-S4 generated in a modified Henderson apparatus (3, 4, 7). [Healthy young adult *M. mulatta*, weighing 3 to 6 kg, were obtained from the Animal Farm, Fort Detrick, Md. Pre-exposure sera did not contain *F. tularensis* agglutinins. Cultures of *F. tularensis* were kindly supplied by H. T. Eigelsbach. They were grown in modified casein hydrolysate medium (Mills et al., Bacteriol. Proc., p. 37, 1949) for 16 hr with continuous shaking at 37 C, and were stored at 4 C until used. The SCHU-S4 strain is sensitive to streptomycin.] The average inhaled dose was 10,000 organisms, a quantity regularly resulting in an acute fatal illness after a short incubation period (Fig. 2). [In addition to twice daily examination and thermometry, serum C-reactive protein was determined and a chest X ray was obtained at weekly intervals (or more frequently upon request of the attending veterinarian). Fever (rectal temperature > 40 C) was the principal criterion of illness.] The results of five schedules of tetracycline prophylaxis are shown in Table 1. In all schedules, the initial dose of drug was given 24 hr after exposure, and prophylactic treatment lasted for 13 days. Illness was suppressed in 10 of the 11 animals receiving the antibiotic at 24- or 36-hr intervals; an unrelated, intercurrent illness cannot be excluded in the one exception. When the interval between doses was increased beyond 36 hr, however, the animals experienced one or more febrile episodes during the treatment period. Because tetracycline administered at 48-hr intervals failed to suppress disease, a different sort of interrupted schedule was tried, i.e., 3-day treatment periods alternating with 2 day periods without drug. Four of the six monkeys were ill during the prophylactic period. Frequent administration of tetracycline, therefore, appeared necessary to limit multiplication of *F. tularensis* so that the infection remained subclinical during the treatment period.

After completion of all of the prophylactic regimens, most of the monkeys became ill (Table 1). Clearly, *F. tularensis* had remained viable in the host tissues throughout the period (13 days) of antibiotic administration. That the duration of persistence could be quite prolonged was demonstrated in another group of monkeys which received tetracycline daily for 6 weeks. Monkeys tolerated prolonged tetracycline treatment well, i.e., weight was maintained and no illnesses

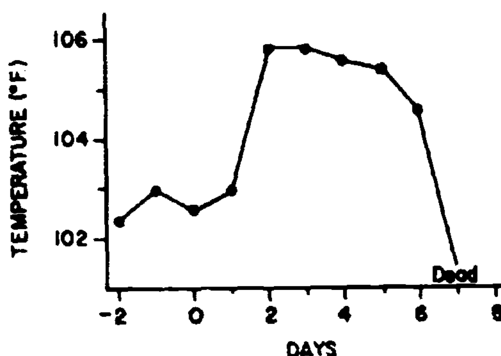


FIG. 2. *Course of fever in experimental simian airborne tularemia.*

TABLE 1. *Tetracycline prophylaxis of airborne tularemia in Macaca mulatta*[a]

| Dosage interval | No. of doses | No. of monkeys | No. ill during treatment | No. ill after treatment | No. of deaths |
|---|---|---|---|---|---|
| *hr* | | | | | |
| 24 | 13 | 5 | 1 | 5 | 0 |
| 36 | 9 | 6 | 0 | 4 | 1 |
| 48 | 7 | 6 | 6 | 6 | 1 |
| 72 | 5 | | 6 | 6 | 0 |
| Intermittent[b] | 9 | 6 | 4 | 2 | 0 |

[a] Each animal received 200 mg of tetracycline intragastrically beginning on day 1 and continued over a period of 13 days. Six of six untreated animals developed fatal tularemia.

[b] Days 1 to 3, 6 to 8, 11 to 13.

attributable to the drug or to "superinfection" were detected. All remained well throughout the treatment period, but two of the six animals developed acute tularemia within 6 days of the last dose of drug. Because rigid precautions were taken to prevent re-exposure to *F. tularensis*, e.g., cross-infection, accidental laboratory aerosol, etc. (6, 7), these illnesses are believed to have resulted from organisms which were not eliminated during the 42 days of tetracycline treatment.

Even with treatment once a day, tissue levels of tetracycline undoubtedly fluctuated considerably, and, when levels were lowest, the organisms might have undergone several cycles of multiplication without yielding a bacterial mass sufficient to produce illness. This seemed unlikely, because agglutinins did not develop in monkeys who remained well during the course of daily prophylaxis (Fig. 3). When these animals became ill after cessation of treatment (see above), agglutinin promptly appeared. Agglutinin titers increased early in monkeys receiving prophylaxis which failed to suppress illness.

Although prophylactic treatment of tularemia with tetracycline failed to prevent illness, it reduced the severity of the disease. Whereas all of the six untreated monkeys died of tularemia, only two of those in the several prophylaxis groups expired within 70 days of exposure, the duration of observation (Table 1).

Because the timing of the institution of treatment may have important bearing on the effectiveness of prophylaxis of intracellular infection [e.g., tetracycline prophylaxis of Q fever merely delays illness if instituted early but is preventative when begun during the last half of the incubation period (18, 19)], initiation of prophylactic treatment was delayed until 60 hr after exposure to *F. tularensis* in one group of monkeys. The results were no better than those obtained with earlier treatment (Table 2). Further delay in initiating prophylaxis was not feasible, because most monkeys became ill between 60 and 72 hr after exposure.

TABLE 2. *Tetracycline prophylaxis of airborne tularemia in Macaca mulatta - Delayed institution of treatment*[a]

| Treatment instituted (hr postexposure) | No. of monkeys | No. ill during treatment | No. ill after treatment | No. of deaths |
|---|---|---|---|---|
| 24 | 5 | 1 | 5 | 0 |
| 60 | 6 | 1 | 6 | 2 |

[a] Each animal received 200 mg intragastrically once daily for 13 doses.

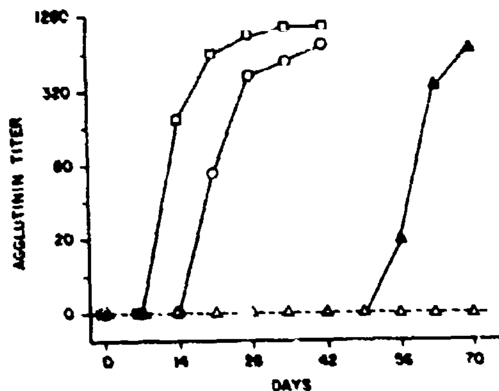


FIG. 3. *Mean Francisella tularensis agglutinin titers of Macaca mulatta receiving tetracycline prophylaxis for airborne tularemia. Symbols:* □ *drug every 48 hr, 6 doses;* ○ *drug daily, 13 doses;* △, *drug daily, 42 doses, animals remaining well;* ▲, *drug daily, 42 doses, animals becoming ill after treatment.*

*Human Tularemia*

The failure of prolonged tetracycline prophylaxis to prevent simian airborne tularemia results from the limited defenses of *M. mulatta* against *F. tularensis*. Although man is quite susceptible to infection with airborne *F. tularensis*, he has better defense mechanisms than *M. mulatta*; e.g., the human respiratory infectious dose is about three times that of monkeys (15), and untreated airborne tularemia has a mortality of less than 50% in man (13) but is usually fatal in monkeys. It seemed likely, therefore, that prophylactic regimens of tetracycline which were only partially successful in monkeys might succeed in man.

The results of trials in volunteers who inhaled 25,000 *F. tularensis* SCHU-S4 confirmed the prediction (Table 3). [Healthy young Seventh Day Adventist soldiers participated on a voluntary basis; they were informed of the nature of the studies prior to volunteering (Army Regulation 70-25, Use of volunteers as subjects of research). The men were observed closely in the hospital before and after exposure. Sera obtained prior to participation did not contain *F. tularensis* agglutinins. The volunteers were examined at least twice daily, and their rectal temperature was recorded every 6 hr. Blood count, erythrocyte sedimentation rate, and serum C-reactive protein were determined and a chest X ray was obtained weekly (more often during periods of illness). Fever (rectal temperature >37.8 C), unassociated with signs of a disease other than tularemia, was the principal criterion of illness.

TABLE 3. *Tetracycline prophylaxis of human airborne tularemia (treatment instituted 24 hr after exposure)*

| Daily dose* | Frequency | Duration | No of subjects | No ill during treatment | No. ill after treatment |
|---|---|---|---|---|---|
| g | | days | | | |
| 1 | Daily | 15 | 10 | 0 | 2 |
| 1 | Daily | 28 | 8 | 0 | 0 |
| 2 | Daily | 14 | 8 | 0 | 0 |
| 1 | Every 2nd day | 19 | 8 | 2 | 8 |

* Divided into morning and evening doses.

Volunteers who developed disease after completion of an experimental schedule of tetracycline were promptly treated with streptomycin, 1 g each 12 hr for 14 doses. All recovered quickly without complications or sequelae. Aerosols of *F. tularensis* were created in a modified Henderson apparatus (3, 4). The men inhaled through the nose and exhaled through the mouth.] All control subjects developed acute tularemia between 2 and 7 days after exposure. (These men participated in studies of therapy; see below). Administration of 1 g of tetracycline each day, beginning 24 hr after exposure, completely suppressed illness during the treatment period, but when treatment was stopped after 15 days, 2 of 10 volunteers developed acute tularemia. Extension of treatment to 28 days prevented illness. Complete protection was also achieved by administration of 2 g of tetracycline daily, even though treatment was terminated after 14 days. Intermittent drug administration, i.e., every other day, failed to protect the volunteers. The pattern of agglutinin response was consistent with the clinical effectiveness of prophylactic therapy, i.e., titers were high (1:1,280) in subjects after overt illness but negative or low (1:80 or less) in the men who remained free from disease.

In contrast to the results in *M. mulatta*, the human studies showed that satisfactory prophylaxis of airborne tularemia could be achieved with tetracycline, the simplest and shortest regimen being 2 g of drug daily for 14 days. With this schedule, disease was completely suppressed both during and after the treatment period; *F. tularensis* agglutinins either did not appear or developed only in low titer.

### TETRACYCLINE THERAPY

The initial objective in the therapy of acute tularemia is the rapid relief of clinical manifestations, an objective readily accomplished with bacteriostatic drugs (see above). Thereafter, the problem is the same as that in prophylaxis—the suppression of multiplication for sufficient time for host mechanisms to eradicate the microorganisms. The major difference, then, in the two situations is the extent of microbial multiplication, and supposedly the degree of stimulation of defense mechanisms, prior to initiation of treatment. Therefore, after control of clinical illness, therapeutic regimens similar to those found effective in prophylaxis should result in a negligible relapse rate, even if therapy is instituted early in the course of disease.

Volunteers exposed to 25,000 airborne *F. tularensis* (see above) became acutely ill after a mean incubation period of 3 days (range of 2 to 7 days). The onset of illness was gradual in 15% of subjects, and a biphasic course was occasionally observed. Treatment was instituted early, within 48 hr of initial signs of illness in 85% of the men, and in no case later than the 5th day after initial signs. Large doses of tetracycline were administered during the first 24 hr, i.e. 1 g every 6 hr, to insure high initial blood levels, and the daily maintenance quantity was administered thereafter in four equal doses.

During the initial phases of evaluation of tetracycline therapy, intermittent treatment schedules were examined. Therapy consisting of three five-day courses of tetracycline (0.5 g every 6 hr) separated by 3 days without drug was efficacious (Fig. 4); the patients responded rapidly and remained well thereafter. These results led

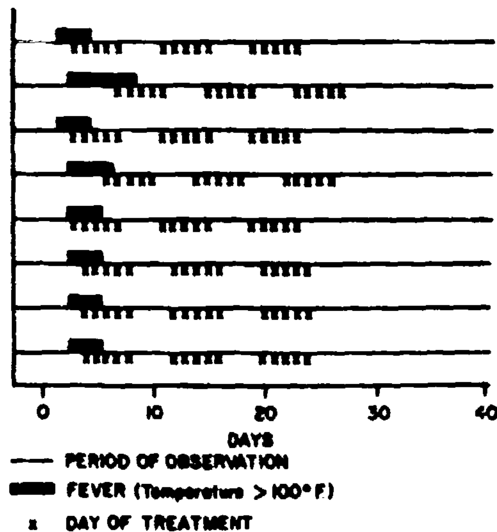


FIG. 4. *Interrupted tetracycline therapy of human airborne tularemia.*

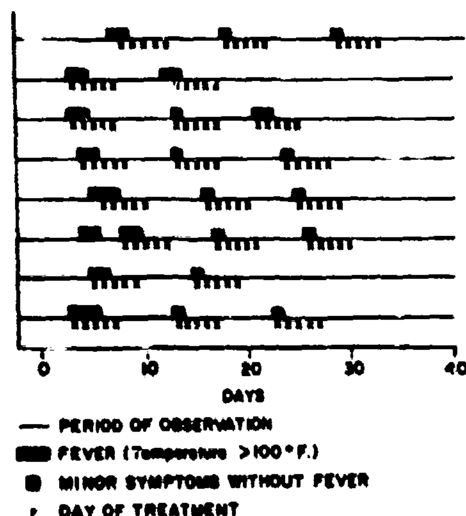


FIG. 5. *Interrupted tetracycline therapy of human airborne tularemia.*

to an attempt to reduce the number of treatment periods. After the initial 5 days of therapy, no additional drug was given to another group of volunteers until ordered by the ward physician, who was instructed to institute treatment at the first sign, no matter how equivocal, of a recurrence. The results were unsatisfactory (Fig. 5). In most instances, the recurrences were so rapid in onset that the men were disabled by the time treatment was effectively instituted; i.e., the time between recognition of possible recurrence and achievement of effective levels of drug in the patient exceeded the time required for the illness to progress from well-being to disability. Moreover, three courses of treatment were administered in 6 of the 8 subjects. It seemed, therefore, that there was little likelihood of developing effective interrupted treatment schedules either employing substantially less drug or of shorter duration than that originally evaluated. Later studies (see below) indicated that continuous tetracycline therapy with similar quantities of drug was equally effective; interrupted therapy, therefore, did not offer any advantage over the simpler continuous treatment schedule.

Prompt clinical improvement was achieved with the continuous therapeutic regimens listed in Table 4. Treatment with 22 g of tetracycline in 10 days resulted in a high incidence of relapse. The same daily dose continued through 15 days was not, however, followed by relapse in any of the 20 patients, 12 infected with the SCHU-S4 strain and 8 infected with the SCHU-S5 strain. The SCHU-S5 strain differs from SCHU-S4 strain only in resistance to streptomycin, SCHU-S5 resisting more than 1,000 μg/ml. When the daily dose was halved, two of eight men had a relapse after 15 days of therapy.

As predicted, the simplest and most successful tetracycline regimen for prophylaxis was very similar to the best therapeutic regimen, i.e., 1 g of tetracycline twice daily for 14 days compared with 4 g of tetracycline the 1st day followed by 0.5 g four times daily for 14 additional days. From a practical standpoint, it would be desirable to have a single schedule of tetracycline administration for both prophylaxis and therapy of airborne tularemia. Therefore, six volunteers with acute illness were treated exactly according to the schedule found successful for prophylaxis; all recovered rapidly and remained well. Thus, a simple schedule of tetracycline treatment was effective in both prophylaxis and therapy of human airborne tularemia; that schedule was 1 g of tetracycline twice daily for 14 days. Because this treatment schedule was suitable for infections induced by exposure to a large number of organisms, the regimen should be satisfactory over the entire range of exposure encountered either in nature or in laboratory accident.

## OTHER ANTIBIOTICS

For infection with streptomycin-sensitive *F. tularensis*, the clinician has a choice of effective antibiotics, particularly streptomycin and tetracycline. In cases of infection by streptomycin-resistant organisms, effective alternatives to tetracycline are needed. Therefore, a number of antibiotics active against *F. tularensis* SCHU-S5 (streptomycin-resistant) in vitro have been evaluated in the therapy of airborne infection of monkeys with the SCHU-S5 strain (Table 5). The inhaled dose was 10,000 organisms; 12 control monkeys became ill within 72 hr and died between the 7th and 15th day after exposure. Therapy was started early, i.e., after 12 hr with a

TABLE 4. *Tetracycline therapy of human airborne tularemia*

| Daily dose[a] | Days of therapy | No. of subjects | No. with relapse |
|---|---|---|---|
| g | | | |
| 2 | 10 | 11 | 5 |
| 2 | 15 | 20[b] | 0 |
| 1 | 15 | 8 | 2 |

[a] All men received 4 g of drug the 1st day of therapy. Daily dose was given at 6-hr intervals.

[b] Twelve men infected with the SCHU-S4 strain and eight with the SCHU-S5 strain of *Francisella tularensis*.

TABLE 5. *Antibiotic therapy of Macaca mulatta infected with Francisella tularensis SCHU S5*

| Antibiotic | Daily dose[a] | No. of monkeys | No. with slow re-[b] | No. with |
|---|---|---|---|---|
| | mg | | | |
| Tetracycline | 225 | 8 | 1 | 8 |
| Kanamycin | 90 | 8 | 2 | 0 |
| Novobiocin | 135 | 6 | 1 | 5 |
| Gentamicin | 9 | 8 | 8 | 3 |

[a] Divided into three doses. Therapy was continued for 7 days or until the animal was afebrile for 72 hr whichever was longer.

[b] An animal was classified as having a slow response if more than 72 hr of treatment were required before it became afebrile.

temperature >40 C or a single temperature of 41 C or greater. Tetracycline treatment resulted in rapid response, but, as expected, relapses followed this short course (*see* Table 5). Kanamycin, which was bactericidal in vitro, was bactericidal in vivo as well, and effected cure, albeit the initial response was somewhat slow in two monkeys. Novobiocin (Eigelsbach, Herring, and Halstead, Bacteriol. Proc., p. 69, 1957) gave results similar to those obtained with tetracycline. Although gentamicin was quite active against *F. tularensis* SCHU-S5 in vitro, therapy with it was disappointing. All monkeys responded, but only slowly; three of the eight had a relapse.

These results suggest that novobiocin may be employed in the therapy of human tularemia, but that prolonged courses, such as those found necessary with other bacteriostatic drugs, are likely to be necessary if therapy is to be completely successful. Although the bactericidal drug kanamycin was highly effective, its toxicity is such that it cannot be recommended for primary treatment. It may, however, be of value as a "backstop" in chronic, recurring infections and as an alternative to the broad-spectrum drugs in the management of infections by streptomycin-resistant organisms.

