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FIRST INTERNATIONAL SYMPOSIUM ON AEROBIOLOGY

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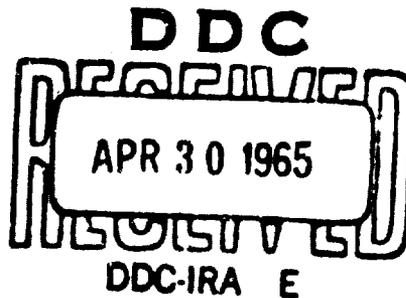
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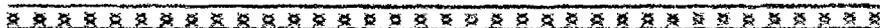
The Naval Biological Laboratory  
School of Public Health  
University of California

1963



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THE FIRST INTERNATIONAL SYMPOSIUM ON AEROBIOLOGY  
was convened  
October 2, 3, 4 and 5, 1963  
at Alumni House  
University of California, Berkeley



UNIVERSITY OF CALIFORNIA, BERKELEY

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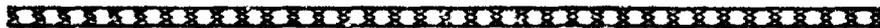
Charles E. Smith, Professor of Public Health  
and Dean of the School of Public Health  
Stanford S. Elberg, Professor of Bacteriology and Immunology  
and Dean of the Graduate Division

The Naval Biological Laboratory

Stewart H. Madin, Professor of Public Health and of Bacteriology  
and Director of the Naval Biological Laboratory

OFFICE OF NAVAL RESEARCH

RADM L. D. Coates, Chief of Naval Research  
CAPT E. J. Hoffman, Deputy



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## FORWORD

The importance of airborne bacteria, or particulate matter in general, to the over-all health and well-being of living things in general and mankind in particular is well-known to both the scientific and lay community. Our understanding of the basic principles of aerobiology, however, is not commensurate with the importance to health and well-being. This deficiency, in part, is because of inadequate communication and coordination of effort between those of us responsible for providing new knowledge. Hence the Symposium on Aerobiology arose as a partial answer to a known deficiency. The most important purpose of a conference is that of strengthening communication, but the results also should include some measure of self-evaluation. In doing so one might well ask what boundaries, if any, delineate the scope of our efforts.

If all aspects of the field of air hygiene were considered as a unit, nearly every field of science would be included in some way or another. Since the field is so broad, it is impossible to divide it into well-defined areas with no overlap, although some distinction between the various areas of interest should be made if we are, individually, interested in the significance of our own contribution. For example, public health sanitarians are concerned with airborne microflora contributing to infection in a given ecological situation; they ascertain by whatever means available the number of infectious particles, attempt to relate these numbers to epidemiological findings, but are usually not interested in the non-pathogenic forms. Often the methodology used is devised by physicists and engineers whose interest is to provide wards and hospital rooms with air containing a minimal number of any kind of airborne bacteria. Also in this group are medical and paramedical investigators interested in discovering mechanisms by which infectious bacteria find their way into hosts to create an infection, as well as in comparing the spread of disease in nature to that found in experimental situations.

Then there are theoretical microbiologists, whose interests, while less well-defined than the above groups, may be described in terms of death or survival mechanisms of airborne microorganisms and

who therefore need to provide a variety of environmental situations in the laboratory. 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.

Finally, in the last few years an interest in airborne bacteria has arisen in those responsible for the "sterility" of extra-terrestrial vehicles, as related to the potential of living forms crossing space, either to yield false positive signals from life-detecting instruments -- of microorganisms actually growing in alien environments.

At the Naval Biological Laboratory we label our studies "aerobiology" as being that part of the whole field concerned with experimental techniques of airborne infection and survival of bacteria. It was, perhaps, an error to have labeled the Symposium one on aerobiology only, for we realize that the subject-matter covered herein includes, and properly so, more than just the experimental techniques required to study such a broad area.

Perhaps, after all, the Symposium's most important contribution, aside from that of providing communication, has been in delineating the variety of interests of those in attendance. For example, air hygiene was well-represented with papers varying from one on the pathology of chronic bronchitis, through epidemiological studies in Navy recruit camps, to wound infection in hospitals. Environmental situations were described ranging from survival of bacteria in desert conditions, through those of constant temperature and relative humidity, and included situations of intense ultraviolet irradiation, as well as certain shifts in water content. Topics ranged from those highly applied; for example, potential contamination of dental suites by commonly employed dental techniques, through another reporting a new sampler, to the highly theoretical question of bound water and protein denaturation as a potential death mechanism. In the included papers there is a little something for everyone.

There is no question in our minds but that the Symposium was worthwhile. We hope that no theory will remain unchallenged, no pathway unexplored, and that there has been sufficient interest aroused and importance demonstrated from this meeting to provide

impetus for future gatherings. We hope that by publishing these proceedings we will have provided a record for those who could not attend -- a record that has posed more potential problems than solutions. There are no easy answers to these problems, so important to the welfare of mankind, but regardless of how difficult they may seem, they must be resolved; and we are proud to have been a part of efforts leading to such solutions.

Stewart H. Madin  
Director  
Naval Biological Laboratory

## P R E F A C E

This book is a compilation of papers presented during a Symposium for Aerobiology held October 2-5, 1963, in Berkeley, California.

The conference was organized into sessions devoted to general aspects of the main topic, and insofar as practicable, the papers were arranged in the order in which they were presented. Verbatim transcripts of informal discussions are included, with changes made only for purposes of clarity, because we wanted to preserve the general tenor of the meetings.

Our initial aims were modest, and we had proposed to limit attendance to approximately 40 persons. As plans progressed, however, the number of interested individuals increased and it was finally decided to include all those who could attend on their own initiative. There were, consequently, over 100 scientists in attendance.

We had hoped that interest in forming a permanent committee for the purpose of continuing such meetings might arise and perhaps bring into being an organization of interested scientists -- a society, as it were, for aerobiology. Although this did not happen, it was informally agreed that Mr. Elwood K. Wolfe of Fort Detrick would arrange for another meeting 2 years from the above date.

If the purpose of the conference was to promote interchange of knowledge on current efforts and to stimulate future research, then I believe the meeting was successful. It was successful because of the enthusiastic participation of those in attendance, the invaluable cooperation of the session chairmen, as well as aid received from many members of the NBL staff.