SUMMARY

Unlike streptomycin, tetracyline and the other broad-spectrum antibiotics do not kill susceptible *F. tularensis* in vitro or in vivo. The broad-spectrum drugs owe their effectiveness to their bacteriostatic action; they check multiplication of the invading organisms until host defense mechanisms can eliminate the bacteria. Elimination of *F tularensis* within cells proceeds slowly, and organisms may persist for many days in man (and many weeks in monkeys) during tetracycline treatment. The results of the present studies of tetracycline treatment indicate that infection with *F. tularensis* can be eradicated through bacteriostatic antibiotic therapy provided (i) that the antibiotic is administered in amounts sufficient to obtain continuous suppression of growth of intracellular organisms, and (ii) that the regimen is maintained for a sufficient period of time. These objectives have been met by a regimen of 2 g of tetracycline daily for 14 days. This regimen may be employed both for prophylaxis and for therapy of human airborne tularemia.

ACKNOWLEDGMENTS

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## Discussion

MARK H. LEPPER

*University of Illinois College of Medicine, Chicago, Illinois*

The elegant work of Dr. Sawyer and his colleagues presents data which are of considerable importance, not only to those dealing with tularemia and related diseases, but also to many investigators who are interested in the general principles of antibiotic therapy and prophylaxis. This discussion is based on a general perspective, with emphasis on the prophylactic aspects.

All chemotherapeutic activity must be viewed in terms of the therapeutic ratio concept. Since there is rarely an assurance in natural situations that any given individual upon exposure will develop a clinical illness, the therapeutic ratio in the prophylactic situation has to be expressed in terms of group risk, group results, and group toxicity. Thus, if only one-half of an exposed population is destined to become ill and the prophylactic regimen gives no better end results than the treatment of half the group of subjects who actually become ill, the toxicity is doubled for the group as a whole and the results are no better than those of therapy; hence, the therapeutic ratio is less favorable. Since the accentuation of toxicity is the most marked effect of prophylaxis, prophylaxis is usually attempted with the least toxic drugs or with a reduced dose. In addition, because it is often not possible to determine with accuracy the expected infection rate in the natural situation, it has been more difficult to measure prophylactic than therapeutic benefits. Clearly, the animal and volunteer studies of Sawyer meet the problem of evaluation well and thus provide important insight. Unfortunately, even the results of treatment of a random sample of an exposed population in a semiclosed situation, when the infection rate is unpredictable, may be difficult to interpret, since the treatment of some members of the group may influence the infection rate among the untreated.

The most important principle illustrated by these data is the primary importance of the host defense mechanisms. The superior results in man as compared with monkeys correlates well with the higher spontaneous recovery rates among the former. It is possible that much of what has been demonstrated is interpretable in terms of the

natural history of the disease and the defense mechanisms. That the agglutinins which were demonstrated were not capable of terminating the disease, per se, appears clear from the fact that the titers appeared early in those animals in which the prophylactic regimen failed to suppress symptoms even after the response had begun. On the other hand, in animals, given the 42-day course of treatment, which remained well thereafter, antibodies were not demonstrated. Measurement of a single type of antibody does not, however, preclude the presence or absence of others. A difference in the effectiveness of antibody as an anti-infectious agent may be part of the explanation for the differences between the success of the intermittent regimens in the rickettsioses in contrast to its failure here. The studies on vaccines, including those reported at this conference, do not lend much support to this hypothesis. Another factor accounting for the difference may be the relatively short incubation period of this disease; hence symptoms reappear in the brief drug-free interval, whereas the longer period in rickettsial disease may place the second course within the relapse incubation time. It would appear unlikely that intracellular parasitism accounts for the difference, since both organisms are intracellular and tetracycline, apparently at least, can influence them there. Perhaps extracellular phases may be of importance.

With respect to the type of stimulation of the defense mechanism, the attenuated nature of the post treatment relapses is of interest. This appears to be evidence of a partial stimulation of host immunity. It is conceivable, however, that in effect there has been a reduction in the surviving inoculum, so that the infection observed is of the same type as would have resulted from a sublethal dose without drug administration.

This study, of course, is immediately applicable to the disease studied, but is also probably a guide to other infections in which organisms survive within host cells. Perhaps the most important disease is tuberculosis. Can one reconcile with the data of Sawyer the results in the field trials of isoniazid prophylaxis in the person who has recently begun to react to tuberculin? In view of the fact that in tuberculosis the expected symptomatic infection rate is well below that seen in these volunteer infections, one might attribute more to the host defenses than to success of the regimen. That some break-throughs do occur even when there is good evidence that the drug is being taken, but that many more occur when it is stopped prematurely, suggests that the situation is similar, even though the rates are proportionally much lower. This similarity is observed in spite of the fact that isoniazid has equally favorable distribution characteristics and is, in addition, more bactericidal than the tetracyclines used in these experiments. The question may well be raised as to what the intracellular location offers in the way of protection to the pathogens. In the tularemia model, at least, there is no reason to suspect that tetracycline becomes ineffective by causing the production of wall-less forms, but a relatively dormant state might well persist, sheltered intracellularly from host destruction until after the drug dissassociates from the anabolic mechanisms and multiplication of the organisms begins again. If this is so, one might expect a difference between prophylaxis preceding inoculation and treatment during the incubation period. Effective treatment of tuberculin-negative children who have contact with infectious persons suggests that this may be so. Dr. Sawyer's model could give some guidance on this point, which is not well established by the field trials. By extending the number treated to a still lower risk group, the therapeutic ratio is made less favorable, so more precise data would be appropriate.

Of interest is the fact that tetracycline is bacteriostatic, and the results are not as satisfactory as those with kanamycin, which is bactericidal. Several of the most successful prophylactic regimens have been the bacteriostatic sulfonamides used in relatively small doses against susceptible strains of meningococci and group A streptococci. Tetracycline prophylaxis also appears to be effective against the latter, but less so against the former. These unexpectedly good results may be related to inoculum size or to the extracellular nature of these infections, or both. In the case of the meningococcus, the sulfonamides work both pre- and postinoculation, even in quite small doses for short periods. The least explained exception to the rule of relatively incomplete activity of bacteriostatic drugs is the very low relapse rate among patients with *Haemophilus influenzae* meningitis, even though there is little reason to suspect great recovery powers on the part of the host, as judged by the natural history of the untreated disease. Similarly, we observed tetracycline to be quite successful in preventing *Haemophilus* infection of the upper airways in patients on a rheumatic fever ward. All of these results suggest a marked difference in those situations in which the defense mechanisms rely heavily on a pyogenic response and those in which they do not.

In many practical applications of prophylaxis, the results are greatly affected by the fact that any one of a number of strains of the same or of different species may give similar difficulty. When

superinfecting strains are more drug-resistant, prophylaxis may even be detrimental.

Since in the "virgin" community the susceptible forms almost always predominate over the resistant types, they must have a survival advantage of some not yet defined sort. Theoretically, such an advantage may be great or small. If it were great, it is likely that a much larger proportion of a population would have to be treated and thus denied to the susceptible strains, in order for the shift to occur toward a resistant population among the untreated. In such a case, the level of use required to initiate such a shift would be a good index of the potential for a drug to cause harm upon mass usage. Our recent experiences in a closed community suggest that the breeding of resistant, aerobic, gram-negative rods of the coliform type and of staphylococci starts when chloramphenicol is used simultaneously in about 5% of the population and increases linearly with usage up to the 30% level. These resistant populations among untreated patients within 2 weeks reach a level as high as 50% of the positive untreated carriers. With *Escherichia coli*, the spread pattern of resistant serotypes is clearly demonstrable as early as 36 hr after the drug has been introduced to subjects on the ward. Careful analysis of the serotypes and phage types indicates that chloramphenicol and tetracycline are good prophylactic agents in these infections when the strains are susceptible, but the effect is masked by the propagation of resistant populations. Against susceptible strains, they are at least as effective as bactericidal agents are against the streptococci.

To meet this problem, there have been several approaches. One is to treat quite briefly at the time of maximal exposure, for example, during surgery. Another consists of using the drug locally in high concentrations often in such a way that resistant strains might not be spread readily, since the area being treated is not one of the primary portals of spread. This type of prophylaxis is best illustrated by the use of antibiotics within the urinary bladder and of sulfamyelon on burns. The situation is less clear for the use of protective nasal ointments, although there have been some successes. Of interest to this conference might be the reinvestigation of aerosol prophylaxis and treatment. Previous poor results might well have been conditioned by improper sterilization of the equipment and the results falsely attributed to failure of the method.

One might predict that, for systemic infections with specific highly virulent strains, systemic prophylaxis will continue to be developed within the limitations of a favorable group therapeutic index. For those situations in which there is a potential for a resistant organism, present among the varied and mixed flora, to become dominant and, in addition, in which there is often a markedly impaired host resistance, a further trial of local regimens will probably be made.

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# Physical and Chemical Stresses of Aerosolization

ROBERT J. ZENTNER

*U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland*

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## INTRODUCTION

Many investigators are concerned with the study of the stresses of aerosolization. As stated by Madin in the foreword to the First International Symposium on Aerobiology (15), they may be said to be engaged in studies oriented toward finding the most tolerable conditions under which bacteria may live in the airborne state and be infective. An entire session of the Berkeley Symposium entitled Survival and Viability was devoted to describing studies dealing with the stress of dehydration and its effect upon the viability of microorganisms. This stress was shown to be of major and primary importance in the study of airborne infection.

Dunklin and Puck (6) suggested that the sensitivity of microorganisms increases at a critical degree of dehydration. They based their suggestion on observations on the variation of the death rate of airborne bacteria with variation in relative humidity (RH). Webb (24) attributed the lethal effects of dehydration in the aerosol to a physical change in the structure of an essential macromolecule when water bound to this molecule is removed. Later, Webb (25, 26) stated that the bound water molecules appear to occupy strategic positions in the molecule, and only certain chemicals capable of forming hydrogen bonds of the correct type can replace them and maintain the biological integrity of the macromolecule. Zimmerman (29), reporting studies on both freeze-drying and aerosolization, proposed that nonpermeable sugars counteract the stress of aerosolization through a plasmolytic dehydration of the organism. Conversely, freely penetrating sugars are required to minimize the effects of the stress of freeze-drying.

Monk and McCaffrey (16) showed that the death rate of rehydrated *Serratia marcescens* is maximal at a water content of 33%, but stated that the effects of oxygen on the death rate had not been determined (17). Heckley and Dimmick (9) remarked that a study of lyophilized organisms has a place in aerobiology because freeze-dried organisms are similar to airborne cells in that they are essentially naked and in direct contact with the atmosphere. Earlier, Davis and Bateman (2, 3) had investigated the killing of freeze-dried *Escherichia coli*, *Micrococcus lysodeikticus*, and *S. marcescens* upon exposure to water vapor in vacuo, and had made a qualitative correlation between susceptibility to injury and surviving oxidative metabolism of the cell types. The assumption common to most of this work has been that a change in cellular water (dehydration or rehydration) causes the death of the cell by the physical disruption of vital structures, by concentration of toxic chemical material within the cell, or by creating an imbalance in metabolic activity.

## EFFECT OF OXYGEN

Rogers (19) was one of the first investigators to recognize the lethal effects of oxygen on lyophilized organisms. Naylor and Smith (18) have reported results in agreement with those of Rogers. These investigators reported that survival is highest for organisms stored under vacuum and lowest for those stored in air or oxygen. Atmospheres of nitrogen, hydrogen, and carbon dioxide yield intermediate results. Scott (20) reported that the effect of the atmosphere upon the survival of dried bacteria depends upon the nature of the suspending medium and its moisture context. Recently Lion and Bergmann (12, 13) listed numerous substances that protect lyophilized *E. coli* against the lethal effects of oxygen. Lion (14) suggested that a prerequisite for effective protection against oxygen in the dry state is the accumulation of the solute around the bacteria, which he assumed to occur during lyophilization. Benedict et al. (1) reported that atmospheric oxygen kills 95% of dried *S. marcescens* in 10 min, that certain reducing agents prevent the action of the oxygen, and that humidity seems to play no role in the phenom-

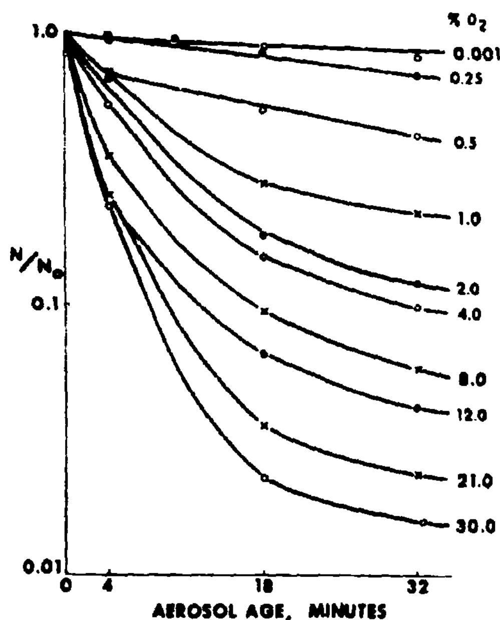


FIG. 1. *Survival of aerosolized Serratia marcescens after contact with various concentrations of $O_2$ at 40% RH, 25 C. Suspensions contain washed cells in water with 20 × $10^9$ viable S. marcescens cells per milliliter and 2.0 × $10^9$ viable Bacillus subtilis spores per milliliter. $N/N_0$ = ratio between viable S. marcescens and B. subtilis spores collected from the same aerosols, corrected for differences in control viable-cell counts: $P_0$ = atmospheric pressure, P = partial pressure of $O_2$. Results of one typical set of trials.*

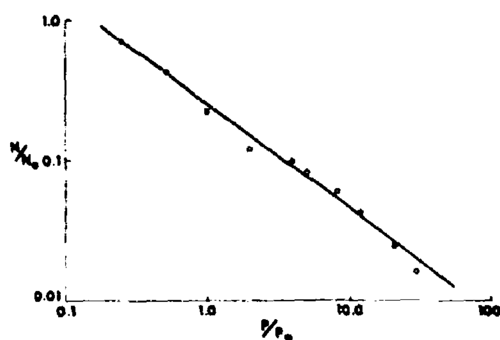


FIG. 2. *Survival of aerosolized washed Serratia marcescens after 32 min versus $O_2$ concentration. Same symbols as for Fig. 1.*

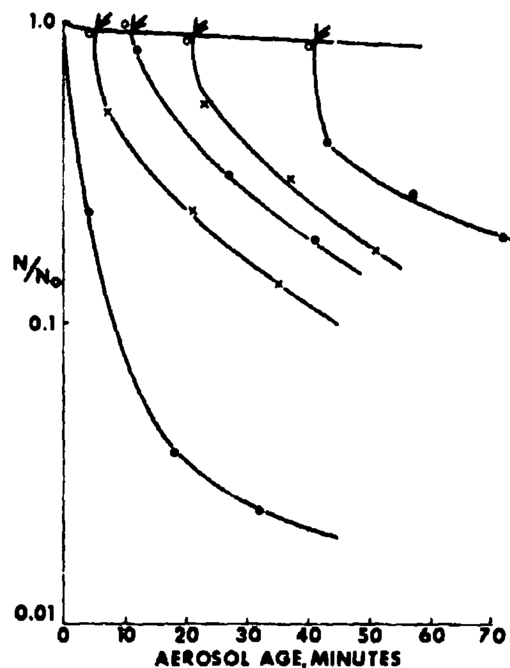


FIG. 3. *Survival of washed Serratia marcescens in nitrogen with 5% $O_2$ added at times indicated by arrows. Same symbols as for Fig. 1.*

TABLE 1. *Survival of washed and unwashed Serratia marcescens aerosolized into air or $N_2$*[a]

| Aerosol age | $N/N_0$[b] | | | |
|---|---|---|---|---|
| | Air | | $N_2$ | |
| | Unwashed | Washed | Unwashed | Washed |
| *min* | | | | |
| 4 | 0.009 | 0.230 | 1.0 | 1.0 |
| 18 | 0.0007 | 0.040 | 0.92 | 0.95 |
| 32 | 0.0001 | 0.024 | 0.90 | 0.90 |
| 150 | —[c] | 0.007 | 0.72 | 0.70 |
| 300 | —[c] | 0.004 | 0.70 | 0.69 |

[a] Suspensions contained 20 × $10^9$ *S. marcescens* cells per milliliter and 2 × $10^9$ *Bacillus subtilis* var. *niger* spores per milliliter. All aerosols generated at 40% RH, 25 C.

[b] Ratio of *S. marcescens* to *B. subtilis* spores in the same aerosol, corrected for differences in control counts.

[c] Too low to assay.

enon. Wagman (23), however, demonstrated a marked dependence of survival upon residual moisture in studies of circulating-gas (air) freeze-drying of water-washed *S. marcescens* and *E. coli*.

Studies of the effects of ascorbic acid on aerosolized *S. marcescens* in our laboratory suggested that interaction between the cells and atmospheric oxygen may contribute to the death

TABLE 2. *Effects of various compounds on survival of aerosolized Serratia marcescens*[a]

| Compound | Concn | $N/N_0$[b] | | |
|---|---|---|---|---|
| | | 4 min[c] | 18 min | 32 min |
| | *moles/liter* | | | |
| None (air, 97% RH) | — | 1.07 | 1.03 | 1.00 |
| None (air, 40% RH) | — | 0.23 | 0.04 | 0.02 |
| None ($N_2$) | — | 1.00 | 0.95 | 0.90 |
| $MnSO_4$ | $5 \times 10^{-3}$ | 0.77 | 0.51 | 0.48 |
| $MnCl_2$ | $3 \times 10^{-3}$ | 0.47 | 0.37 | 0.34 |
| $Mn(NO_3)_2$ | $2 \times 10^{-4}$ | 0.41 | 0.35 | 0.19 |
| $MgSO_4$ | $2 \times 10^{-3}$ | 0.28 | 0.11 | 0.07 |
| $CoCl_2$ | $2 \times 10^{-4}$[d] | 0.52 | 0.22 | 0.13 |
| NaCl | $8 \times 10^{-3}$ | 0.06 | 0.03 | 0.01 |
| $CuSO_4$ | $2.5 \times 10^{-5}$[d] | 0.20 | 0.04 | 0.03 |
| Glycerol | $1 \times 10^{-3}$ | 0.47 | 0.28 | 0.18 |
| Thiourea | $1 \times 10^{-3}$ | 0.44 | 0.25 | 0.21 |
| Cysteine-HCl[e] | $5 \times 10^{-3}$ | 0.26 | 0.10 | 0.06 |
| *N*-ethylmaleimide (air) | $1 \times 10^{-4}$[d] | 0.20 | 0.07 | 0.05 |
| *N*-ethylmaleimide ($N_2$) | $1 \times 10^{-4}$[d] | 0.75 | 0.44 | 0.40 |

[a] Suspensions contained $20 \times 10^8$ *S. marcescens* cells per milliliter and $2 \times 10^8$ *Bacillus subtilis* var. *niger* spores per milliliter. All aerosols were generated in air at 40% RH, 25 C, except as noted.