To mention the specific contributions of those involved would require more space than can reasonably be allotted. However, I will use my editorial prerogative to extend my heartfelt thanks and appreciation, with a brief indication of the part they played, to those who helped so willingly to make the conference a success, and so patiently aided in assembling this manuscript. They were:

CONFERENCE ASSISTANTS

Robert E. Durham	Finance
Dr. E. Frank Deig T. R. Wilkinson, LTJG USNR	Housing and Entertainment
J. E. Campbell S. A. Dunn	Liaison
Agnes H. Allison Judith A. Fabian Anna A. Hunrick Vivian A.D. Scudder	Secretarial
George Chu Peter J. Garrow John N. Schutz Richard I. Werre	Recording and Reproduction
Dr. Robert L. Miller	Editorial Assistance

Finally, we wish to extend our sincere appreciation to the Office of Naval Research and the University of California for their financial support, and administrative aid, which together made the accomplishment of this Symposium possible.

R. L. Dimmick  
Chairman of the Symposium Committee  
Editor

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## OPENING REMARKS

E. J. HOFFMAN, CAPT MC USN

Office of Naval Research  
Department of the Navy  
Washington 25, D. C.

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On behalf of the Office of Naval Research I want to welcome you to this Symposium in Aerobiology conducted by the Naval Biological Laboratory, a unique ONR research facility operated under contract by the School of Public Health of the University of California at Berkeley. Aerobiology is a new field in which much of the research is involved in defense against bacteriological warfare. It is a field which has burgeoned recently since exposure to airborne microorganisms is a subject not only of military interest but of increasing concern to those involved in industrial health and epidemiology. Research conducted at NBL concerns fundamental investigations in the general areas of both aerobiology and experimental pathology of infectious diseases as they apply to public health and to medical problems of the Navy.

Biological research is of obvious concern to the Navy, and one of the six major research divisions of ONR is the Biological Sciences. Our program primarily focuses on supporting research which extends knowledge of fundamental life processes and utilizing this knowledge to solve problems with which the Navy is now -- or may some day -- be confronted.

The broad needs of the Navy in the biological area can be described briefly. First of all, naval personnel must be protected from the stresses imposed by new weaponry, vehicles and unusual environments. The combat effectiveness of the Navy can only be assured if

its men can function efficiently while undergoing such stresses.

Second, the design of advanced ship types, aircraft, and weapons requires in part a basic understanding of biological phenomena. New means of propulsion may derive from studies of hydrodynamic characteristics and the methods of propulsion of marine animals. Longer range underwater detection and navigational devices could result from studies of the communication and acoustic signals emitted by marine life.

Another need is that the Navy's ships, docks, cables, mines and electronic equipment must be protected from the ravages of marine-fouling organisms. This requires research on the organisms responsible for fouling and deterioration, including basic research on the reproduction, physiology and genetics of the organisms involved.

Finally, basic research in biology is supported in order to fill gaps in our knowledge of biological systems with a potential for affecting naval situations. This research is not necessarily related to specific or immediate problems but may be applied at a later date to solve presently unrecognized problems. Incidentally, this is a major facet of all ONR's research programs.

It is plain, of course, that biological problems are not confined to the Navy, but are military-wide. Our last two conflicts, World War II and the Korean fighting, produced a toll of casualties which could easily be dwarfed by a future nuclear war. The treatment of these casualties and the rehabilitation of the personnel is a challenge we must be prepared to meet. The Navy and the other military services have a responsibility to maintain a firm research program in the fundamental scientific disciplines that will lead to the control or prevention of injuries to which military personnel are subject, including both traumatic injuries and debilitating diseases.

Aside from this urgent need, biological research is essential if we are to accomplish our ultimate goals in space, in which all three services have a stake. If men are ever to journey to the planets and stars, we must know the biological effects of cosmic radiation, meteoric collision, synthetic air and food-waste cycling. We must determine the biophysical effects, not only of these factors but also of disorientation and illusions of space and time. Indeed, the powerful engines and intricate devices needed to send and guide space satellite vehicles are relatively simple in comparison with the biological or

life-support systems involved in the successful transportation of man on extended trips into outer space. But more important to us -- the knowledge and skills used in space will inevitably improve naval operations!

Research in aerobiology is a significant part of the spectrum of overall biological research which is aiding man, as much as any of the physical sciences, to control and master his environment. Every bit of this knowledge, no matter what its immediate objective may be, will contribute something to man's future benefit.

## WELCOME ADDRESS

Charles E. Smith  
Dean, School of Public Health  
University of California, Berkeley

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Thank you Dr. Madin, Captain Hoffman and fellow participants. Fourteen years ago I left a very happy association as a student and subsequent faculty member of Stanford to come to the University of California. Amidst the travails and problems of adjusting to this environment, there was one very outstandingly warm and hearty welcome: that of Al Krueger, the organizer and first Commanding Officer of NAMRU #1. Dr. Krueger's genius built this program in parallel with his duties as Chairman of the Department of Bacteriology at the University of California. From both developed the program of the Naval Biological Laboratory which Captain Hoffman has just described. Dr. Krueger's warm welcome to me, I, in turn, have the great privilege of extending to you.

I recall that his welcome of me was especially appreciated because I had just worn out the patience and the good will of my Stanford Alma Mater by being, instead of a model of preventive medicine in my Department of Public Health, undoubtedly the greatest, albeit unwitting, menace to public health in the medical school because of my coccidioid investigations. The climax came when we generated an epidemic through our entire building. Our laboratories were located on the first floor. I had cautiously (I thought) transferred my stock cultures of Coccidioides on Washington's birthday when nobody was around. However, when we reconvened, the epidemic erupted. Fortunately, there

were no disseminated cases, but severe primary coccidioidal illnesses developed in a colleague professor of radiology and in one laboratory technician with one lung out of commission because of a thoracoplasty. As a sequel, I heard muttered, "Well, if you hadn't decided to go to the University of California, we would have sent you there anyway!" There, at the University, in stark contrast was Dr. Krueger's welcome, "Bring on your spores--we're happy to have these kinds of aerobiology problems at U.C."