[b] Ratio of *S. marcescens* to *B. subtilis* spores in the same aerosol, corrected for differences in control counts.

[c] Aerosol age.

[d] Higher concentrations were toxic to control suspensions.

[e] Brought to *p*H 7.0 with NaOH.

of cells. Preliminary experiments indicated that, when *S. marcescens* is aerosolized into air diluted with nitrogen, the death rate increases with oxygen concentration.

Work was undertaken to test the possibility that drying sensitizes organisms to lethal effects of oxygen but in itself is not the direct cause of death of the microorganisms. Hess (10) tested the effects of oxygen on aerosolized *S. marcescens*, and Dewald (5) has studied the kinetics of the effects of oxygen on lyophilized *S. marcescens*. It is appropriate to describe the results of their studies in some detail.

## AEROSOL EXPERIMENT

Hess (10) aerosolized water suspensions of *S. marcescens* (ATCC strain 14041) in a rotating drum of 86.6-liter capacity revolving at 5 rev/min (8). *Bacillus subtilis* spores were used as a tracer to indicate maximal viable-cell recovery. Ratios of viable *S. marcescens* and *B. subtilis* spores from aerosols stored in various concentrations of oxygen are shown in Fig. 1. Maximal survival of the *S. marcescens* occurred at the minimal oxygen concentration attained, and at that point was nearly equivalent to spore survival. As the oxygen concentration was increased, loss in viability increased so that $\log N/N_0 = k \log P/P_0 + C$ (Fig. 2). All aerosols were generated from thoroughly washed cells free from added solutes. The tests were performed at 40% RH because aerosols of this organism routinely yield minimal survival in air at this humidity.

Lethal effects were observed when $O_2$ was added to aerosols originally disseminated into $N_2$ (Fig. 3). Although only 30% loss in viability occurred during 5 hr in nitrogen (Table 1), the addition of 5% oxygen resulted in at least 80% loss in viability within 30 min. This effect was noticed at oxygen concentrations as low as 0.25%, and became greater as oxygen concentration increased. The addition of 5% oxygen after aerosols had been stored in nitrogen for up to 40 min was selected as an arbitrary example of this system.

Unwashed, unstarved *S. marcescens* cells were more sensitive to storage as aerosols in air (Table 1) and respired 5 to 10 times faster than washed shaken cells. Stability in nitrogen was unimpaired, however, indicating a relationship between respiration and sensitivity to oxygen.

Effects of several additives on the stability of aerosolized *S. marcescens* are shown in Table 2. These compounds were selected because of their demonstrated influence on the stability of enzymes in organisms exposed to oxygen in other systems. The concentration of each compound inducing

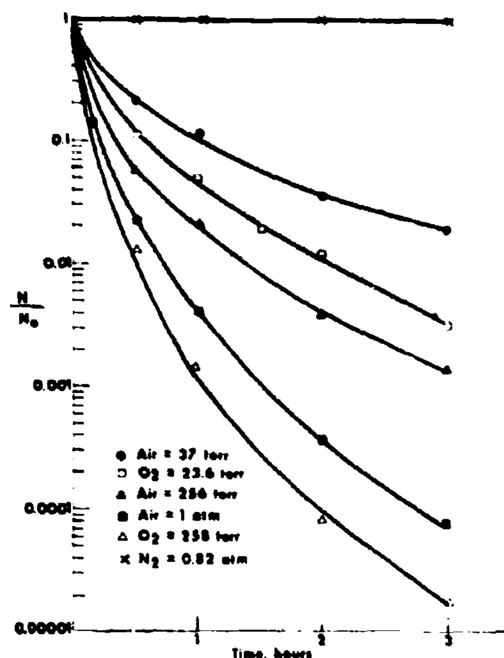


FIG. 4. *Semi-log plot of survival versus time after exposure of lyophilized Serratia marcescens to various pressures of oxygen dry air, or purified nitrogen. $N_0$ and N are the number of viable organisms before and after the exposure, respectively.*

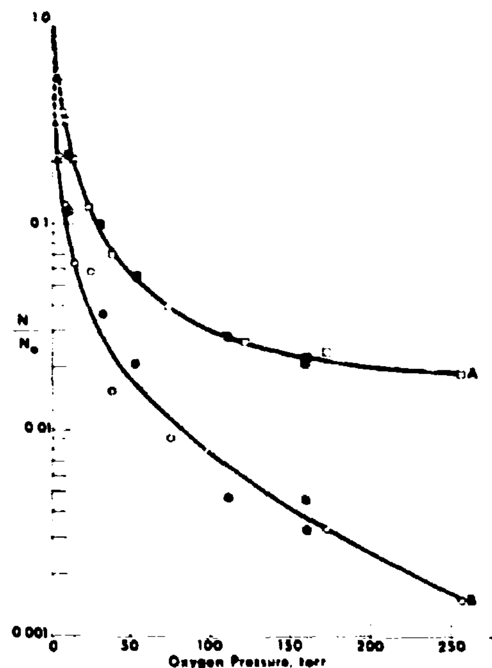


FIG. 5. *Semi-log plot of survival versus oxygen pressure after 0.5- (line A) and 1-hr (line B) exposures to oxygen or partial pressures of oxygen in dry air; $N_0$ and N are the number of viable organisms before and after exposure, respectively. Open circles and squares, pure oxygen; closed circles and squares, partial pressure of oxygen in dry air.*

optimal stability in the aerosol was determined empirically.

Experiments were performed to determine the sensitivity of completely hydrated *S. marcescens* to oxygen. Completely hydrated organisms were insensitive to oxygen at pressures up to 100 psi for 4 hr, and no viability loss occurred in aerosols of washed cells in air at 97% RH.

### EXPOSURE OF LYOPHILIZED ORGANISMS

Dewald (4) has developed a high-vacuum method of lyophilization of *S. marcescens* that yields 45 to 70% survival of the parent suspension; he has used this material to study effects of exposure to oxygen and air (5).

Data on survival versus time, obtained by exposing lyophilized *S. marcescens* at various pressures of oxygen, dry air, and nitrogen, indicate a dependence upon partial pressure of oxygen similar to that observed in the aerosolization studies of Hess (Fig. 4). No loss in viability could be detected when the dried organisms were held under vacuum for periods up to 3 hr at pressures less than $10^{-5}$ torr. The dependence of viability upon the partial pressure of oxygen after 0.5- and 1-hr exposures at 25 C was demonstrated (Fig. 5). Another representation of the inactivation curves (Fig. 6) shows that the survival data can be linearized by plotting log $N/N_0$ versus $(\text{time})^{1/2}$, leading to a rate expression, $-\ln N/N_0 = Kt^{1/2}$, where $K$ is a pseudo rate constant that in turn can be related to the oxygen concentration by $K = k[O_2]^n$ or $\log_{10} K = n \log_{10} [O_2] + \log_{10} k$.

The pseudo rate constants, $K$, for all the inactivation data determined by least-squares fit and related $k$ values are given in Table 3. The log of the pseudo rate constant versus log oxygen concentration is given in Fig. 7, and leads to the following expression for the inactivation of the freeze-dried material by oxygen:

$$-\ln N/N_0 = K[O_2]^{1/2}t^{1/2}$$

where $k = 276 \pm 36$ moles $^{-1/2}$ cc$^{1/2}$ hr$^{-1/2}$ at 25 C.

The Arrhenius function was determined for the dried organisms exposed to dry air at atmospheric pressure for 1 hr at temperatures ranging from

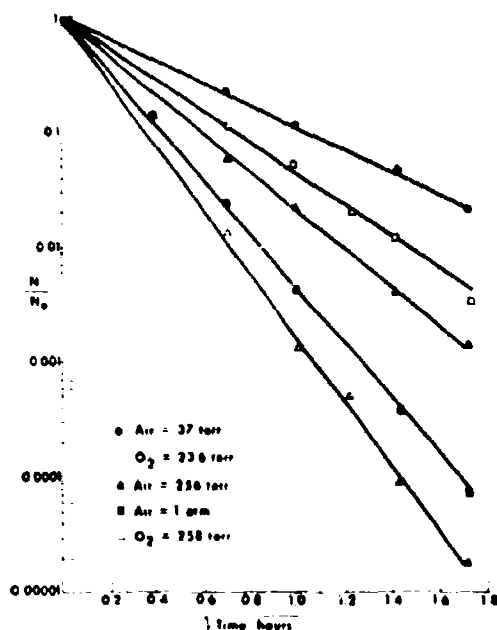


FIG. 6. *Semi-log plot of survival versus (time)$^{1/2}$ after exposure of lyophilized Serratia marcescens to various pressures of oxygen or dry air. $N_0$ and N are the number of viable organisms before and after the exposure, respectively.*

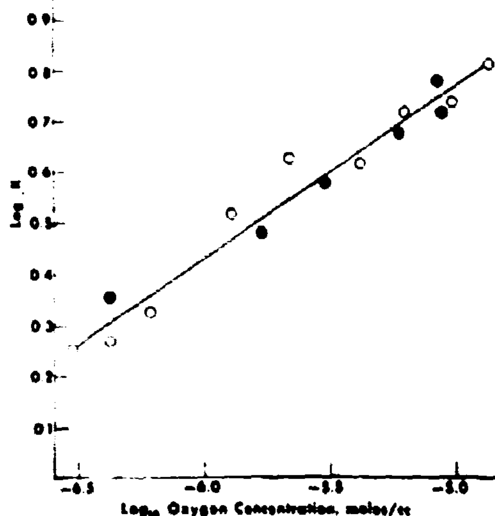


FIG. 7. *$Log_{10}$ of the pseudo rate constant, K, versus $log_{10}$ of the oxygen concentration. Open circles, pure oxygen; closed circles, dry air.*

**TABLE 3.** *Kinetic data for the inactivation of Serratia marcescens by oxygen*

| Oxygen pressure (torr) | Oxygen concn[a] ($10^{-7}$ mole/cc) | K pseudo, ($hr^{-1/2}$) | k (moles$^{-1/3}$ cc$^{1/3}$ hr$^{-1/2}$) |
|---|---|---|---|
| 258 | 139 | 6.51 | 271 |
| 172 | 92.5 | 5.50 | 261 |
| 160[b] | 86.1 | 5.17 | 252 |
| 159[b] | 85.5 | 6.13 | 300 |
| 121 | 65.0 | 5.27 | 283 |
| 110[b] | 59.2 | 4.79 | 275 |
| 71.6 | 38.5 | 4.15 | 264 |
| 53.7[b] | 28.9 | 3.85 | 271 |
| 37.7 | 20.3 | 4.28 | 337 |
| 30.8[b] | 16.6 | 3.02 | 258 |
| 23.6 | 12.7 | 3.31 | 306 |
| 11.0 | 5.92 | 2.11 | 251 |
| 8.0 | 4.30 | 1.85 | 245 |
| 7.8[b] | 4.20 | 2.29 | 306 |
| 5.5 | 2.96 | 1.78 | 267 |

[a] Calculated by use of the ideal gas law.
[b] Dry air used as source of oxygen.

−78 to 40 C. The results, plotted as Fig. 8, yield, by least-squares fit:

$$k = 10^{[illegible] \pm 0.43} \exp[(-430 \pm 26) \text{ cal}/RT] \text{ moles}^{-1/3} \text{ cc}^{1/3} \text{ hr}^{-1/2}$$

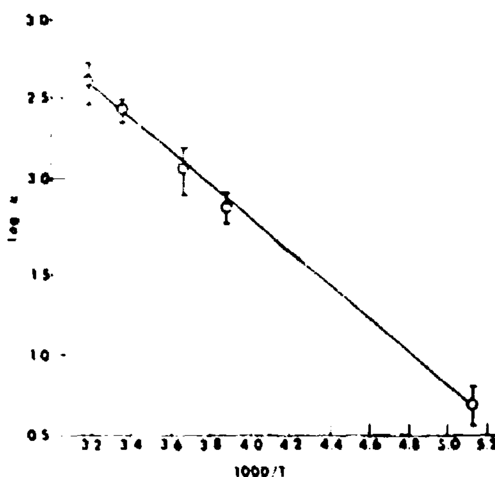


FIG. 8. *Arrhenius plot for the inactivation of Serratia marcescens by oxygen. On the abscissa, T is in degrees Kelvin.*

## DISCUSSION AND OUTLOOK

The results here can be compared with those of aerosolization inactivation (Fig. 9). There appears to be no pronounced difference in the degree of inactivation after 0.5 hr as a function of the partial pressure in the two systems. The data of Hess were obtained at 40% RH; 0% RH was used in the lyophilization-exposure studies;

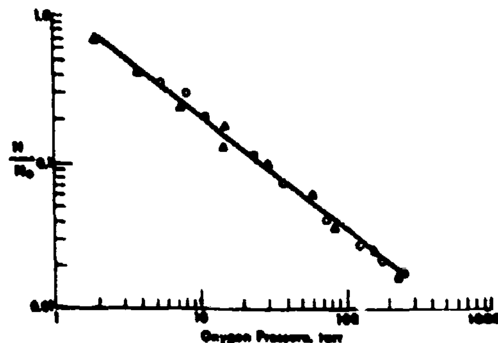


FIG. 9. *Log-log plot of survival of Serratia marcescens versus partial pressure of oxygen. $N_o$ and N are the number of viable organisms before and after the stress. Open circles, this work, lyophilized organisms after 0.5-hr exposure; closed triangles, aerosolized organisms after 32 min.*

hence, direct comparison is not completely valid. Additional and preliminary studies indicate that the survival of washed, lyophilized *S. marcescens* exposed to oxygen in humidified air is essentially independent of RH between 0 and 85%, whereas the survival of the organisms exposed under the same conditions, but lyophilized from suspensions containing 0.05% NaCl, showed a marked dependence on RH. The comparison does indicate a remarkable similarity in degree of inactivation in the two systems. Although the mechanism resulting in death is not known, the data require a consideration of oxygen effects in explaining the stress of dehydration. Hess (10) has made a comparison with other systems, and he points out similarities with studies by Hollaender, Stapleton, and Martin (11) and Tallentire (21, 22) on the lethal effects of oxygen on irradiated organisms. Further, he recognized the hypothesis of Gerschman et al. (7) concerning a common mechanism between oxygen poisoning and the initial effects of X irradiation in biological systems, and he proposed that $O_2$ poisoning and death of airborne bacteria may be similarly related. Webb (27, 28) examined the effects of ultraviolet light and X rays upon air-dried microorganisms, and proposed that, in aerosols below 80% RH, death due to toxic chemicals, irradiation, or desiccation alone is the direct result of removal or reorientation of bound water.

It is clear that the stress of dehydration and the events that are initiated by application of this stress remain to be adequately defined. A complete description of the behavior of microorganisms and their reactions to the environment is required to understand airborne organisms and the complexities of airborne infection.

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## Discussion

MYLES MAXFIELD

*Biophysics Program, University of Southern California, Los Angeles, California*

The experiments on the disinfection by oxygen of bacteria dried either by aerosolization or by lyophilization presented by Mr. Zentner are delightfully clean and precise, and are adequate for some kinetic studies. This fact relieves us of the relatively unrewarding task of discussing the experimental techniques, accuracy of the data, and validity of the conclusions. It makes it possible to attempt to place the conclusions in broader perspective, to attempt to interpret them, and to examine whether they suggest or point the way to further investigation.

It is perhaps surprising at first glance that oxygen is toxic to *Serratia marcescens* under the experimental conditions described. Mr. Zentner has, I think quite correctly, related this implicitly to dehydration of the bacteria. The observation has been amply confirmed and is even intuitively reasonable in terms of several hypothetical mechanisms; for example, the concentration of some toxic metabolite may increase at a critical location because diffusion is limited by dehydration, or dehydration may distort some structure and render it more susceptible to oxidation.

It is even more surprising that the biological reactions observed follow so nicely the kinetic laws described by the figures; in particular, there is no threshold concentration for the toxicity of oxygen (Fig. 2, 5, 7, and 9), and the data form a straight line on the Arrhenius plot (Fig. 8) over an extraordinarily wide range of temperature (−78 to +40 C). Data on disinfection with a variety of disinfectants commonly follow similar kinetic laws (see, for example, F. H. Johnson, H. Eyring, and M. J. Polissar, *The Kinetic Basis of Molecular Biology*, p. 453 ff. John Wiley & Sons, Inc., New York, 1954). The generality of this type of biological data simply emphasizes the importance of understanding why the biological data follow these chemical laws.

The Arrhenius relation is usually derived from a thermodynamic consideration of a chemical reaction at equilibrium or from a statistical mechanical consideration of reaction rates. In the latter case, it is found necessary to introduce the concept of an "activated state" in or near equilibrium with the chemical reactants of the system. Each molecule in an activated state has a certain probability of decaying into its products (or, by analogy, dying). The logical essence of the reasoning leading to the Arrhenius relation, then, is the existence of two states of the system at, or nearly at, equilibrium with each other. One of these states may be the "activated state" in which

the organism has a certain probability of dying. It places less strain on our credulity to accept a rather abstract interpretation of the experimental results such as this than to believe that each organism behaves like or is dependent upon a single molecule.

The very abstractness or generality of this interpretation of the kinetic data is a serious handicap, because it does not point the way toward a critical chemical substance or chemical or physical reaction, nor does it even say whether there are one or more "activated states" of the bacterium. In fact J. B. Bateman has gathered evidence that several different "activated states" may be involved in bacterial death due to dehydration. We may hope that the situation with regard to oxygen toxicity is not as complex, and hence as depressing, as that pictured by Bateman for dehydration disinfection. Continuing in this happy but somewhat illogical vein, we may say that the data presented by Zentner are consistent with the existence of a single "activated state," and even suggest that the "activated state" is related to the accumulation of some intermediary metabolite closely involved with oxidative metabolism. In this way, the experiments presented by Zentner do suggest new experiments through which our understanding of oxygen toxicity might be improved, but they do not exclude wholly different types of possible mechanisms, such as free radical production, direct oxidation of critical material, or structural change in the bacterium induced by oxygen.

It is possible that a clever guess may permit an experiment which will elucidate the nature of an "activated state." Otherwise, before the disinfection of dried bacteria by oxygen or by other disinfectants can be understood and adequately described, a great number of experiments and measurements on many independent variables will be necessary, followed by a complex relaxation analysis of the kind outlined by Bateman.

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# Multistage Liquid Impinger

K. R. MAY

*Microbiological Research Establishment, Porton, Wiltshire, England*

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## INTRODUCTION

Instruments which collect viable airborne particles by inertial impact may be divided into two classes. Class I embraces instruments which project the viable particles straight onto the surface of a nutrient agar gel. Here they grow during incubation into one colony per viable *particle*, regardless of the size of the particle or the number of viable cells it contained. In class I are the slit-sampler, cascaded slit-sampler, sieve sampler, and the cascaded sieve or Andersen sampler, the features of all of which are conveniently summarized by Decker et al. (1). There is also the design of Lidwell (4). Class II samplers, with which we are concerned in this paper, project the particles into liquid, where they are broken up into their individual component cells. The liquid is serially diluted, plated out, and incubated to give a colony count and an estimation of the total viable *cells* in the original sample.

Typical of class II is the widely used Porton or capillary impinger (6, 14) in which a jet of air, accelerated to sonic velocity by suction in excess of 15 inches (38.1 cm) of mercury depression, impinges into liquid where small particles are collected with high efficiency. The sonic velocity jet serves also to limit the flow to a constant value.

The impinger is simple, cheap, convenient, easily sterilized, and, by the addition of a pre-impinger (5, 7), becomes an approximate simulant of the upper and lower parts of the respiratory system. Class II devices have the following features not possessed by those of class I. (i) They can cope with any high concentration of aerosol by virtue of the serial dilution process, whereas class I devices are easily overloaded. (ii) They permit counts of several different organisms from

the same sample, by means of differential counts or selective media. (iii) They are very convenient when nondecaying tracers are added as one component in an artificial aerosol. The tracer, such as a spore or radioactive element, allows the rate of decay of another component to be estimated, such as that of an organism under radiation stress. This estimation is independent of changes in the aerosol concentration. (iv) Virus aerosols can be estimated. (v) When the cascade system is used to give particle size discrimination and the aerosol of interest is highly skewed in size distribution, with many small particles and very few large ones, class I devices are subject to an error which in class II is negligible. This error arises from the shape of the cut-off curves for each stage of the system (see Fig. 3). The lower tail of the curve tends to flatten off along the abscissa, so that a few of the small particles will be caught on the large particle stage(s). In class I devices, these few particles will be recorded as large weighty particles which are in fact fictitious, but in class II their effect will be negligible in the final count of total cells per stage. Therefore, when the large particle count is of interest, it should be treated with reserve in class I devices, when there are many small particles present.

The special features of the impinger system are sometimes offset by its limitations. It is not very good with very dilute aerosols, in which it yields but a few cells widely dispersed in a large volume (10 to 20 ml) of liquid; the liquid, which is under low pressure, evaporates rather quickly and also tends to freeze in cool dry air; the violent impingement can kill delicate cells (6, 14).

It was deemed desirable to design a sampler which minimized the limitations of the Porton impinger and could be more widely used. The outcome is the subject of this paper. The new sampler has a higher sampling rate and gives a greater concentration of cells per unit volume of collecting fluid. The rates of evaporation and freezing are low and the impingement is gentle. The particle size discrimination is in three stages and is intended to simulate some of the finer details of the respiratory tract, where similar processes of inertial collection of particles operate. Also, the new sampler is more robust and more easily portable than the Porton impinger-preimpinger combination and is not subject to loss of collecting fluid through spilling as is the preimpinger. Finally, prolonged sampling periods do not result in as high a proportion of organism death as does the Porton impinger.

## BASIC DESCRIPTION OF DESIGN

### *Standard Model*

Drawings of the form of instrument most commonly used to date are shown in Fig. 1. A and B are sectional side elevations at right angles to each other in the directions I-I and II-II, respectively. The body is entirely of Pyrex glass, the outer parallel portions being blown from thick-walled tubing of 70-mm outer diameter. The sampler has three chambers or stages, 1, 2, and 3, in serial order vertically. The air inlet tube 4 has a smoothly curved entry to promote laminar flow, a flat ground lower end, and a bore of 15 mm. The straight tube 5, also with a smoothly curved bell-mouth, a flat ground lower end, and a bore of 10 mm is sealed into the flat floor of stage 1. This tube provides air-flow connection with the next stage. The tube 6, again with a smooth bell-mouth and bore of 10 mm, is sealed into the floor of stage 2. At its lower end, it bends and tapers smoothly and continuously to the nozzle 7, which has an internal diameter of 3.3 mm. The nozzle is close to the bottom of the annular well 8, formed as shown, and the axis of the nozzle lies in a plane tangential to the wall of the well and makes an angle of 45° to the vertical. Two circular discs, 9 and 10, made from coarse sintered glass 3 mm thick, are held about 1 mm above the flat floor of their respective chambers by the pairs of curved glass rods, 11 and 12, fused to the external walls of tubes 4 and 5, respectively. The discs 9 and 10 are twice the diameter of the bores of their respective tubes, 4 and 5, and are separated from the flat ends of these tubes by a distance equal to three-eighths of the bore. In use, the discs are constantly wetted by sampling fluid in each chamber. Access holes to each chamber are sealed by the rubber bungs, 13, 14, and 15. The lowest bung, 15, is fitted with a tube, 16, for connection to a suitable pump. This tube 16, which may be of any suitable material, projects into the center of the lower chamber and, as drawn in Fig. 1, may embody a flow-controlling critical orifice to give a constant throughput of 55 liters per min.

When operating the sampler in a cross-draft or wind, a hemicylindrical metal shield, 17, is clipped over the top so that the concave side faces upwind.

## PRINCIPLES AND METHODS OF OPERATION

### *First Stage*

When air is drawn through the instrument, air enters the intake tube, 4 (Fig. 1), and flows over the disc, 9, where some of the larger aerosol particles impact on the wet surface. To minimize

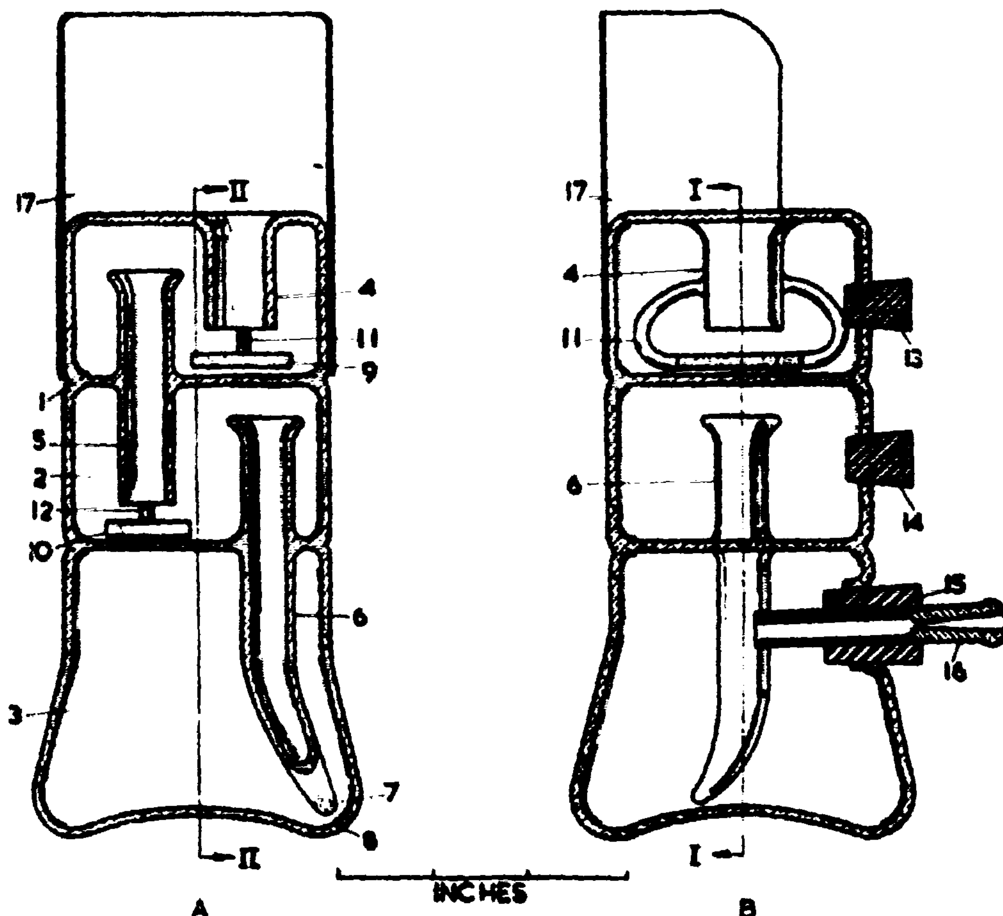


FIG. 1. *Sectional elevations of standard design.*

particle loss inside the intake tube, it should always be kept clean and dry. The ratio of 3:8 for (distance from tube to disc)/(tube bore) is that which is considered by Mitchell and Pilcher (11), and also by Mercer (9, 10), to give the steepest cut-off curve (Fig. 3), i.e., the sharpest particle-size cut-off. When the flow rate is 55 liters per min, 50% of bacterial particles 6 $\mu$ in diameter ($d_{50}$) with a specific gravity of about 1.5 will be trapped on the first sintered disc, and the proportion of particles of other sizes caught may be judged from Fig. 3. The rather high specific gravity is that measured for the dry bacterial clusters used in the calibration.

The air then flows outwards over the disc and liquid surface. It is particularly important that the sampler should be vertical and that the liquid should be nowhere higher than the upper surface of the sinters; otherwise, the outflowing film of air will encounter a standing liquid wave which will trap many particles intended for the next stage.

*Second Stage*

The same process of impaction and particle selection takes place at the second stage. With the smaller bore and higher velocity, the $d_{50}$ is 3.3 $\mu$, as shown in Fig. 3

*Third Stage*

The remaining particles now pass down the third tube, 6, and through the jet 7 (Fig. 1). It is important that tube 6 be smoothly tapered to the jet without any sharp bends; otherwise, particle loss on the inner walls will be excessive. For the same reason, it is important that tubes 4 and 5 should be quite smooth internally.

The tangential component of the jet direction

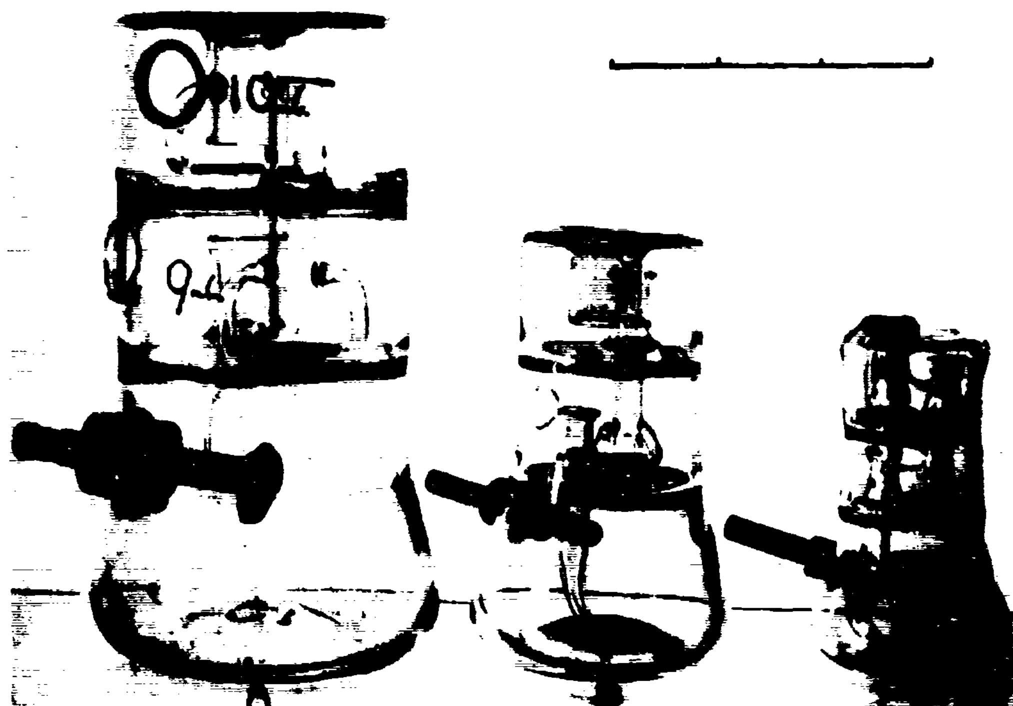

FIG. 2. *Three variations of basic design: (A) 50 liters per min model; (B) 20 liters per min model; (C) 10 liters per min model. Scale is in inches. Crown copyright reserved. Reproduced by permission of Her Majesty's Stationery Office.*

imparts a vigorous swirl to the liquid, which ensures that impingement is always on a wetted surface, provided that the liquid volume is not less than 5 ml. The diameter of the jet was determined on the basis of the performance of the "sub-critical" impinger described by May and Harper (6), and the idea of liquid swirl came from the Shipe sampler (14).

The jet gives no more than just sufficient velocity to ensure the capture of most single bacterial cells. Tests by G. J. Harper (*personal communication*) established that 80 to 90% of single cells of *Bacillus subtilis* and *Escherichia coli* were retained. The design has three advantages over the standard critical orifice impinger. First, the minimal violence of the impingement minimizes or perhaps eliminates kill of delicate cells. Second, splashing and frothing are minimized, so that a high air flow can be maintained through the compact third stage chamber without liquid loss by entrainment (note that the extract tube, Fig. 1 and 2, extracts air from the center of the chamber). Third, the critical orifice or other flow control system is downstream of the extract point, so that the pressure drop on the liquid surface is only about 2 inches (5 cm) of mercury below ambient. In this way, both the rate of liquid evaporation and the possibility of freezing are very much reduced.

### *Liquid Loss by Splashing*

Sometimes splashed liquid may collect on the roof of the third chamber and drip down on tube 16, whence it is entrained to waste through the tube. Such loss may be avoided by fitting the rubber drip ring seen in Fig. 2A.

### *Liquid Loss by Evaporation*

If a long-period sample is taken, evaporation of the sampling fluid will occur. Evaporation is permissible to about 5 ml per stage, and the gap under the sintered discs will ensure circulation of liquid so that the disc remains moist. If evaporation threatens beyond this level, the lost liquid may be made up. With air at normal living conditions, topping-up may be necessary every 0.75 hr or so. About the same quantity will be lost from each of the three stages.

*Sampling in Still Air*

When the air to be sampled is calm or nearly so, as in a room, the sampler is used as it stands (Fig. 2). The smooth entrance, large diameter, shortness and smooth straight bore of the entry tube, 4, will ensure a high efficiency of collection of airborne particles.

*Sampling in a Crosswind*

In this case, the shield, 17, is fitted to stop cross-flow above the entry tube, thus approaching the still-air conditions. The system, which might be termed "stagnation-point sampling," is an entirely different concept from "isokinetic sampling" where air enters a knife-edged sampling tube with no change of speed and direction from that of the wind. Obviously, isokinetic sampling cannot be achieved from a horizontally moving air stream with impaction onto a horizontal liquid surface, as a 90° turn must intervene. In fact, true isokinetic conditions cannot be achieved outside a laminar-flow wind tunnel because of air turbulence. In stagnation-point sampling, the requirement for high intake efficiency would seem to be that the intake zone should be large compared with the particle's "stopping distance." The stopping distance is the distance of projection of the particle into still air from a given initial velocity, and varies as the square of the particle diameter and the velocity of projection. The validity of this concept is discussed below in the Calibration and Testing sections.

*Sampling from a Tube*

It may be required to withdraw aerosol samples from a chamber via a tube. In such a case, the connecting tube must never be inserted into the intake tube of the sampler by means of a bung, etc., as the jet effect from the narrow tube would completely alter the collection characteristics of the first stage. Ideally a 2.75-inch (7-cm) bore rubber hose would be pressed over the whole of the top of the sampler, maintaining the same wide bore to its source. If this is difficult, a tube at least as wide as the intake tube over the whole of its length should be used for the connection, with a wide metal flanged piece at its end. A sponge-rubber ring should be fitted under the flange, and the whole should be pressed firmly on top of the sampler.

*Filling with Sampling Fluid*

Fluid suitable for the organisms of interest is pipetted into each stage through the bung holes. The two upper stages are filled until the liquid surface is just below the upper surface of the sintered disc when the sampler is standing vertically. The upper surface of the disc will always be wet by capillarity, provided that the sinter is maintained in a grease-free condition by appropriate cleaning procedures. The standard model requires between 7 and 10 ml, varying from sampler to sampler, because of the vagaries of glass-blowing. It is convenient to mark indelibly by the side of each bung hole the volume required, as shown in Fig. 2A. Into the lower stage, 10 ml is pipetted.

*Emptying*

After use, the sampler may be shaken gently to mix the liquid, which is then withdrawn by a 10-ml calibrated pipette, to measure the volume. The liquid should be squirted a few times over the sintered disc to ensure removal of all organisms. This, and subsequent pipette mixing, must be done vigorously to ensure breakdown of clumps into single cells.

*Sterilization and Cleaning*

Before autoclaving or heat-sterilizing the sampler, the rubber bungs must be removed. When collecting fluid containing dissolved solids has been used, the sintered discs should be washed thoroughly with distilled water before heating, so that solids do not get baked into the pores. The sintered glass must always be kept chemically clean.