Through the years, Dr. Krueger's microbiological interest combined with Walter Leif's remarkable abilities in aerobiological engineering, attracted uniquely competent colleagues. It would be tempting to trace and discuss the remarkable program which has ensued. I shall say only that it has had, and continues to enjoy, the full support of the University of California Administration. Dr. Krueger initiated the program under the Presidency of Robert Gordon Sproul. When Clark Kerr became the first Chancellor of the Berkeley campus, he continued and extended this support and personally carried through very careful negotiations and understandings with Admiral Bennett, Admiral Coates' predecessor. When Chancellor Kerr succeeded to Dr. Sproul's Presidency, he continued his allegiance, joined first by his successor, Chancellor Glenn Seaborg and then, when Dr. Seaborg became Chairman of the Atomic Energy Commission, by our present Chancellor, Dr. Edward Strong.

Thus the University of California affords strongest support for this program. Here is the happy combination of the flexibility and vision of ONR, which Captain Hoffman has indicated, together with the capabilities and critically important participation of the Bureau of Medicine and Surgery, including the unique resources of NAMRU #1. Now, recently sparked by our outstanding Director, Dr. Stewart Madin, the National Institutes of Health of the U.S. Public Health Service is participating.

The late Dr. Morris Stewart, as Dean of the Graduate Division, was a key figure in integrating this Navy-University program. Now an organized research unit of our School of Public Health, the liaison with the Graduate Division is assured by the service of its Dean, Dr. Sanford Elberg, as Co-investigator with Dr. Madin and me. Dean Elberg succeeded Dr. Krueger as Chairman of the Department of Bacteriology and served a critically important period as Scientific Director of the

Naval Biological Laboratory. As Dean of the Graduate Division he continues to have a deep interest in, and provides the strongest support for, this unique program which is proceeding so effectively under Dr. Madin's direction. We are deeply grateful to you scientists from all over the world for the interchange of knowledge in this Symposium. As Captain Hoffman has indicated, these discussions of airborne infections are of vital concern in every-day living.

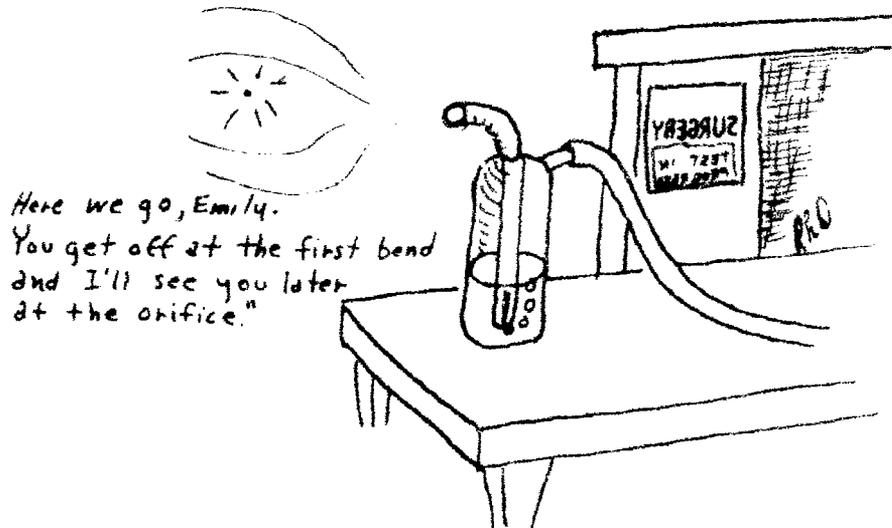
We are very proud that among you renowned scientists assembled here we may claim as "alumni" two individuals who formerly headed the medical programs in NAMRU #1, Dr. Lloyd Miller and Dr. Jack Millar. As outstanding epidemiologists, their considerations of viral and meningococcal infections can be counted on to underscore the problems of the environment. As epidemiologists we do have to consider the host, the parasite and the environment. Not one of these can stand by itself, but all are in dynamic interrelationship. The recrudescence of meningococcus meningitis as a military problem underscores the fact that the objective of nature is survival. In this goal nature plays no favorites. She is just as happy to help out the meningococcus as she is to help out man. Therefore, in our investigations of airborne infections, the patterns of epidemiology must constantly be before us. We must make sure that we do use all the resources at our disposal, including the application of rapidly emerging scientific sophistication in the field of Engineering, Physics and Chemistry in aerobiology.

On behalf of the University of California, I do want to reiterate the very stimulating comments of Captain Hoffman and express our deep appreciation to all of you.

# MEDICAL AND PUBLIC HEALTH SECTION

Phillip S. Brachman, Moderator

Application of research to enhance knowledge or to better mankind is a major reward in our effort. In this section, the results of clinical and microbiological techniques applied to problems of epidemiology and public health are reported.



## ACUTE RESPIRATORY INFECTIONS IN NAVAL PERSONNEL

(Published in part in Military Med. 129:526-532, 1964)

Lloyd F. Miller, CAPT MC USN\*

Officer in Charge, Naval Medical Research Unit No. 4  
U. S. Naval Hospital, Great Lakes, Illinois

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The intent of this presentation is to review the magnitude and extent of the acute respiratory disease problem in the Navy and to consider epidemiologic observations and other research data pertaining to airborne infection. Most of the information considered will be that obtained by the Naval Medical Research Unit No. 4 during the course of studies into the etiology, epidemiology, and prevention of acute respiratory disease. Since one of the objectives of this conference is to stimulate discussion, considerable latitude will be exercised regarding speculation on the information presented, with the view towards indicating areas for further research on the relative roles of airborne droplet nuclei and direct contact including droplets of respiratory secretion in the transmission of acute respiratory disease.

Acute respiratory infections rank first as a cause of total hospital and dispensary admissions in the Navy and Marine Corps<sup>(1)</sup>. As can be seen in Table 1, approximately 32,000 admissions of Acute Respiratory Disease (ARD) and 7,000 of pneumonia occurred in 1961. These two causes of admissions represented approximately 20% of the total admissions from all causes for the Navy and Marine Corps, and accounted for approximately 5% of the total sick days.

---

\* Present address: Commanding Officer, U.S. Naval Medical Research Unit No. 3, Navy 540, Fleet Post Office, New York, New York.