*Flow-rate Control*

Any system of flow control may be used, but the Hartshorn or venturi-shaped critical orifice (16 in Fig. 1) is perhaps the most convenient. These orifices constrict in a smooth curve to the throat, and then expand at an included angle of 4° over a length of about 1 inch (2.5 cm). As pointed out by Druett (2), this orifice geometry allows critical flow to commence at a very small depression, 4 to 5 inches (10.2 to 12.7 cm) of mercury compared with 15 inches (38.1 cm) for a conventional parallel-sided orifice. A substantial economy of pumping power is thus afforded. The addition of the 2-inch pressure drop across the sampler gives a safe minimum of only 7 inches of mercury depression required from the pump at the full flow rate of the sampler. Critical orifices are made slightly undersize and then reamed out until, in situ in the sampler, the desired throughput is achieved. In the present case, the initial bore is 2.5 mm for final adjustment to 55 liters per min. Systems of control not embodying a critical orifice are (i) a flowmeter with valve, downstream of the sampler; (ii) a compressed air or steam ejector operating at a constant pressure

(although these can only give a small pressure drop on their suction side, it is adequate for the 2 inches of Hg that this sampler requires); (iii) a sensitive pressure gauge just downstream of the sampler followed by a valved T-piece in the pump suction line. The valve is adjusted to bleed ambient air into the line until the depression across the sampler is that which in prior calibration gave the correct flow. The latter system makes the least demands of all on the pump but must be watched to maintain steady flow unless a sensitive pressure-regulated bleed valve is built into the T-piece.

## OTHER FEATURES OF THE DESIGN

### *Wet-disc Collection Surfaces*

The disc surfaces are moist, as are particle deposition areas in the respiratory system. This similarity is important in that the retention capability of a surface undoubtedly depends on its physical state as well as on the physical state of the surface of the particle.

Another valuable feature of the sintered discs is that their geometry, hence, impaction efficiency, is unaffected by the gradual evaporation of the liquid surface. Cells remain viable on the moist surface when the optimal collecting fluid is used, permitting prolonged sampling.

### *Portability*

The sampler has been designed so that it has a smooth external surface with no projecting glassware which might be damaged by chance knocks. The rubber bungs and rubber-mounted suction tube act also as fenders, and more such protection could be added if desired. The thick-walled glass tubing has considerable mechanical strength, provided that the whole has been correctly annealed. The sampler will stand up to prolonged usage and repeated sterilization when reasonable care is employed and use is made of properly fitted storage boxes for transportation. It will stand firmly on a bench of its own accord but will not resist being dropped on a hard surface.

### *Nonspill Property*

When the sampler is charged with sampling fluid and has the bungs in place, liquid cannot be spilled or transferred from one stage to another, however much the sampler is turned or inverted. The principle is similar to that of the unspillable ink-well.

### *Variations of the Basic Design*

The basic design may easily be varied. In addition to the standard 55 liter per min model (A in Fig. 2), two other models have been constructed (B and C in Fig. 2).

The 20 liter per min model (B) has stage characteristics somewhat different from A. Its first stage was designed to collect "fall-out" particles and has a 50% cut-off at 10 $\mu$; the second stage has a similar cut off to the standard "pre-impinger" (50% at 4 $\mu$), as described by May and Druett (5), so that its collection resembles upper respiratory retention, whereas the third stage resembles lower respiratory retention. The first-stage tube bore is 15 mm, and the jet impulse is so slight that no sintered disc is needed as the liquid surface remains undisturbed by the flow; nor is any filling hole required in the top stage as filling and emptying can be performed through the entry tube. A small dent in the glass floor of the top stage immediately under the entry tube acts as a sump. The clearance between the liquid surface and the bottom of the entry tube is 4 mm. The change in this as the liquid evaporates is unimportant. The second-stage jet diameter is 7.5 mm and the third, 2 mm. Each stage holds 4 ml of sampling fluid.

The small model (C), which is only 3.5 inches (8.9 cm) high, is operated at 10 liters per min, and was designed to have the same particle size range per stage as the large model (A). The jet bores are: first stage, 8 mm; second stage, 5 mm; third stage, 1.42 mm. The ratio of disc diameter to jet bore and the ratio of the clearance between the disc surface and the end of the tubes is as already described. This model is very much more compact than the standard Porton impinger which functions at about the same flow rate, yet it is capable of yielding more information and has the other advantages of the new design. It holds 2 ml of sampling fluid per stage.

## CALIBRATION AND TESTING

### *Particle Intake Efficiency*

Wind-tunnel tests on the sampler were carried out by J. Edwards (*personal communication*). With the hemicylindrical baffle in place (17 in Fig. 1), the sampler was exposed to wind-borne aerosols of dyed, involatile, and uniform droplets generated by a spinning-top atomizer (8). An array of knife-edged isokinetic orifices around the sampler measured the absolute dosages. Estimations were by colorimetry.

Table 1 shows that the intake efficiency with the baffle in place is high, except for the largest particles and highest wind speed. The latter figures can be greatly improved by employing a larger baffle. For example, with no baffle, the intake efficiency was found to be only 9.6% at 15 $\mu$ and 10 mph but was raised to 99% by using

Table 1. *Per cent intake efficiency*[a]

| Drop size | Wind speed | | | | |
|---|---|---|---|---|---|
| | 2 mph | 5 mph | 10 mph | 15 mph | 20 mph |
| μ | | | | | |
| 10 | 93 (0.3) | 106 (0.7) | 87 (1.5) | 73 (2.2) | 64 (2.9) |
| 15 | 102 (0.6) | 102 (1.6) | 69 (3) | 61 (4.5) | 57 (6) |
| 20 | 98 (1.1) | 89 (2.8) | 56 (5.6) | 39 (8.3) | 27 (11) |

[a] Particle stopping distance (in millimeters) is given in parentheses.

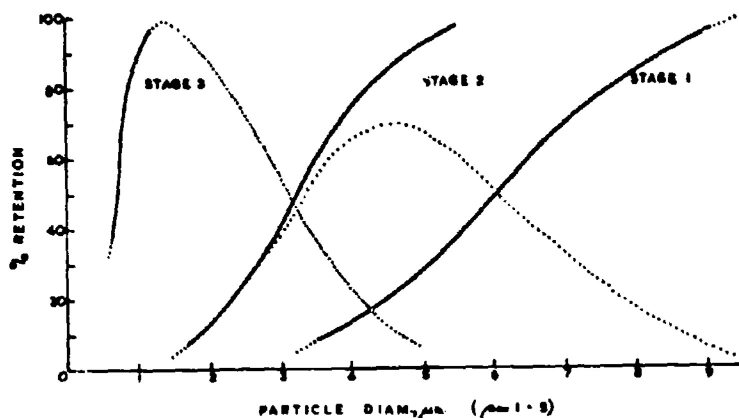


Fig. 3. *Individual stage cut-off curves (solid lines) and relative distribution of particles among all stages (dotted lines).*

a 6-inch square concave baffle, compared with the efficiency of 69% in Table 1, obtained with the Fig. 1 baffle. It is clear that a baffle is an essential addition to the sampler when there is a wind or cross draft. Note also that in Table 1 there is a good inverse correlation between the stopping distance (given in parentheses) and the intake efficiency, as predicted. With the particular conditions of the Table 1 tests, the intake efficiency is less than 50% when the stopping distance is greater than the radius of the intake tube.

### *Stage Cut-off Curves*

It is essential to know the performance of each stage in terms of particles of each size retained; one must also know the relationship of this parameter to the dimensions of the jets and the throughput of sampled air. For this work, dry spherical airborne particles, nearly uniform in particle size, were generated from solutions of the intense blue dye Chlorazol Sky Blue (Imperial Chemical Industries Ltd.) sprayed from a spinning-top atomizer (8). The atomizer was mounted in a vertical wind tunnel similar to that described by Druett and May (3). In this, the upflow of air permits the rather large droplets generated to dry down to their final size without serious wall loss. The size of the dry particles is determined by the initial wet droplet size and the concentration of the dye solution. In this way, the range 2.5 to 10 μ was studied in small intervals, the relative proportion of particles caught in each stage being obtained by colorimetric estimation of the dye collected in the water with which the stage was filled. The spherical dye particles had about the same density as the particles obtained by spraying suspensions of bacterial cells (ca. 1.5 g/cc). The results for the standard 55 liters per min model are shown in Fig. 3, where the continuous lines are the cut-off curves for each stage considered individually, and the dotted lines (which in parts coincide with the continuous lines) show the percentage of all particles entering the sampler collected by each stage.

The cut-off curves of Fig. 3 are not as steep as those presented for the same optimal geometry by Mitchell and Pilcher (11) and by Mercer (9). There are two possible reasons for this. First, their methods were different, giving aerosols

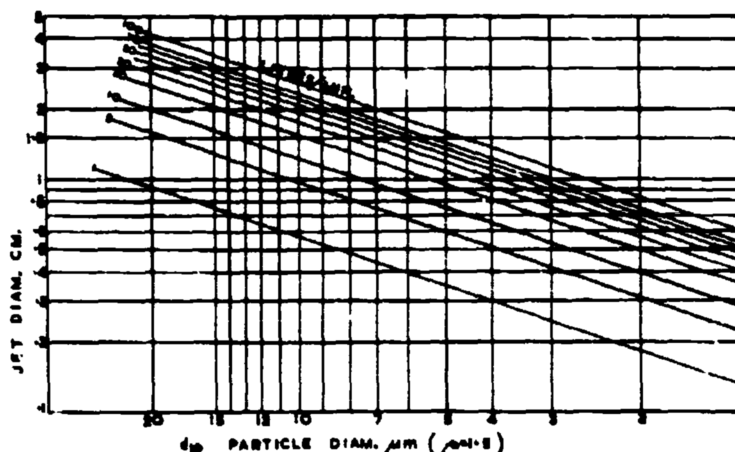


FIG. 4. *Relationship between jet diameter, particle diameter at 50% retention, and flow rate.*

which may have been more truly homogeneous in particle size than those used in the present work, where a spread in particle size tends to flatten the cut-off curves and reduces the precision. Second, the flow in the two upper chambers is rather complex. The air, after flowing out over the sintered discs, passes over a rather extensive liquid surface, and then turns upwards and inwards to enter the next jet tube. This causes turbulence and vortices which could throw out particles, though wall losses have not actually been noted other than to a limited extent in the tubes. Although the cut-off curves are less steep than hoped for, they are in fact as sharp as those of the preimpinger (5, 7) and the cascade impactor, and certainly not less sharp than the parts of the respiratory system that the sampler could simulate.

### *Effect of Varying Jet Sizes and Flow Rate*

Workers wishing to adapt performance to their own requirements, with jet sizes or flow rates different from those described here, may use Fig. 4 as a guide. The chart derives from the expression for the dimensionless impaction parameters $P = k\rho V d^2/\eta l$, where $k$ is a constant which in practice varies from system to system, $\rho$ is the particle density, $V$ the jet velocity, $d$ the particle diameter, $\eta$ the gas viscosity, and $l$ the jet diameter (or characteristic length). For a given efficiency of impaction of particles, $P$ is constant, and in the present case we are concerned with its value at $d_{50}$, the 50% cut-off diameter. For operation in air at normal temperature and pressure, we can also take $\rho$ and $\eta$ as constants so that, as in Fig. 4, we can relate $d_{50}$, $l$, and $V$ (transformed into the more convenient flow rate parameter). The foundation of Fig. 4 was the set of six values for $d_{50}$ $(v/l)^{1/2}$ obtained from the calibration, as described in the preceding section, of the top two stages of the three models shown in Fig. 2. The values were 113, 108, 105, 122, 118, and 122 $\times$ $10^{-4}$ calculated in units of centimeters and seconds.

For any given value of $d_{50}$, the shape of the cut-off curve will be geometrically similar to those of Fig. 3.

### *Testing with Bacterial Aerosols*

To study the performance of the sampler under rigorous conditions, a procedure identical to that previously described (6, 7) was adopted. In the relatively warm and dry conditions of summer daylight, a mixed suspension in known ratio of vegetative organisms (*E. coli*), which lose viability rapidly under such conditions, particularly in full sunlight, and of nondecaying spores (*B. subtilis var. niger*) was sprayed by a pneumatic hand spray and sampled at sufficient distance downwind for appreciable cell death to have taken place. By comparing the ratio of the two components recovered from the several stages of the sampler with their ratio before spraying, the percentage of *E. coli* cells remaining viable could be determined.

In the sampling array, two pairs of samplers were employed, each shielded from direct light. Each pair consisted of a three-stage glass sampler and a "tilting" preimpinger plus impinger unit (7). The latter unit was known to have a high efficiency of intake of aerosol particles and was used as a standard reference sampler for comparison with the three-stage sampler. The two pairs were within a few yards of each other, close enough to ensure that they were exposed to comparable, but not necessarily the same, dosages.

TABLE 2. *Dosages[a] given by adjacent "tilting" and three-stage samplers in the field[b]*

| (1) Test no. | (2) Length of time airborne | "Tilting" sampler's total dosage | | Three-stage sampler | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Dosage of *E. coli* cells | | | | Dosage of tracer spores[c] | | | | Percentage of *E. coli* remaining viable[d] | | |
| | | (3) *Escherichia coli* | (4) Trace spores[c] | (5) Stage 1 | (6) Stage 2 | (7) Stage 3 | (8) Total | (9) Stage 1 | (10) Stage 2 | (11) Stage 3 | (12) Total | (13) Stage 1 | (14) Stage 2 | (15) Stage 3 |
| 1 | 60 sec | 22 | 76 | 14.3 | 2.1 | 3.1 | 19.5 | 46 | 16 | 24 | 86 | 31 | 13 | 13 |
| | | 24 | 128 | 20.5 | 9.5 | 5.0 | 35 | 52 | 40 | 17 | 109 | 41 | 24 | 29 |
| 2 | 70 sec | 374 | 1,700 | 399 | 150 | 62 | 611 | 910 | 610 | 565 | 2,085 | 44 | 25 | 11 |
| | | 398 | 1,745 | 273 | 119 | 22 | 414 | 748 | 615 | 404 | 1,767 | 37 | 19 | 5.4 |
| 3 | 65 sec | 259 | 1,559 | 390 | 121 | 93 | 604 | 933 | 298 | 398 | 1,629 | 42 | 40 | 23 |
| | | 249 | 2,160 | 220 | 118 | 64 | 402 | 625 | 384 | 255 | 1,264 | 35 | 31 | 25 |
| 4 | 55 sec | 3,774 | 9,380 | 2,310 | 990 | 1,230 | 4,530 | 4,200 | 2,600 | 3,520 | 10,320 | 55 | 38 | 35 |
| | | 3,772 | 9,720 | 1,045 | 940 | 540 | 2,525 | 3,220 | 2,630 | 1,710 | 7,550 | 35 | 36 | 31 |
| 5 | 3 min | 754 | 4,070 | 410 | 290 | 107 | 807 | 1,330 | 1,030 | 1,060 | 3,420 | 30 | 28 | 10 |
| | | 350 | 3,360 | 255 | 225 | 26 | 506 | 800 | 1,060 | 665 | 2,525 | 32 | 21 | 4 |
| 6 | 50 sec | 2,550 | 8,940 | 1,315 | 510 | 323 | 2,348 | 3,140 | 1,820 | 3,150 | 8,110 | 42 | 28 | 17 |
| | | 2,588 | 8,650 | —[e] | — | — | — | — | — | — | — | — | — | — |
| 7 | 4.5 min | 19 | 198 | 25.8 | 16.7 | 6.1 | 48.6 | 77 | 64 | 61 | 202 | 34 | 26 | 10 |
| | | 28 | 208 | 35.5 | 11.5 | 2.2 | 49.1 | 122 | 60 | 33 | 215 | 29 | 19 | 7 |
| 8 | 60 sec | 1,776 | 6,790 | 856 | 463 | 480 | 1,799 | 2,380 | 1,830 | 2,470 | 6,680 | 36 | 25 | 20 |
| | | 1,589 | 6,520 | 617 | 453 | 437 | 1,507 | 2,080 | 1,910 | 2,380 | 6,370 | 30 | 24 | 18 |
| 9 | 3.5 min | 202 | 1,192 | 100 | 58 | 6 | 164 | 365 | 280 | 205 | 850 | 27 | 21 | 2.9 |
| | | 130 | 873 | 84 | 26 | 3 | 113 | 375 | 237 | 137 | 749 | 23 | 11 | 2.2 |

[a] "Dosage" in units of cells. Minutes per liter is the total number of cells in the sample divided by the sampling rate.

[b] The three-stage sampler recorded in the second line of each test was allowed to run for 30 min after receiving its sample. The other three samplers in each test ran for only about 5 min.

[c] The recorded tracer spore dosages have been adjusted to be equivalent to a starting ratio of unity for concentration of viable *E. coli*/tracer in the sprayed suspension.

[d] Column 13 is the percentage of column 5/column 9, and so on.

[e] Pump failed.

TABLE 3. *Atmospheric conditions during field tests of Table 2*

| Test no. | Temp | Humidity (% RH) | Illumination perpendicular to sun (ft-c) | |
|---|---|---|---|---|
| | *C* | | | |
| 1 | 17.8 | 56 | 8,000 | (clear sky) |
| 2 | 18.9 | 48 | 2,000–8,000 | (5/10 cloud) |
| 3 | 19.4 | 60–65 | 800–2,000 | (overcast) |
| 4 | 16.1 | 68 | 1,500 | (overcast) |
| 5 | 18.9 | 57–61 | 2,000–8,000 | (5/10 cloud) |
| 6 | 17.2 | 53 | 2,000–7,500 | (5/10 cloud) |
| 7 | 15.6 | 58 | 2,000 | (overcast) |
| 8 | 18.3 | 54 | 8,000 | (clear sky) |
| 9 | 19.4 | 54 | 8,000 | (clear sky) |

The first pair of samplers was switched off after sampling the aerosol for a few minutes, and in the second pair the three-stage sampler was left running for 0.5 hr to test whether the continuous aspiration of the dry air over the collected particles affected their viability. During this time, about 40% of the sampling fluid (phosphate buffer plus 1 M sucrose plus antifoam) evaporated.

## *Results*

The results from nine tests are summarized in Table 2. Each test has two lines of dosage figures, one line for each pair of samplers. Table 3 gives the atmospheric conditions of the tests.

*Effect of running for 0.5 hr.* This may be judged by comparing the ratios of total dosages measured by each type of sampler in the first pair (top line of each test) with corresponding ratios in the second pair (bottom line) after extended operation of the three-stage sampler. If the extended operation caused a loss of viability, the ratio in the second pair would be less, on the average, than in the first. These ratios have been extracted, and are presented in Table 4 for the *E. coli* and in Table 5 for the spore tracer. Although in both tables the means from the second pair are slightly lower numerically, statistically they do not differ significantly from each other in either pair. Neither organism, therefore, shows appreciable loss after 30 min of aspiration in the conditions of these tests.

*Overall recovery of viable cells.* The intake and yield of viable cells in the three-stage sampler as compared with the tilting unit may also be judged from Tables 4 and 5. Means C and D in Table 5 are not significantly different from unity for the very robust spore tracer. We may conclude from this that both types of sampler in the pairs have the same intake efficiency and subsequently yield the same dosage of viable cells. In Table 4, the means A and B are significantly greater than unity, indicating that the three-stage sampler yields more viable cells of *E. coli*. This is due to the absence in the three-stage sampler of the violent sonic velocity impingement in the Porton impinger, which is known to cause death of sensitive cells (6, 14). The tests do not tell us anything about the absolute recovery of *E. coli*, as one cannot assert from the figures presented that cells are not being killed in both samplers. However, in recent work by K. P. Norris (*personal communication*), the three-stage sampler in some winter conditions of less light, higher humidity, and long aspiration times has yielded viability figures for *E. coli* in the region of 100% on all stages. This indicates that there was no killing effect in the sampler. It is reasonable to conclude from accumulated experience with various other types of sampler, as well as with the new sampler, that neither the systems of impaction nor the washing-off process from the sintered discs results in appreciable loss of viable cells. Nevertheless, workers proposing to use the sampler with very delicate organisms would be well advised to carry out viability tests under their experimental conditions.

*Effect of particle size on viability.* As one of the main functions of this sampler is to yield estimates of organism viability within the various particle size classes, it is of value to examine the information in columns 13, 14, and 15 of Table 2, relative to this point. The periods during which the cells were airborne in the daylight was either close to 1 min or 3 to 4.5 min (column 2). The percentage of *E. coli* cells remaining viable after these periods has been averaged in Table 6. After 1 min, the small particles contained only half the proportion of viable cells that the large ones did. In the longer period, the small particles have continued to die off more rapidly, to about one-fifth the viability, on the average, of the large particle figure. In the full sunlight of the final test recorded in Table 2, nearly all the *E. coli* cells in small particles succumbed in 3.5 min.

## DISCUSSION AND CONCLUSIONS

Morrow (12) has reviewed the recent position on the relationship of particle size in toxic dusts to respiratory deposition. He stresses the importance of selective sampling, which is usually designed to accept the small, lung-attacking particles and to reject the harmless large ones. For infective aerosols, selective sampling seems even more important because of two factors. First, it has been shown in the laboratory that with many infective diseases the smallest particles are the most dangerous, but it is also considered that in those infections which start in the upper respiratory tract,

Table 4. *Ratios of total Escherichia coli dosage in three-stage sampler to total in tilting sampler*

| Pair | Test no. | | | | | | | | | Geometric means[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| 1 | 0.89 | 1.64 | 2.33 | 1.20 | 1.07 | 0.92 | 2.57 | 1.01 | 0.81 | (A) 1.27 |
| 2 | 1.48 | 1.04 | 1.61 | 0.67 | 1.45 | — | 1.75 | 0.95 | 0.87 | (B) 1.17 |

[a] Means A and B do not differ significantly from each other, but each is significantly greater than unity.

Table 5. *Ratios of total tracer spore dosage in three-stage sampler to total in tilting sampler*

| Pair | Test no. | | | | | | | | | Geometric means[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| 1 | 1.17 | 1.22 | 1.02 | 1.10 | 0.83 | 0.91 | 1.02 | 0.97 | 0.72 | (C) 0.98 |
| 2 | 0.86 | 1.01 | 0.59 | 0.78 | 0.75 | — | 1.03 | 0.98 | 0.85 | (D) 0.86 |

[a] Means D and D are not significantly different from unity.

or in open wounds, etc., large particles are the most dangerous. Second, as Table 6 indicates, maintenance of viability in the airborne state may be markedly dependent upon the size of the airborne particle.

We therefore need to know the airborne concentration and state of viability of cells in various particle size ranges. It is not necessary that these ranges be narrow, sharply defined, and many in number, because physiological response to particle size effects cannot have sharp boundaries. For example, all sizes of particles can probably infect a wound, though large ones are much more likely to fall out than small ones; particles of say 6 to 7 μ are found in the alveoli, but those of 1 to 3 μ can penetrate in proportionately much greater numbers; persons differ in structure and breathing patterns, and flow rates vary widely. A selective sampler can, therefore, do no better than give a performance resembling a human average which can be generally accepted. The present model attempts this in its selection of three ranges: "large" particles, i.e., those greater than 6 μ in diameter ($\rho = 1.5$) which are normally retained in the upper respiratory tract; an intermediate range of 3- to 6-μ particles which are likely to lodge in the bronchi or bronchioles; and the fine range, 3 μ to single cells, which penetrate to the alveoli. Probably many bacterial particles are hygroscopic and will have time to increase in size in the depths of the lung. Lung retention of 1-μ viable particles should therefore be much higher than it is for dusts, many of which are expired at this size, so that the retention of 80 to 90% of single cells (*E. coli*) given in Fig. 3 seems reasonable. Single virus particles, ca. 0.3 μ and

Table 6. *Means of results from Table 2, columns 14, 15, and 16*

| Stage | Percentage of *Escherichia coli* viable | |
|---|---|---|
| | at 1 min airborne | at 3 min plus |
| Top | 38 | 29 |
| Middle | 27 | 21 |
| Bottom | 19 | 6 |

smaller, would not be retained in the sampler, whereas in the lung a high proportion would be retained by another process, diffusion to the walls. It seems unlikely, however, that such small isolated virus units can ever be generated in quantity by natural processes in air. The writer knows of no facts to confirm or refute this important point, which may be extremely difficult to resolve.

It is not claimed that the size ranges of the new sampler are necessarily the best. Further knowledge may require them to be altered in cut-off or in number, or both, and this can readily be done by use of the data in Fig. 4. The standard design might be modified by adding a top stage with similar characteristics to the top stage of model B, Fig. 2. This could distinguish large wound-infecting particles from respirable ones. In other work, a simple two-stage "upper" and "lower" respiratory tract simulation, as in stages 2 and 3 of model B, might be quite adequate. Selective sampling is based on the mass of work done on the effects of toxic industrial dusts on the relatively few workers exposed to them, whereas much less seems to be known about the equivalent

properties of the causative agents of airborne infection, to which all members of society are exposed. It is hoped that the new sampler, modified if necessary and used perhaps in conjunction with those of class I, will extend the latter field of knowledge.

The usefulness of the sampler is not necessarily confined to viable aerosols. It might well be used in air pollution work, indoors and out, and anywhere where respiratory irritation is related to particle size.

Finally, it may be mentioned that the gap between class I and class II samplers may be bridged by using soluble gel collection surfaces (gelatin with glycerol), which may afterwards be washed off in warm water for dilution and plating to give a single cell count. Both the Andersen (1) and Battelle (11) impactor configurations can easily be converted to this use. Studies on these lines were carried out in the present work, and were quite promising, except that the problem of finding a sufficiently stiff yet soluble gel to resist the high velocity impingement in the final stage was not solved. Microscope studies showed that impinged cells could not be effectively washed off agar surfaces, and dry surfaces cannot be used because of death of cells by desiccation. Also, the preparation of gel surfaces requires extra work, and it was concluded that the glass-liquid model is preferable because it offers greater simplicity in use, gives a reliable performance, and is inexpensive to manufacture.

## AVAILABILITY

The sampler may be obtained from A. W. Dixon and Co., England. The design is patented (No. 1964 65) by the British Government.

## ACKNOWLEDGMENTS

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# Air Sampling for Respiratory Disease Agents in Army Recruits

MALCOLM S. ARTENSTEIN AND WILLIAM S. MILLER

*Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D.C., and U.S. Army Biological Center, Fort Detrick, Frederick, Maryland*

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## INTRODUCTION

Among the few reports relating to the quantitative parameters of the transmission of many types of respiratory infection, are those of Riley for tuberculosis (4) and Tigertt et al. for Q fever (5). These reports suggest that adequate quantitative information can be obtained in natural situations only if it is possible to sample volumes of air which are very large in relation to the respiratory volume of man.

In a recent epidemiological study, we had the opportunity to employ the Large Volume Air Sampler (LVS) described informally by William Perkins at this meeting. The device has been described in detail by the manufacturer (Rept. 2586 of Litton Industries, Inc., Minneapolis, Minn.). The present report indicates a technological potential of importance, in spite of the preliminary nature of the data and the present lack of estimates of quantitative reliability.

## EPIDEMIOLOGICAL PROBLEM

Acute respiratory disease (ARD) in Army recruits occurs in epidemic form in basic training centers all over the United States. The disease is caused mainly by adenoviruses, especially type 4, and occurs regularly each winter in new recruits during the 2nd, 3rd, and 4th weeks of the basic training cycle. Meningococci and group A streptococci are other common respiratory pathogens which may produce epidemic disease in recruits. Rates of infection and febrile illness vary during the year and from year to year, owing to a number of poorly understood factors, including physical and emotional stresses.

The pattern of a typical ARD outbreak at a training camp is depicted in Fig. 1. Adenovirus infections began to occur during the second week of training. Viral isolation attempts were performed at weekly intervals and yielded positive results from 37 of the 48 men (77%). Of the remainder, five had type-specific serum antibody at the time of first sampling and did not develop the infection, and six men showed rises in antibody titers, but without illness. Thus, of the susceptible subjects, all responded either by shedding virus or with antibody production. Twenty-seven of the infections were associated with febrile illness.

Bacterial studies of the throat and nasopharynx showed that meningococcal carrier rates among recruits increased from 42% upon entry in the Army to a peak of 90% during the 5th week of training. The curve for meningococcal incidence lagged about 1 week behind that of adenovirus type 4. We questioned whether adenovirus infections were responsible for the spread of meningococci in a manner analogous to the "cloud baby" spread of staphylococci (2). Sulfonamide-resistant strains of meningococci were initially absent, but accounted for 25 to 30% of strains isolated in the 6th through 8th weeks. Group A streptococcus carrier rates were rather stable and at a low level during the period of observation. (The excess cases shown in Fig. 1 in the final 2 weeks were associated with nontypable strains.) In the population under study, then, there were at least three different respiratory pathogens, each of which appeared to have a different pattern of transmission.

These recurring epidemiological features presented an excellent opportunity for the study of microbial transmission. The initial studies were concerned with detection of aerosols of the agents, which might serve as a source of infection. The LVS was employed in order to have maximal sensitivity of detection.

## LVS STUDIES

In January 1966, during a period when attack rates were high, attempts were made to sample air in the vicinity of ill recruits. A number of patients were studied individually in a hospital room of 1,440 $ft^3$ capacity. Attempts were made to isolate adenovirus type 4 and meningococci from throat gargles and throat swabs, respectively, and from the collecting fluid of the LVS.

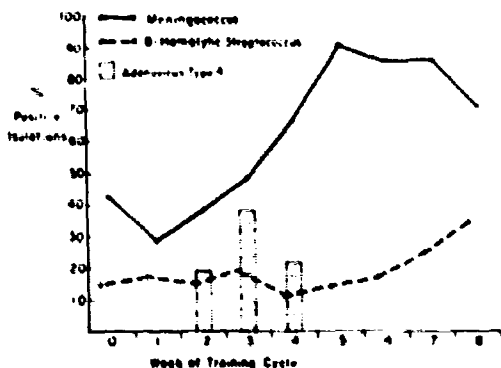

FIG. 1. *Pattern of a typical ARD outbreak among army recruits.*

In one such study, the patient was in the room for 10 min coughing frequently, before sampling began. A nasopharyngeal swab sample was found to be positive for group B meningococci, and a throat wash was positive for adenovirus type 4.

In 5 min of sampling, a total of 1,785 ft³ of air was drawn through the LVS, and the particulate content was collected in a total of 180 ml of sampling fluid. One group B meningococcal cell was recovered for every 99 ft³ of air, and 1 adenovirus unit per 277 ft³ of air. The latter unit was a tissue-culture infective dose employing 1 ml of sampling fluid as inoculum

Several attempts at sampling for adenovirus type 4 in barracks were made in training companies in which epidemic disease was beginning. Samples were collected during early evening when activity was at a peak, and again later after "lights out" when recruits were in bed. Results to date are incomplete. Data on adenovirus type 4 recoveries in two trials, however, are given in Table 1.

TABLE 1. *Results of air sampling for adenovirus type 4 in barracks[a]*

| Trial no. | Activity | Vol of air sampled | Recovery |
|---|---|---|---|
| | | ft³ | |
| 31 | 15 men making beds, coughing frequently | 6,700 | 1 tissue culture infective unit[b] per 920 ft³ |
| 32 | 15 men in bed, coughing frequently | 5,700 | 1 tissue culture infective unit per 2,820 ft³ |

[a] Room size, 13,347 ft³.

[b] Infected tissue culture after inoculation of 1 ml of sampler collecting fluid.

## DISCUSSION AND CONCLUSIONS

These examples indicate that the LVS can provide bacterial and viral isolations from air collected in field situations. Meningococci were found in a concentration of one viable particle per 100 ft³ of air, whereas with adenoviruses one tissue culture infective dose was found in 300 to 3,000 ft³ of air. Although the results presented above can only be considered as preliminary data, they do indicate the need for sampling large volumes of air in studies of naturally produced aerosols. It is readily apparent that an all glass impinger, operating at 12.5 liters per min (6), is inadequate for collecting such low concentrations. These results may explain our failure in the past to detect infective particles in epidemiological sampling with an all glass impinger (1).

The experiments provide some of the information that Morton (3) had in mind in 1963 when he proposed four "postulates" relating to the epidemiology of airborne infection. They were as follows: (i) one must demonstrate the presence of airborne viable infective organisms; (ii) one must measure concentrations and particle sizes; (iii) one must demonstrate experimentally that concentrations and particles of this sort can cause infection; and (iv) one ought to show directly where the particles have come from. The present experiments show that the LVS can recover airborne, viable organisms at very low concentrations in natural aerosols. These studies have not demonstrated infectivity for man of the organisms collected, nor have they proved the source of the organisms.

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James Rust and the Department of Bacteriology, Walter Reed Army Institute of Research, performed the bacterial studies. Thomas Lemson, Preventive Medicine Officer, Fort Dix, N.J., collaborated.

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# Discussion

O. M. LIDWELL

*Central Public Health Laboratory, Colindale, London, England*

## INTRODUCTION

In any discussion of air sampling methods, the practical consequences of the divergent interests of the participants quickly become apparent.

One group of workers, exemplified by the Microbiological Research Establishment at Porton, England, and by the Army and Navy Biological Laboratories here in the United States, are primarily concerned in the sampling of artificially generated clouds. This often involves higher air concentration, single species of microorganism, small particle sizes, and relatively little extraneous organic material.

Another, more diffuse group, to which I belong, is concerned with the sampling of the airborne flora of occupied places and the relation of this to infection and disease in ordinary life.

The airborne organisms of interest in these environments are usually present in small numbers only, accompanied by much larger numbers of a wide variety of other species; particle sizes extend above 20 μ equivalent particle diameter, and the typical particle is probably principally composed of dried secretion or cellular debris.

In spite of these differences, however, the technical problems involved have sufficient fundamental similarity for me to hope that the topics I am going to discuss briefly are of concern to both.

## COLLECTION OF THE SAMPLE

### *Separation of the Particulates from the Air*

This is the primary problem of all air sampling. The physical principles involved are now generally understood (3), but there are two particular points worthy of consideration.

First, when looking for a particular species in high dilution, it is helpful to collect the organisms into as small an amount of medium as possible. The level of interest may be as low as one or two viable cells in 100 ft³ of air or more. We have experimented on two approaches with a view to sampling for respiratory virus. In contrast to May's experience (10), we have found it possible to recover the great majority of the cells collected by impingement onto an agar surface by washing with a small volume of fluid while rubbing with a glass rod, both with and without previous coating of the agar surface (6). Alternatively, the airborne particles have been impinged onto the surface of a stainless-steel cylinder which is kept moist by continual rotation in fluid medium (Fig. 1). A more complex principle, which has been employed in some commercial air cleaners (Cyclone type air cleaners with separated air-flow—Rotonamic, Farr Co., Los Angeles, Calif.), is to concentrate the particulate material into a part of the air stream which is then bled off into the sampler proper.

The second general point concerns the provision of efficient, quiet air suction devices. The energy required to extract 10 liters of air per min against 10 cm of water pressure is only ⅙ of a watt. Even 600 liters per min at 30 cm of water pressure represents only 30 w. (These figures represent the two extremes in the air samplers we use.) Measured against these figures, the pumps we commonly employ are both noisy and very inefficient. At the lower end of the scale, we have just succeeded in building a pump with a 4-inch neoprene diaphragm having a mechanical efficiency of about 60%.

Its present motor, about 35% efficient, takes 150 ma at 6 v or 0.9 w, an overall efficiency of nearly 20%. Motors with 60 to 70% efficiencies are now available in this size. A quiet, efficient pump handling several hundreds of liters of air per minute at back pressures up to 25 to 35 cm of water would be a real asset.

## SIZE DISTRIBUTION OF THE PARTICLES

Selective impaction, either onto solid or liquid medium, is probably the best method we have. Very elegant methods employing sedimentation over fixed distances through nonturbulent air streams, sometimes in a centrifugal field, have been employed for dust analysis (14, 15), but not, so far as I know, for microorganisms. These lead to much sharper size separations than the impaction devices, where the overlap between the size groups is uncomfortably large. The restricted working size range is a further limitation to several designs. In occupied places, microorganisms are commonly associated with particles whose median equivalent diameter is about 12 to 15 μ (11). Such clouds can only be adequately sized if the first stage cut-off of the sampler approaches 20 μ or larger.