TABLE 1. Leading causes of new admissions and sick days, Navy and Marine Corps, 1961\*

Diagnostic Group	New Admissions		Sick Days	
	Rank order	No. in 1000's (approx.)	Rank order	No. in 1000's
ARD <sup>†</sup>	1	32	4	147
Injuries	2	24	1	636
Pneumonia	3	7	3	162
Character and behavior disorders	4	5	2	172
Rubella	5	5	-	17

\* Adapted from Med. Stat. U. S. Navy, 97th Report Surgeon General, 97:2. 1961.

† Acute respiratory disease exclusive of pneumonia.

Recruits have a disproportionately high incidence of ARD and pneumonia. Figure 1 shows that in 1959, recruits, which made up approximately 4% of the total enlisted population, accounted for 9% of the total sick days, and nearly 50% of respiratory disease admissions<sup>(2)</sup>. It should be pointed out that a recruit population is unique in that it consists of an open-ended population with a continuous input of susceptibles to begin training and removal of immunes at the time of graduation 9-11 weeks later. The population turnover approximates 30-50%/month.

Intensive studies of ARD have been undertaken since 1948 by Naval Medical Research Unit No. 4 (NAMRU-4) at the Naval Training Center, Great Lakes. They have revealed an increased incidence in ARD, including pneumonia, since the Asian influenza pandemic in 1957. The occurrence of ARD and pneumonia is shown in Table 2. The number of recruits trained per year since 1954 has increased and this increase has been accompanied by a higher incidence of ARD and pneumonia. In addition, it should be noted that the prevalence of adenoviral disease also increased, as determined by serologic study of ill recruits. The increased population size may have been at least partially responsible for the higher incidence; thus, crowding could be suspected as having played a role.

Crowding increases effective contact rates between infected and susceptibles by airborne droplet nuclei spread, as well as through

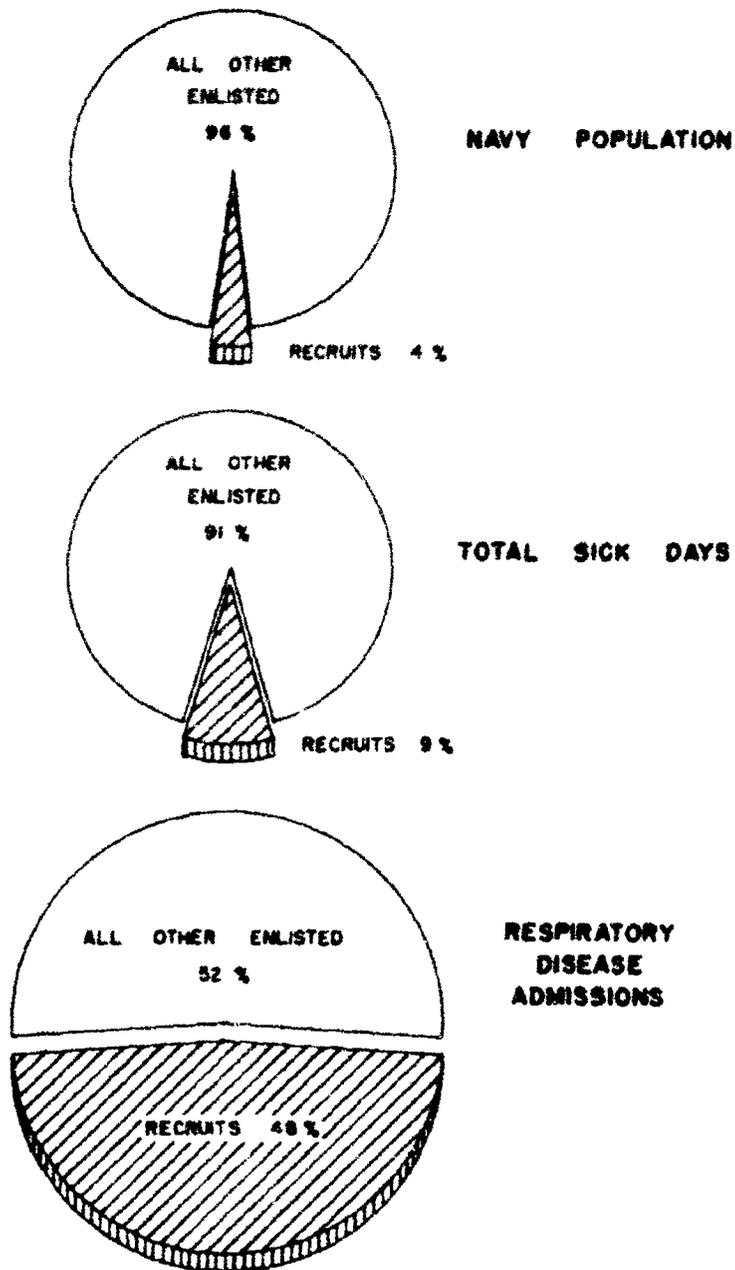


FIG. 1. Recruit contribution to incidence of acute respiratory disease admissions and total sick days in Naval and Marine Corps enlisted personnel in 1959 (Statistics of Navy Medicine, May 1961)

direct contact. It is interesting to consider available evidence pertaining to the relative importance of airborne spread by droplet nuclei in naval recruits.

Studies on the effectiveness of ultraviolet (UV) light in the barracks on reduction of ARD and streptococcal disease incidence have been revealing. Table 3 summarizes the results of the UV light studies which were performed over a period of 6 yr<sup>(3)</sup>. A rather consistent reduction in incidence of undifferentiated ARD and streptococcal illness occurred usually ranging from 20-30%. Irradiation at bactericidal levels was restricted to the ceiling areas and thus would have had limited effect on direct contact spread by droplets. Consequently, it is easier to conceive of any reduction of illness as being due to lethal effects on infectious agents present in droplet nuclei. The degree of reduction of viral agents in the air was not possible to determine, due to lack of adequate technics. A reduction of 50-75% of alpha streptococci did occur, indicating a killing effect on bacteria having their origin in the respiratory tract. If viruses are no more susceptible to irradiation than bacteria, the reduction of viral agents was less than optimal and perhaps more efficient methods of irradiation could have resulted in a considerably greater improvement in air hygiene and a further reduction in illness. It appears that spread by airborne droplet nuclei was of considerable importance.