## COMPOSITION OF THE PARTICLES

This is not often considered as a sampling problem, but it may often have important epidemiological implications. Rubbo and his co-workers (13) attempted to demonstrate an association between cotton fiber and airborne bacteria in hospital

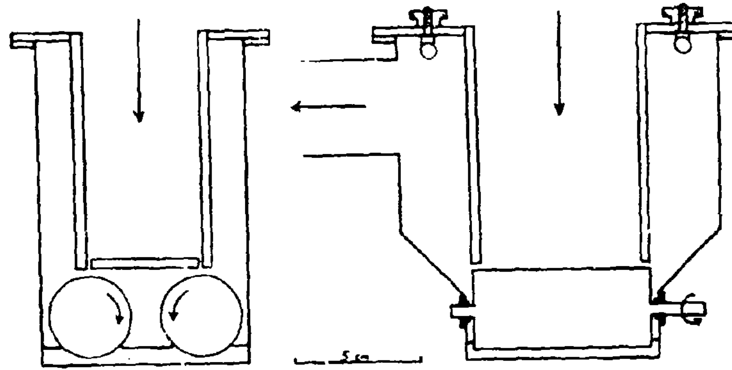

FIG. 1. *"Stamp-licker" air sampler; two sections at right angles. The perspex entry duct terminates in two slits 1 mm wide by 5 cm long, each with their lower faces 2 mm above the top of a stainless-steel cylinder 2.5 cm in diameter. The matt-surfaced cylinders are rotated in polytetrafluoroethylene bushes by an external motor at 6 rev/min and dip into the collecting fluid. The main body of the sampler is fabricated from stainless-steel sheet, and the entry duct is clamped down onto locating blocks, with a sponge rubber sealing gasket. Directions of air flow and of rotation of the cylinders are shown by arrows.*

wards. Davies and Noble (4) suggested that *Staphylococcus aureus* is often found associated with skin fragments. Some indication of the effective disseminating sources of, for example, *Clostridium welchii* might be found along these lines. Another aspect of the composition of the airborne particles is the number of viable cells contained in it. The clumps of cells in the larger particles of artificially generated clouds will often be dispersed in liquid medium. In this case, comparison between colony counts obtained by impaction on solid media and the recovery from fluids enables an estimate of the mean number of viable cells per clump to be obtained. Species, such as certain streptococci, whose cells do not separate easily during multiplication, obviously cannot be examined in this fashion. More generally, it appears that the viable cells in many, if not most, naturally occurring particles cannot be easily separated (1). Dispersion by mechanical methods or by chemical (e.g., enzymatic) action has been suggested but not adequately explored (7, 9). Estimation of clump size from the shape of the dose-survival curve resulting from exposure to a sterilizing process with a constant chance of cell death per unit dose increment is essentially laborious, and a suitable sterilizing process cannot always be found. Electron bombardment has been used for staphylococcus-carrying particles (8), but the killing action of this and other radiations is often complex. Knowledge of the clump size is important in infectivity studies. The dose may differ substantially from the particle estimate, and this difference may vary with, for example, the age of the suspension, as death of cells reduces the average number of viable units per particle.

## SELECTIVE CULTURAL METHODS

The viable cells carried on dry airborne particles differ from those present in liquid cultures. They appear to be more resistant to the action of ultraviolet radiation, although it is not clear to what degree this is a property of the cells themselves or to what degree it depends on the nature of the matrix in which they are embedded (2). They may differ in infectivity and virulence (5, 12), and they may differ in antigenic structure and power to combine with immunofluorescent reagents. More pertinent here, they are often less able to grow on selective media (16). How far this relative disability is affected by a preliminary hold time in a collecting fluid I do not know. In practice, this cell defect, whatever its nature or cause may be, is a severe limitation. Sometimes a useful discrimination in favor of the species of interest without significant loss can be obtained by reduction in the concentrations of the selective agents. An alternative approach is to grow the organisms to microcolony size on an unselective medium and then to replicate onto one or a variety of selective media (16). The transferred cells are then in their growth phase and are better able to continue growth in the inhibitory medium.

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# Assessment of Experimental and Natural Viral Aerosols

PETER J. GERONE, ROBERT B. COUCH, GARRETT V. KEEFER, R. GORDON DOUGLAS, EDWARD B. DERRENBACHER, AND VERNON KNIGHT

*U.S. Army Biological Center, Fort Detrick, Frederick, Maryland, and Laboratory of Clinical Investigations, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Bethesda, Maryland*

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## INTRODUCTION

This report describes a number of characteristics of artificially prepared aerosols containing coxsackievirus A, type 21, a virus that causes respiratory illness in man. Studies on natural aerosols produced by subjects who have been infected with this virus are also described. The findings are part of a continuing program of investigation of the role of aerosols in human viral respiratory disease conducted as a joint undertaking by the U.S. Army Biological Center, Fort Detrick, Frederick, Md., and the Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

The report is divided into two sections. The first deals with observations on the properties of laboratory-generated viral aerosols used for inoculation purposes, and the second covers the production of viral aerosols by experimentally infected subjects and the contamination of air in rooms occupied by them.

The program has availed itself of a large body of information concerning bacterial aerosols and was aided by some new techniques pertinent to viral aerosols. The work so far has provided a sound experimental basis for a broad approach to the problem of the role of viral aerosols in human respiratory disease, and the information already gained has indicated a possible significance for this mode of dissemination of these infections.

## RESULTS

### *Preparation and Properties of a Small-Particle Viral Aerosol*

Studies with artificially prepared small-particle aerosols were undertaken to provide better control of the site of inoculation than was possible with liquid suspensions instilled into the noes. Opportunity was also provided to make observations on virological and physical properties of this form of viral suspension. The results to date are limited to findings with coxsackievirus A, type 21, but the methodology is applicable to agents belonging to three other major virus groups: adenoviruses, rhinoviruses, and influenza viruses.

An aerosol apparatus originally designed for use with a bacterial organism (5, 8, 11) and the Collison atomizer (2, 9) were selected for evaluation. The aerosol was generated from a safety-tested, tissue culture suspension of virus (4, 10). The equipment produced a heterogeneously sized, small-particle aerosol under the conditions in which it was used. The sampling instrument used in these studies was the Shipe impinger (16). It contained 5 to 10 ml of a suitable cell culture medium that could be used directly in the selected assay system. The high efficiency of the Shipe impinger for the collection of virus from these aerosols has been established. About 50% of the total virus atomized was recovered.

Preliminary experiments were performed to determine the relationship between the concentration of the viral suspension to be sprayed and the viral concentration of the resulting aerosol. This information was essential to provide a degree of control over doses of virus to be administered. Figure 1 shows data collected with coxsackievirus A-21. It is apparent that a direct relationship exists between the concentration of the virus in the spray suspensions and that of the aerosol. With this information, it was possible to estimate, within an acceptable range, doses of virus to be administered to volunteers by appropriate dilution of the spray suspension. The actual dose administered was determined at the time of each inoculation (4).

Another factor of concern with both the experi-

mental and natural aerosols was the distribution of virus in aerosols of heterogeneous particle size. It was important to know whether virus concentration followed the volume distribution of the aerosol or whether some unknown selective force caused an unexpected concentration of virus in particles of one size or another. To answer this question, the concentration of virus was measured in aerosol particles of various size ranges. The particle-size distribution of the aerosol was determined by direct microscopic measurement, and virus was collected in an Andersen sampler (1). The plates were prepared by pouring a 21-ml base layer of hard agar and, after this solidified, an overlay of 6 ml of 12% gelatin was added (6). The agar served to place the gelatin surface at the proper level below each sieve plate. After sampling, the gelatin in the plates was liquefied at 37 C and was removed for virus assay. Figure 2 shows the results of one of these experiments. As can be seen, the virus concentration appeared to be more closely related to the volume distribution rather than the particle number distribution of the aerosol. Similar findings (1) have been reported for bacterial aerosols.

Particle sizing of virus aerosols, both experimental and natural, presented no unique problems. Standard techniques with use of cascade impactors, membrane filters, and settling slides were used without modification (14).

*Viral Aerosols Produced by Infected Persons*

For present purposes, natural aerosols are defined as those arising directly or indirectly from infected volunteers. The events that were considered to be possible sources of viral aerosols

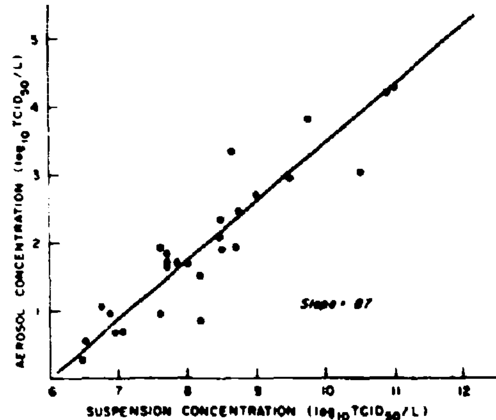


FIG. 1. *Relationship of coxsackievirus A-21 concentrations in spray suspensions and aerosols. Reproduced by permission from reference (4).*

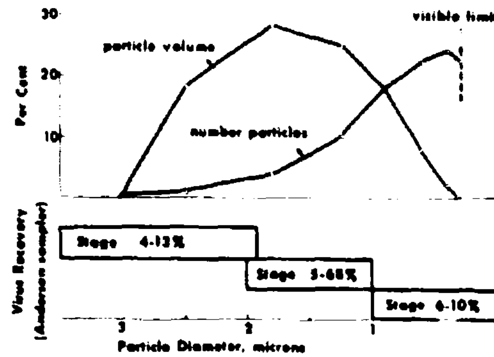


FIG. 2. *Distribution of coxsackievirus A-21 in an aerosol heterogeneous in particle size. Reproduced by permission from reference (4).*

included sneezes, coughs, talking, and breathing. Because talking and breathing produced relatively few particles, our studies were concentrated on the sneeze and cough.

Two procedures were devised to examine the aerosols produced in coughs and sneezes by infected volunteers. One was used to recover virus from coughs and sneezes, whereas the second was principally concerned with sizing and distribution of particles in the aerosol.

Recovery of virus from aerosols and droplets produced by coughs and sneezes was accomplished by having the volunteer sneeze or cough into a deflated weather balloon (Fig. 3). The balloons were washed several times to remove as much talc as possible. They were sterilized while submerged in buffered saline and then stored in a refrigerator. Prior to use, the excess fluid was removed and replaced with 10 ml of cell culture medium. The balloon was attached to a face mask that provided a tight fit around the nose and mouth of the volunteer. After the volunteer sneezed or coughed, the neck of the balloon was clamped off. By use of a Shipe impinger, the air phase of the balloon was immediately sampled. The inlet on the critical orifice was modified from the usual blunt-end capillary to a funnel shape to reduce the loss of larger particles (>5 $\mu$) by impaction (12). The balloon was reinflated with laboratory air, and the wall inside was carefully rinsed with 10 ml of medium. The impinger fluid was assayed for virus directly. The wash medium from the balloon was clarified by centrifugation, and the supernatant fluid was assayed for virus. This procedure gave the approximate amount of total virus in a sneeze or cough, and roughly defined the airborne component as distinct from the portion that either impacted on the inner wall of the balloon or immediately fell out because of large-particle size.

FIG. 3. *Use of a weather balloon for the entrapment of sneezes and coughs.*

Some examples of results obtained by use of this technique on volunteers infected with coxsackievirus A-21 are given in Tables 1, 2, and 3. These results are presented to illustrate that the procedure can be used for detecting virus in these expiratory events. Although the quantities of virus recovered range from a few $TCID_{50}$ to several thousand, the results cannot be considered in absolute quantitative terms. There is little doubt, however, that virus can be aerosolized in the process of sneezing or coughing, and that, in some instances, sufficient quantities are expelled which could account for infection of susceptible individuals in the environment.

Particle-size analyses were made on sneezes and coughs collected in a 127-liter stainless-steel chamber. The chamber was shaped as a truncated cone to minimize impaction of particles on its sides (Fig. 4). It was equipped at the small end with a pneumatic tube that tightly fit the facial contour around the nose and mouth. At the opposite end of the chamber were several sampling ports that would accommodate impingers, impactors, Andersen samplers, and a particle-size analyzer (13). A large weather balloon could be inserted into the chamber with its mouth open to the outside. This balloon would inflate as the aerosol was sampled, avoiding the dilution of the aerosol with outside air. Preliminary particle-size analyses showed that the particle content of room air obscured the particles produced by the sneeze or cough. To circumvent this problem, the volunteer was placed in a plastic tent that was continuously purged with filtered air, as was the chamber. After several minutes of deep breathing in this environment, the particles were almost completely removed and reliable measurements could be made.

An example of the particle-size distribution of aerosols from sneezes and coughs, by use of this equipment, is shown in Table 4. In comparing the sneeze and cough from a single volunteer, it may be noted that the particle-size distributions were similar. The sneeze produced 18 times more particles than did the cough. The volume of the sneeze was about 30 times that of the cough.

Particles above 15 $\mu$ in diameter presented a special problem which has not been successfully solved. Because of their high settling rate and low concentration, no attempt was made to enumerate these particles.

### *Air Sampling in the Environs of Infected Volunteers*

After it was established that the infected human volunteer did produce airborne virus, it was of interest to determine whether virus could be recovered from the room air surrounding the subjects. Preliminary calculations were based on the average volume of oral secretions in a sneeze, the expected titer of virus in oral secretions, and the volume of the room. If volunteers harbored $10^4$ $TCID_{50}$ of virus per milliliter of oral secretions, sneezed 100 times in a closed room (70,000 liters in volume), and atomized $5.9 \times 10^{-4}$ ml of secretions with each sneeze, 12,000 liters of air would have to be sampled to recover 1 $TCID_{50}$ of virus. Any biological and physical losses of airborne particles would tend to increase the volume of air that must be sampled. It was apparent, therefore, that devices that sampled 10 to 30 liters of air per minute were impractical for use in these studies. This eliminated from consideration virtually every commonly used sampling device.

The apparatus that was selected for these studies was a newly developed large-volume sampler (LVS; designed by Litton Systems, Inc., Minneapolis, Minn., under contract with Fort Detrick) that functioned by electrostatic precipitation (Fig. 5 and 6). It is capable of drawing air flows up to 10,000 liters per minute. The air passes through a high-voltage corona that charges particulate matter, causing it to precipitate on a grounded disc. The disc rotates at 200 to 300 rev/min and is covered with a thin, flowing film of collecting fluid. The diluent used in

TABLE 1. *Recovery of coxsackievirus A-21 from coughs of volunteers by use of the balloon technique*

| Volunteer no. | Source | $TCID_{50}$ of virus | | | | | | | Positive tests[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | 4 days[a] | 5 days | 6 days | 7 days | 11 days | 14 days | 29 days | |
| 1 | Air | 30 | 48 | 25 | 0 | 25 | 10 | 0 | 6/7 |
| | Wall | 0 | 0 | 260 | 30 | 0 | 0 | 0 | |
| 2 | Air | 90 | 0 | 0 | 0 | 0 | 0 | —[c] | 1/7 |
| | Wall | 0 | 0 | 0 | 0 | 0 | 0 | — | |
| 3 | Air | 0 | 0 | 0 | 0 | 0 | 0 | — | 1/7 |
| | Wall | 0 | 0 | 0 | 30 | 0 | 0 | 0 | |
| 4 | Air | 90 | 0 | 0 | 10 | 0 | 0 | 0 | 2/7 |
| | Wall | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |

[a] Days after exposure.
[b] Number of positive coughs/total tested.
[c] Not tested.

TABLE 2. *Shedding of coxsackievirus A-21 by human volunteers*

| Volunteer no.[a] | Specimen | $TCID_{50}$ | | | | | Positive/total[c] |
|---|---|---|---|---|---|---|---|
| | | 3 days[b] | 4 days | 5 days | 6 days | 7 days | |
| 1 | Oral secretion[d] | >32,000[d] | 30 | 100 | 3,200 | 100 | 5/5 |
| | Cough | | | | | | |
| | Air[e] | 90 | 0 | 0 | 0 | 0 | 1/5 |
| | Wall[e] | 30 | 0 | 0 | 0 | 0 | |
| | Sneeze | | | | | | |
| | Air | —[f] | 0 | 0 | 0 | — | 2/3 |
| | Wall | — | 0 | 30 | 15 | — | |
| 2[g] | Oral secretion | | | 0 | 100 | 3,200 | 2/3 |
| | Cough | | | | | | |
| | Air | | | 5 | 15 | 0 | 2/3 |
| | Wall | | | 0 | 0 | 0 | |
| | Sneeze | | | | | | |
| | Air | | | 0 | 0 | 90 | 1/3 |
| | Wall | | | 0 | 0 | 800 | |

[a] In a third volunteer, all specimens were negative (not infected).
[b] Days after exposure.
[c] Number of positive specimens/total tested.
[d] $TCID_{50}$ per 0.2 ml of secretion.
[e] Balloon technique (see text).
[f] Not tested.
[g] Began shedding virus on day 5.

our experiments was Eagle's basal medium containing 20% calf serum, and antibiotics to reduce bacterial and fungal contamination. About 125 ml of medium was recirculated through the apparatus. Evaporation over a 3.5 min period caused a loss of about 25% of the fluid.

Preliminary tests to determine the efficacy of the sampler were carried out in a room with a volume of 32,800 liters. A suspension of coxsackievirus A-21 was atomized into the room by a University of Chicago Toxicity Laboratories (UCTL) atomizer (15), and the aerosol was circulated by a 15-inch fan directed toward the aerosol stream at a 90° angle (Fig. 7).