It is well to recall the theoretical importance of size of groups on the occurrence of epidemic disease before considering the observations made by several investigators on the relationship of barracks space available to recruits and the number of recruits within a space. With a constant effective contact rate between susceptibles and infected, the occurrence of a propagative-type epidemic may depend upon the number of susceptibles present. The larger the number, the more likely an epidemic would occur. Crowding, in contrast to group size, would have a more direct influence upon effective contact rate, although an increase in crowding also increases the number of aggregated susceptibles if available space remains the same.

The relationship between barracks space per recruit, i.e., degree of crowding and incidence of ARD, when the size of the epidemiologic unit (the recruit company) was kept constant, is shown in Table 4<sup>(4)</sup>.

TABLE 2. Relationship of population size to adenovirus infections and acute respiratory disease (ARD) and pneumonia rates, naval recruits, Great Lakes, Illinois

Year	% Administered adenovirus vaccine (Approx.)	% Adenovirus positive in ill recruits (Approx.) <sup>*</sup>	Population	No. pneumonia	%	No. ARD	%
1954	0	13	21,730	95	0.4	1,058	4.8
1955 <sup>‡</sup>	0	40	43,626	291	0.7	4,649	10.6
1956	25	20	47,377	215	0.5	2,917	6.1
1957	< 10	65	30,239	572	1.9	4,190	13.9
1958	< 50	50	42,094	1,461	3.4	7,503	17.4
1959	0	75	47,778	1,484	3.1	11,304	23.6
1960	0	70	54,439	1,723	3.2	12,948	23.7
1961	75 <sup>‡</sup>	80	49,314	2,478	5.0	13,701	27.7
1962	< 50	75	54,036	2,078	3.8	9,878	18.2

\* Non-systematic sampling.

‡ Streptococcal epidemic.

‡ Vaccine of questionable potency.

TABLE 3. Effects of ultraviolet irradiation in barracks on incidence of acute respiratory disease (ARD)

ARD season	USN Training Center	Population size	% reduction of ARD in test group	
			Undifferentiated	Streptococcal
43-44	Sampson, N. Y.	4800	25	--
44-45	Sampson, N. Y.	?	20	--
45-46 <sup>*</sup>	Great Lakes, Ill.	4000	20	24
46-47	Great Lakes, Ill.	1000	50 (0% flu)	--
47-48	Great Lakes, Ill.	3200	25-30	40
48-49 <sup>‡</sup>	Great Lakes, Ill.	3-4000	22	22

\* Also irradiation of other areas (class rooms, mess halls, dispensary waiting rooms) and oiling with Triton-13-mineral oil of all barracks and decks.

‡ Similar to 45-46 design except oil treatment of blankets in irradiated group only. Decks not oiled.

TABLE 4. Acute respiratory disease (ARD) incidence and barracks space per recruit\* (old and new barracks) May-December 1959, Naval Training Center, Great Lakes.

	Space per recruit		Population Man Weeks	Number admissions	Admissions per 1000	ARD admissions		Per Cent relative reduction	
	Ft <sup>2</sup>	Ft <sup>3</sup>				Per Cent + adenovirus <sup>‡</sup>	Rate	Positive adenovirus	
Old	32	286	57,360	1501	26.2	60			
New	51	460	57,480	1329	23.1	53	11.8 <sup>‡</sup>	11.6	

\* Navy standard minimum space = 50 ft<sup>2</sup>; 450 ft<sup>3</sup>.

‡ Admissions for period June-October 1959.

‡ P = < 0.001.

Both the old and new barracks in which the recruits were housed were of the single bay open dormitory-type construction, but the new barracks were considerably larger, making more sq. ft. of floor space and cu. ft. of air space available per recruit. A significantly higher incidence of reported disease occurred in the old barracks along with a greater proportion of adenovirus-associated illness. There is no reason to suggest that the difference in disease incidence was due to a greater effect of crowding or airborne transmission than on direct contact spread. Caution also should be exercised in concluding that there was less illness in recruits housed in the new barracks, since in reported illness motivation to attend sick call is an important factor and living conditions were considerably better in the new barracks.

Studies by Breese et al.<sup>(5)</sup> during World War II indicated that the size of the group housed together influenced the admission rates to a greater degree than did the space available per recruit. This relationship is depicted in Table 5. On the other hand, the common cold-type illness occurring in the first 2 weeks of training, which in retrospect would be considered largely viral in nature, was influenced more by the space available per recruit than by the size of the group. Streptococcal disease and viral disease occurring later in training were associated to a greater degree with group size. The authors were careful to point out that the reasons for the paradoxical occurrence of a higher disease incidence, when more space was available per recruit,

did not imply crowding to be beneficial, but reflected an overriding effect of group size. An analysis of the data reported by Bernstein<sup>(6)</sup> revealed grossly similar types of correlations of group size and space available per recruit with resulting disease (Table 6). His data were obtained in a study of recruit airmen in 1952 and 1953.

TABLE 5. Correlation coefficients of illness to crowding as measured by naval trainees per room, per 450 ft<sup>3</sup> air space and 50 ft<sup>2</sup> floor space

	Study No. 1*		Study No. 2 <sup>†</sup>			
	Total illness	Strep carriage % Positive	Illness first 2 weeks training		Illness last 4 weeks training	
			Non-Strep	Strep	Non-Strep	Strep
No. per room (from 14-185)	+0.54	+	-0.09	+0.14	+0.35	+0.62
Per 450 ft <sup>3</sup> (from .73-1.40)	-0.36	-	+0.07	-0.07	-0.39	-0.54
Per 50 ft <sup>2</sup> (from 1.03-1.77)	-0.18	-	+0.55	+0.25	-0.05	-0.04

\* Observations April-September 1943: Values over .48 significant at 1% level.

† Observations Winter 1942-43: Values over .43 significant at 1% level; over .34 at 5% level.

TABLE 6. Correlation of acute respiratory disease (ARD) in Air Force recruits with space/recruit and number of recruits/room, Sampson Air Force Base, 1952-1954\*

ARD rate/1000/week	No. per Room	ft <sup>2</sup> /man	ft <sup>3</sup> /man
26.4	30-72	59-142	643-1543
23.2	3-6	42-84	510-1020
Correlation with ARD rate	Positive	Negative	Negative

\* Adapted from data by S. H. Bernstein, Am. J. Hyg. 65:162-171. 1957.

Since ARD incidence is, at least in part, a function of effective contact rate and number of aggregated susceptibles, reduction of the former by minimizing the airborne spread through development of improved methods of air sanitation and the latter by housing fewer recruits per living space has considerable practical merit, but would be costly and may not be presently feasible. Recent studies by Ariander et al.<sup>(7)</sup> of naval and marine recruits in training at San Diego have suggested a presently available method of achieving in effect a reduction in size of the aggregate numbers of susceptibles. Segregation of training units accompanied by intermittent, rather than continuous, input of newly arrived recruits into the training units, which is practiced to a greater extent by the Marines than by the Navy, was observed to be associated with a much lower incidence of ARD in the marine recruits. Barracks interviews, serologic and X-ray surveys, ruled out the reduction as a reflection of differences between the two recruit populations in their motivation to attend sick call, or in criteria utilized by medical department personnel in diagnosing and recording illness. The difference in reported ARD incidence between the two populations and presumably of unreported illness has occurred consistently for at least 8 yr<sup>(8)</sup>.

The higher incidence in the winter of specific diseases, such as measles and chickenpox, has been suggested as evidence for airborne droplet nuclei transmission of those diseases, in that peak incidence occurs when ventilation rates of living spaces are at a minimum<sup>(9)</sup>. ARD is characteristically epidemic in military recruits in the winter, and although in recent years it has also occurred in epidemic form at the Great Lakes Naval Training Center in the summer, the highest incidence was in the winter<sup>(10)</sup>.

The temporal association of the seasonal increase of ARD in winter and lower ventilation rates that occur at that time, although ventilation rates have not been quantitated, is perhaps more significant in naval recruits than in most populations since, except for the climatic variables, other factors are more constant throughout the year. For example, training schedules and procedures remain the same. Classes are held indoors throughout the year. In fact, crowding of recruits occurs to a greater extent in the summer than in the winter due to the larger on-board populations in the summer.

Although highly attractive, this apparently straightforward association of ARD with lower ventilation rates as supporting evidence for transmission by droplet nuclei was found to be more complex when the specific etiologic agents involved were considered.

A longitudinal serologic survey of recruits randomly selected for study at the beginning of training revealed adenoviral infections to peak in the late fall and winter and to reach a low in summer as expected<sup>(10)</sup>. Influenza B also peaked in the winter. Of interest, however, was the peaking of Eaton agent (Mycoplasma pneumoniae) infection in the summer and fall with the lowest incidence in winter. The seasonal variations in the incidence of these three agents in the randomly selected recruits are shown in Fig. 2.

The inverse association of M. pneumoniae disease with ventilation rates definitely requires consideration of the effects of relative humidity (RH) and other less apparent seasonal variables upon airborne survival. It is entirely possible that RH effects can override those of ventilation rates for any of the etiologic agents, and if such is the case, the association of ventilation rates and ARD incidence could be secondary and even unrelated. Perhaps direct contact, which is also influenced by crowding, is the predominant factor for spread of M. pneumoniae infection and is the explanation for highest incidence during periods of maximum ventilation. In this connection, it is interesting to recall the observations of Breese and his associates<sup>(5)</sup> discussed earlier in this paper. The incidence of streptococcal, and to a lesser extent non-streptococcal, disease occurring in the last 4 weeks of training was correlated to a higher degree with group size than with space available per recruit, whereas the early, more rapidly acquired infections were more strongly associated with the latter. In addition, Wells<sup>(9)</sup> noted in her studies that although a seasonally negative correlation existed between measles and chickenpox incidence and ventilation rates, as inferred from differences in disease patterns observed in Maine, Virginia and Florida, the seasonal pattern of scarlet fever did not differ significantly in these three states. This suggests that ventilation per se was not of predominant importance in the spread of streptococcal disease. Although, in conjunction with other information obtained by Schulman and Kilbourne<sup>(11)</sup>, droplet nuclei spread of influenza is likely, and may be a major mode of transmission, the need for further research is

obvious in the case of adenovirus and *M. pneumoniae* infections. High priority should be given to studying the influence of RH upon airborne survival of these two agents. Studies such as those by Hammes et al. have demonstrated influenza virus to survive better in the airborne state at lower RH<sup>(12)</sup>.