Since most determinations were made on aerosol concentrations below the threshold of other sampling devices, there was no base line for comparison. It was necessary, therefore, to calcu-

TABLE 3. *Shedding of coxsackievirus A-21 by human volunteers*

| Volunteer no. | Specimen | $TCID_{50}$ | | | | | Positive total[b] |
|---|---|---|---|---|---|---|---|
| | | 3 days[a] | 4 days | 5 days | 6 days | 7 days | |
| 1 | Oral secretion | 10,000[c] | 1,000 | 1 | 10 | 100 | 5/5 |
| | Cough | | | | | | |
| | Air[d] | 10 | 150 | 0 | 0 | 0 | 2/5 |
| | Wall[d] | 0 | 400 | 0 | 0 | 0 | |
| | Sneeze | | | | | | |
| | Air | --[e] | 0 | 0 | 0 | 0 | 0/4 |
| | Wall | -- | 0 | 0 | 0 | 0 | |
| 2[f] | Oral secretion | | 0 | 100 | 10 | 0 | 2/4 |
| | Cough | | | | | | |
| | Air | | 10 | 480 | 0 | 0 | 2/4 |
| | Wall | | 0 | 80 | 0 | 0 | |
| | Sneeze | | | | | | |
| | Air | | 0 | 4,800[g] | 0 | 9 | 2/4 |
| | Wall | | 0 | 500,000[g] | 0 | 1,600 | |
| 3[h] | Oral secretion | | | | 1 | 10 | 2/2 |
| | Cough | | | | | | |
| | Air | | | | 15 | 0 | 2/2 |
| | Wall | | | | 160 | 30 | |
| | Sneeze | | | | | | |
| | Air | | | | - | 0 | 0/1 |
| | Wall | | | | | 0 | |

[a] Days after exposure.
[b] Number of positive specimens/total tested.
[c] $TCID_{50}$ per 0.2 ml of secretion.
[d] Balloon technique (see text).
[e] Not tested.
[f] Began shedding virus on day 4.
[g] Gross nasal secretions were expelled by the sneeze.
[h] Began shedding virus on day 6.

late the efficiency of the apparatus from the amount of virus atomized. Figure 8 shows the results of these experiments. Recoveries ranged as low as 0.6% to as high as 71%, with the vast majority falling between 1 and 20%. It is significant that virus was recovered in all experiments in which the predicted aerosol concentration was 0.001 tissue culture infectious unit (TCIU) per liter or greater. [Concentration was estimated by the dilution method of Fisher and Yates (7).]

In trying to establish the best method for handling the fluid from the LVS prior to assay, a number of techniques were employed in an effort to concentrate the virus and reduce the problem of contamination. These included both high- and low-speed centrifugation, sonic disruption, extraction with trichlorotrifluoroethane, and sometimes no treatment at all. Although these procedures were more or less successful in reducing contamination or reducing the volume of fluid to be tested, they did not seem to alter the per cent recovery.

In the interpretation of these recovery values, several factors must be considered:

(i) The sampling period was based on one turnover of room air through the sampler. Since the effluent air was returned to the room, the maximal efficiency would not be expected to exceed 66%.

(ii) No measurement of biological or physical loss of the aerosol was made. Any losses of this nature would reduce the maximal per cent recovery that would be expected.

(iii) When contamination of the cell cultures occurred, the tubes were eliminated from the assay, and it was noted that a low recovery value was obtained in these instances.

A second series of experiments was done in a similar manner, except that a tracer, sodium fluorescein, was incorporated into the virus suspension to be atomized, and large concentrations of virus were used. With these large concentrations of virus, it was possible to make direct comparisons between the LVS and the Porton all-glass impinger (AGI), a common laboratory

FIG. 4. *A stainless-steel, 127-liter chamber for the collection of sneezes and coughs.*

TABLE 4. *Airborne portion of a representative sneeze and a representative cough*

| Particle diam | Sneeze | | Cough | |
|---|---|---|---|---|
| | No. of particles | Vol | No. of particles | Vol |
| μ | | $μ^3$ | | $μ^3$ |
| <1 1 | 800,000 | 167,000 | 66,000 | 13,860 |
| 1-2 | 686,000 | 1,210,000 | 21,300 | 37,701 |
| 2-4 | 101,000 | 1,427,000 | 2,800 | 39,564 |
| 4-8 | 16,000 | 1,800,000 | 700 | 79,100 |
| 8-15 | 1,600 | 1,270,000 | 38 | 30,248 |
| Total | 1,604,600 | 5,874,000 | 90,838 | 200,473 |

* Ratio of number of particles in a sneeze to number of particles in a cough was 17.6:1; the ratio of volume of a sneeze to volume of a cough was 29.3:1.

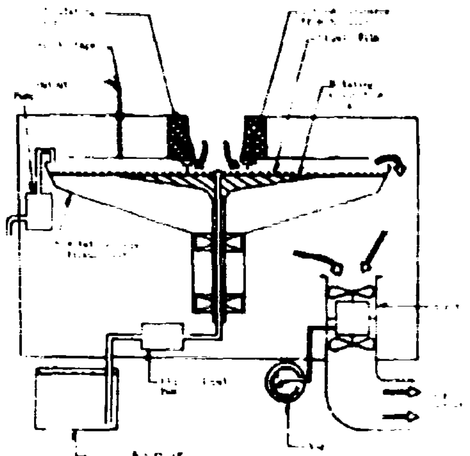

FIG. 5. *Schematic diagram of the air and liquid flow systems of the large-volume air sampler.*

aerosol sampler. The LVS was operated for a 3.5-min period, whereas the AGI were operated for 1 min (12.5 liters per minute of flow). Based on the total amount of virus and fluorescein aerosolized into the room and the amounts recovered in the samplers, recovery rates were calculated. Table 5 shows that the LVS consistently recovered more fluorescein than the AGI. The virus recovery

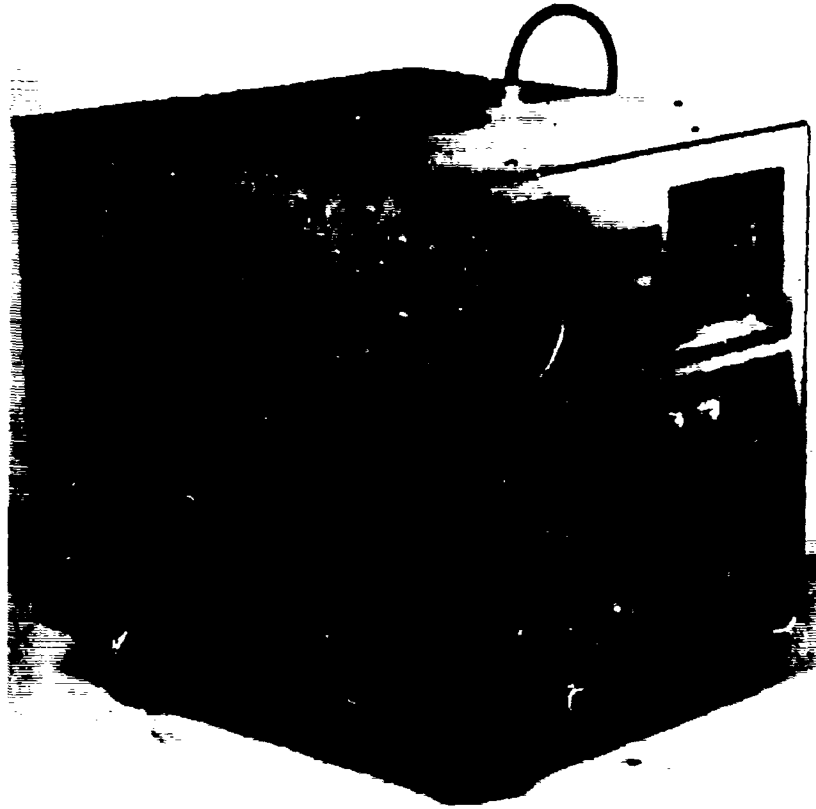

FIG. 6. *Photograph of a large-volume air sampler.*

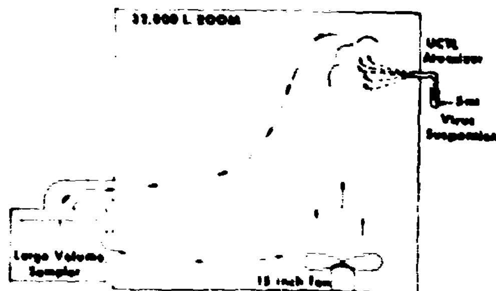

FIG. 7. *Sampling arrangement for testing the efficiency of the large-volume air sampler.*

rates exhibited variability between samples. It was also significant that the recovery rates of the samplers were not changed in situations where sampling was started after the aerosol generator was stopped. These results suggest that the LVS is a highly efficient sampler and that biological inactivation of the virus did contribute to the low recoveries in earlier experiments (Fig. 8).

The large-volume sampler was used for the detection of virus in the air of rooms occupied by volunteers experimentally infected with aerosols of coxsackievirus A-21. Prior to sampling, the

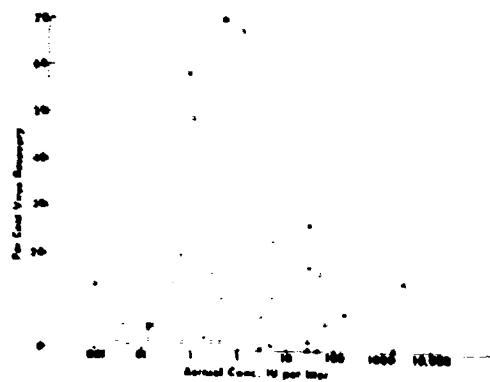

FIG 8. *Recovery of coxsackievirus A-21 from aerosols of varying concentrations by use of the large-volume air sampler.*

ventilation was turned off for a 2- to 4-hr period. The room was closed except for entry for the sampling. During the 2- to 4-hr period, no restrictions were imposed on the volunteers, and routine activity was normal. The sampler was operated for a 12-min period, which amounted to sampling 120,000 liters of air. The room volume was 70,000 liters. It was estimated that about 82% of the room air was sampled by this procedure. The sampling fluid was immediately frozen and stored for subsequent assay in cell cultures.

The results of one experiment in which two rooms were sampled twice daily for 5 days are shown in Table 6. Virus was recovered from 5 of these 16 samples tested. Overall recovery rates revealed a distinct relationship between the quantity of virus in secretions and recovery of virus in the LVS (3).

## DISCUSSION

The purpose of these studies was to describe procedures employed in studies on the role of viral aerosols in human viral respiratory disease. The results showed that viral aerosols prepared with the Collison atomizer can be adjusted to a desired content of virus, and that the size distribution of such aerosols coincides to most particles produced in sneezes and coughs from infected volunteers. Thus, the convenience and precision of the technique and its resemblance, at least in part, to natural viral aerosols indicate its potential utility for studies of this kind.

TABLE 5. *Recovery of coxsackievirus A-21 and fluorescein from room aerosols*

| Expt no. | Conditions of sampling | Sampler | Per cent recovery: Virus | Per cent recovery: Fluorescein |
|---|---|---|---|---|
| 7 | During spraying | LVS | 1.2 | 64 |
| | | $AGI_1$ | 2.5 | 46 |
| | | $AGI_2$ | 6.0 | 45 |
| | | $AGI_3$ | 2.5 | 41 |
| 8 | After spraying | LVS | 16.0 | 64 |
| | | $AGI_1$ | 0 | 42 |
| | | $AGI_2$ | 32.0 | 43 |
| | | $AGI_3$ | 16.5 | 39 |
| 11 | After spraying | LVS | 18.8 | 74 |
| | | $AGI_1$ | 2.5 | 42 |
| | | $AGI_2$ | 3.0 | 47 |
| | | $AGI_3$ | 2.5 | 47 |
| 12 | During spraying | LVS | 7.0 | 65 |
| | | $AGI_1$ | 5.4 | 46 |
| | | $AGI_2$ | 3.0 | 52 |
| | | $AGI_3$ | 2.5 | 50 |
| Avg | | LVS | 10.75 | 66.8 |
| | | AGI | 7.13 | 45.0 |

TABLE 6. *Recovery of coxsackievirus A-21 from room air by use of the large-volume sampler*

| Room no. | Sampling time | Titers of virus by days after exposure: 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| 211 | 7:00 AM | [a] | 0 | 185 | 5 | 0 |
| | 10:00 PM | [a] | 0 | 0 | 0 | – |
| | No. positive/no. tested[b] | 1/[illegible] | 1/3 | 2/3 | 2/3 | 2/3 |
| 215 | 7:00 AM | [a] | 0 | 0 | 90 | 90 |
| | 10:00 PM | 0 | [a] | 75 | 0 | |
| | No. positive/no. tested[b] | 1/3 | 2/[illegible] | 3/3 | 3/3 | 3/3 |

[a] Not done.

[b] Number of volunteers having virus-positive saliva, cough, or sneeze, or all three, over total in the room.

Virus was recovered from coughs and sneezes by collection in a weather balloon. The disadvantages of this procedure were that only a rough approximation of airborne virus could be obtained and that it was not practical to measure the size of the airborne particles.

The particle-size studies were best performed in a rigid, stainless-steel chamber. These were accomplished by a combined use of a cascade impactor and a particle-size analyzer. The larger particles were not measured by these procedures, because they did not remain airborne long enough and because they were present in relatively low concentrations.

The use of a large-volume sampler to detect virus aerosols in room air proved to be useful, and the presence of virus in the environmental air of infected subjects was demonstrated. When these studies were performed, the apparatus was used essentially as it was originally designed. It is conceivable that, with additional work and modifications, the LVS can be used for quantitative determinations of airborne virus in a natural environment. In this regard, it was of interest to find that the greatest number of positive LVS samples occurred in the room with patients that shed the larger amount of virus (3). With due regard to the inefficiency of present recovery methods, evidence given here and from another study from this laboratory (4) suggests that infected persons may discharge sufficient virus into their environment to account for airborne transmission of this disease.

The full significance of these studies will not be realized until investigations of this nature are extended to other respiratory virus diseases. By examining viruses of varying epidemic potential and comparing such factors as infectious dose, clinical illness, virus-shedding patterns, airborne survival, etc. on a quantitative basis, a better knowledge of the underlying mechanisms of airborne transmission of virus will be gained. This information will be helpful in approaches to environmental control of respiratory disease.

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## Discussion

WILLIAM S. GOCHENOUR, JR.

*Walter Reed Army Institute of Research, Washington, D.C.*

Gerone and his associates have presented observations on the production of small-particle virus aerosols with a Collison atomizer in a modified Henderson apparatus, information on the production of viral aerosols by persons infected with coxsackievirus A-21, and data acquired by large-volume air sampling in the environs of infected volunteers. Portions of those studies have been published in greater detail elsewhere (1, 2).

From these observations, certain inferences are made as to the significance of small particles in the transmission of naturally occurring disease due to coxsackievirus A-21, as to the suitability of

the exposure method for the inoculation of volunteers, and as to the usefulness of the large-volume air sampler in demonstrating virus in the environment of infected persons.

The purposes of large-volume air sampling may be twofold. First, one might theoretically detect airborne agents in one-thousandth the concentration detectable by conventional samplers. Second, the larger volume sampled might give one more confidence in an estimate of concentration than in that derived by the smaller sample size conventionally obtained. The large-volume sampler has two inherent limitations. First, because of problems of evaporation of collecting fluid, only relatively short periods of sampling are possible. Second, no estimate is possible of particle size distribution of the aerosol sampled. The first problem might be solved by the introduction of sterile distilled water at a rate equal to the loss by evaporation, thus maintaining the integrity of the composition of the collecting fluid. The second, that of particle sizing, appears to be insoluble with this equipment.

The results presented indicate that the device has not attained its theoretical capability to meaningfully quantitate airborne virus. A 100-fold variation between estimates of the concentration of a given aerosol and a 20-fold average variation over the concentrations sampled reduce the device in its present state to a qualitative sampler whose negative results would be suspect.

The presented results of comparison of the large-volume sampler with the Porton all-glass impinger raise more questions than they answer. Greater than 10-fold differences in virus recovery versus tracer recovery are indicated for both samplers, under conditions where tracer recovery was remarkably consistent. These are not compatible with the stated accuracy and reproducibility of the virus assay procedure (standard deviation, 0.25 $\log_{10}$ $TCID_{50}$ per ml).

The studies on sneezes and coughs establish two main points. First, the particle size distributions and particle volume distributions are markedly unlike that of the artificially generated aerosol used to infect volunteers. Second, no correlation can be made between titer of oral secretions and the amount of virus in a sneeze or cough.

The suitability of any method for the inoculation of volunteers by inhalation can be defined in terms of predictability of the dose to be administered and the site of deposition desired for the purpose of the experiment being conducted.

Predictability of the dose administered to man is influenced by stability of the agent in the spray suspension and in the airborne particulates generated, the uniformity of the aerosol generated, both qualitatively and quantitatively, and by the rate, manner, and volume of breathing of the test subject. It is also obviously dependent upon the accuracy of the assay procedures employed.

The lengthy training required to accustom volunteers to the highly stylized breathing cycle of nasal inhalation and oral exhalation required for mask exposures has long been recognized. Without this, marked variation in respiratory rates and tidal volumes will materially affect sites of deposition of airborne particles, yet not be reflected in the presented dose.

Data have been presented showing a not unreasonable relationship between the concentration of coxsackievirus A-21 in spray fluids and in the aerosol generated in the device employed. The inconstancy of the relationship, as demonstrated by these data, deserves consideration. As examples, aerosol concentrations of approximately 10 $TCID_{50}$ per liter were obtained with spray suspensions with concentrations ranging from 6.9 × $10^{10}$ to 8.2 × $10^{10}$ $TCID_{50}$ per liter, and aerosols containing approximately 1,000 $TCID_{50}$ per liter were obtained from suspensions containing 8.6, 9.5, and 10.5 × $10^{10}$ $TCID_{50}$ per liter. Conversely, from a single spray fluid concentration were generated aerosols containing less than 100 and over 1,000 $TCID_{50}$ per liter. Thus, although the relationship, or so-called "spray factor" may be useful in generalized predictions, it does not have the constancy and precision required for individual dose determination. Reliance must still be placed upon after-the-fact estimation of doses presented by assessment of samples collected over the same periods as the volunteer exposures, from samplers located in immediate proximity to the exposure port of the aerosol-generating device.

Even were the problems of dose predictability resolved, suitability of an exposure method still remains dependent upon the purpose of the experiment. If the objective of a study is to determine whether or not man may be infected by an airborne agent in an essentially small-particle aerosol, the method employed by Gerone and his associates is quite appropriate. Similarly, the capacity of a virus to initiate disease in the lungs and tracheobronchial tree is susceptible to examination by this method, the artificially induced pneumonia and tracheobronchitis with strain 49882 HEK affirmatively answers such a question. Elucidation of the mechanisms of naturally acquired coxsackievirus A-21 infection and of the significance of particles of various sizes in natural transmission of disease is an altogether different matter.

Naturally occurring coxsackievirus A 21 illness