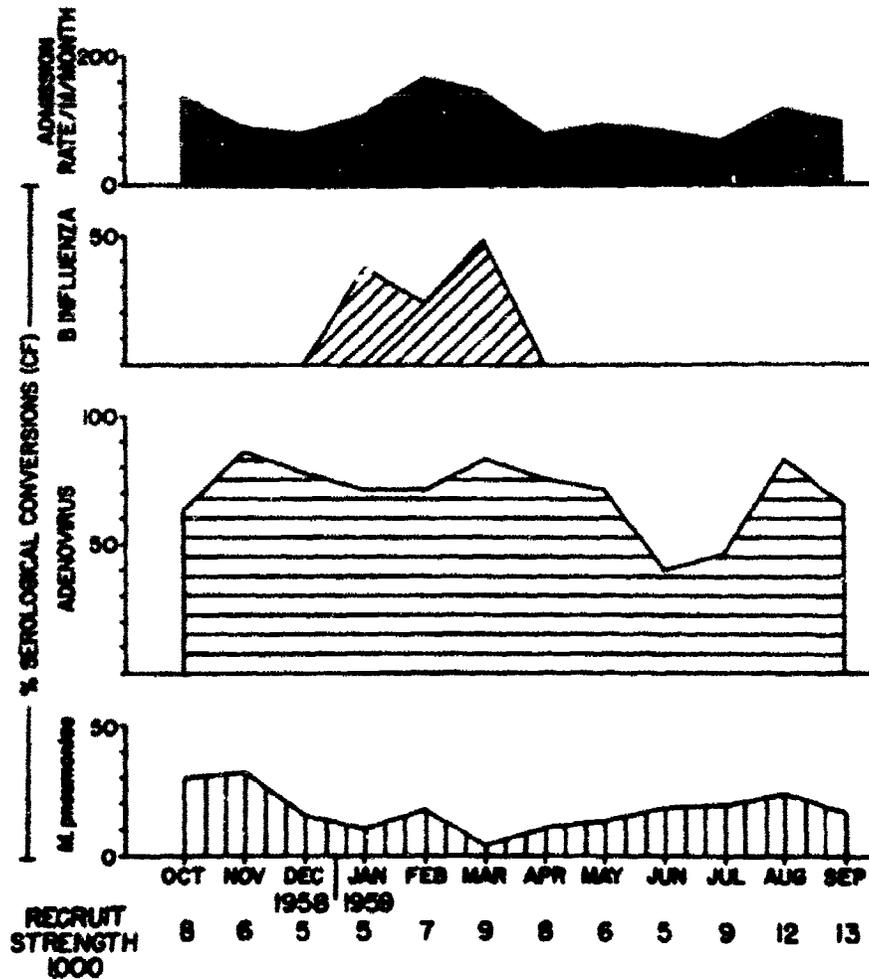


FIG. 2. Seasonal relationship of admission rates acute respiratory disease (ARD) and *Mycoplasma pneumoniae* in randomly selected navy recruits, Naval Training Center, Great Lakes, Illinois. 1958-1959.

In summary, the problem of ARD is of major importance and constitutes the principle cause of hospital and dispensary admissions in the Navy and Marine Corps. Recruits experience a disproportionate incidence of this illness. Evidence obtained by NAMRU-4 strongly supports the supposition that airborne spread is an important means of transmission of ARD agents. UV light irradiation studies have provided the most unequivocal evidence that droplet nuclei play an important role in recruit disease. Delineation of the comparative roles of direct contact, including droplet spread, and of spread by droplet nuclei, requires further research. The availability of newer techniques and, in particular, methods for studying airborne Mycoplasma and viruses, makes further study attractive. A relatively high priority should be given to a study of survival of adenovirus and M. pneumoniae agents under conditions of varying RH. Perhaps such a study would explain, at least in part, the reason these two agents have different seasonal peaks of incidence and serve as a partial explanation for the apparent occurrence of high M. pneumoniae incidence in naval recruits at Great Lakes during periods of higher ventilation rates in their living spaces.

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#### DISCUSSION

Schulman: There are a few points I would like you to cover. Without a question of crowding in the two different types of barracks situation -- the old barracks and the new one -- mention was made as to differences in ventilation for the two barracks, so that one can't really know which effect is of most importance, the effect of crowding or the effect of ventilation.

L. Miller: That is entirely right. I don't have good information on that, except that in the new barracks they had thermostatically controlled heat and did not run into the problem where it got so unbearably hot that they had to open the windows wide. In other words, there may have been a lower ventilation rate in the new barracks. It is common to get so hot in the old barracks that all windows are opened for awhile and thereby increased ventilation would result. I think the point I really wanted to make was not the relative importance of ventilation rate or crowding but the fact that the size of the groups was comparable, so that the amount of direct contact, not considering droplet infection now, should have been very similar in the two groups. This, by exclusion then, leaves you with the impression that perhaps other methods of spread were important; perhaps the airborne route. It is not real good information.

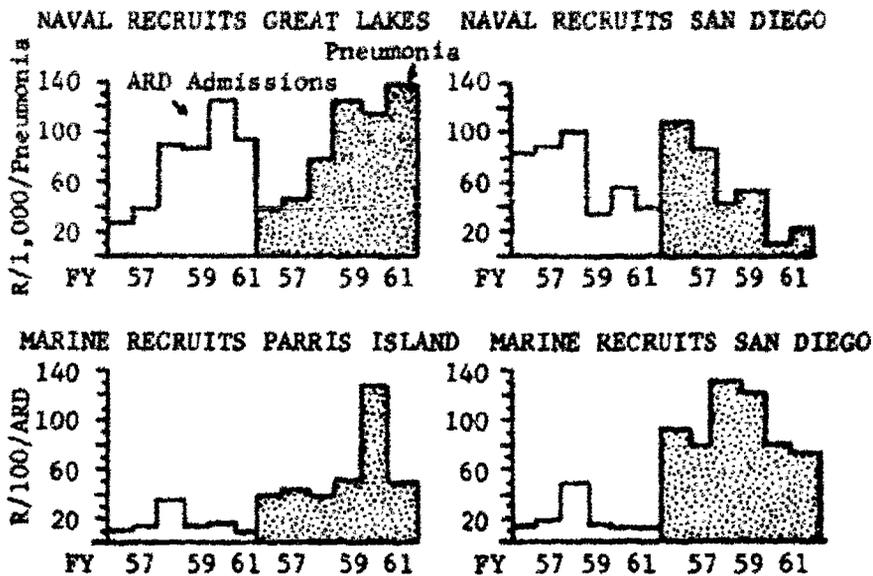
Bloom: I understand the NAMEU-4 is studying the comparative respiratory disease incidence in different military populations in the same geographical location.

L. Miller: Yes. We have noticed for some time that there has been more reported illness in the Navy recruits in San Diego; that is,

a higher incidence than in the Marine recruits at San Diego. They are in the same geographic area, right across the fence from each other. I expected this question so I have some slides, if I can take the time.

VFTF\*: The Marines are supposed to be tougher, anyway.

L. Miller: Well, by the time I show you this information you will see that although they may be tougher individuals, that is not the reason they have less reported illness.



**FIG. 1-D.** Incidence acute respiratory disease (ARD) and pneumonia.  
Naval and Marine recruits FY 1956-61

This slide (Fig. 1-D) shows what occurred over a number of years in the Naval recruits at San Diego and the Marine recruits at San Diego. This is the incidence per 100 men of admitted ARD. You will see that there is consistently a considerably less reported ARD incidence in the Marine recruits compared to the Naval recruits. When you come to the pneumonias, they are more comparable. As a matter of fact, there is actually a somewhat higher admission rate for pneumonias in the Marines, as seen in the next slide (Table 1-D).

\* VFTF = Voice from the floor.

TABLE 1-D. Rubella rate/1000/average strength

Fiscal Year	NAVY			MARINES	
	Bainbridge	Great Lakes	San Diego	Parris Island	San Diego
1962	--	140	178	0	49
1961	--	195	189	1	37
1960	--	156	141	29	109
1959	--	184	168	6	62
1958	--	191	182	25	47
1957	52	152	182	1	17
1956	6	142	123	4	34
1955	4	47	138	0	20

This slide shows the rubella incidence at the four training centers (excluding Bainbridge). Rubella is a pretty good disease index because a recruit, whether he is Navy or Marine, will report his illness when he gets a rash. If he doesn't report it, his buddies will make him go to Sick Call. You'll notice particularly in the Navy and Marines at San Diego that there is a consistently higher rate for rubella in the Naval recruits.

TABLE 2-D. Meningococcal meningitis, Navy and Marine Corps recruits, San Diego, California 1961-1963

	1961	1962	1963 (to Sept)
Navy	9	25	33
Marines	1	0	1

Table 2-D shows the cases of meningococcal meningitis occurring at San Diego -- this is the number of cases, not a rate, and the Marines usually have about 1/3 the population size. You will notice that meningitis was primarily a problem in the Navy.

Table 3-D shows that acute rheumatic fever was again primarily a problem in the Navy.

TABLE 3-D. Rheumatic fever cases, Recruit Training Commands, San Diego, California

Calendar year	NAVY	MARINES
1960	1	1
1961	18	8
1962	34	2

Figure 2-D shows the total incidence of reported illness; that is, admissions and febrile respiratory disease episodes reported by the Navy and Marine recruits to Sick Call. A sharp increase occurs in the early weeks of training in the Navy recruits. The Marine recruits have a delayed incidence, and respiratory disease does not occur to the same extent.

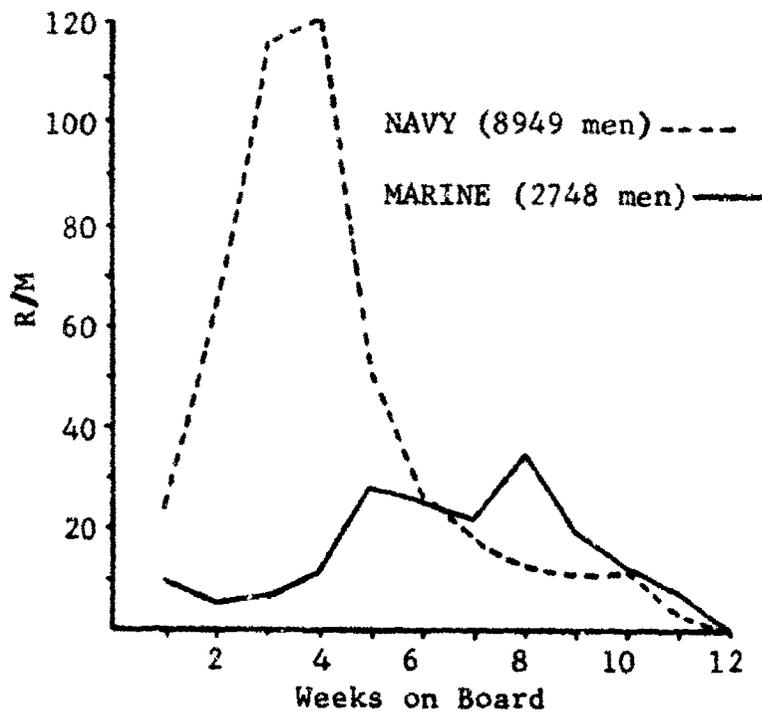


FIG. 2-D. Febrile respiratory episodes reported to sick call. Rates per 1000 plotted by weeks on board.

Table 4-D shows the number of men, Marines and Navy, we were studying and the man-weeks of exposure; the febrile and afebrile episodes of ARD and the incidence of pneumonia are shown. You will see that the Navy has higher rates in respiratory disease until we get to pneumonia. There the rates were almost identical -- all this referred to so far is reported illness. It is necessary to survey men in the barracks to determine the extent of unreported illness. Six Marine platoons and six Navy companies were surveyed -- they were seen a couple of times a week. On surveyed illness you get away from the factor of motivation to report their illness.

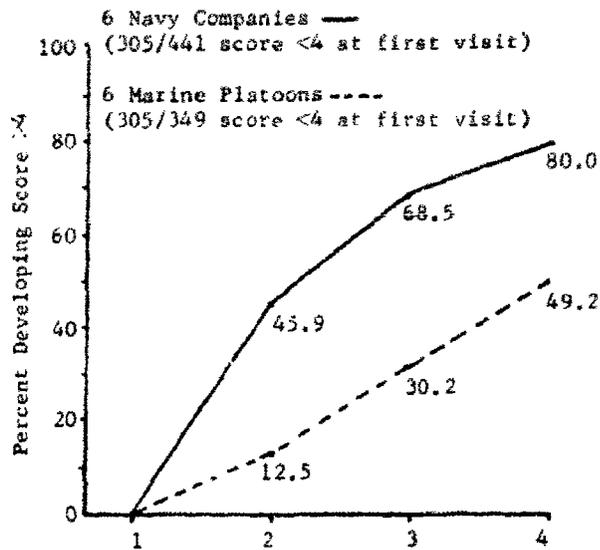
TABLE 4-D. Summary of admitted respiratory illness

	NAVY	MARINE
Number of men	8949	2789
Man weeks	89490	33468
ARD admissions	983	117
ARD admissions/M man weeks	11.0	3.5
Pneumonia admissions	270	120
Pneumonia admissions/M man weeks	3.0	3.6

Figure 3-D shows a larger percentage with respiratory disease in the Navy compared to the Marines. So the Marines actually do have less respiratory disease.

Table 5-D shows the serology on the surveyed groups regardless of their illness status. You will notice for Eaton agent that there is a considerably higher CF conversion rate in the first 4 weeks in the Navy than in the Marines. Later on in training, the Marines begin to catch up and by the end of training the Marines are more comparable. I think this may possibly provide a partial explanation as to why the pneumonia rates were more similar than the ARD rates in the Marine and Navy recruits. Table 6-D shows the same trend for streptococcal disease as indicated by the ASO responses. The conversion rate is higher and earlier in the Navy compared to the Marines -- yet toward the end of the 9 weeks total period there is not a great deal of difference. In

other words, there was a slower spread of streptococcus in the Marine recruits.



Time for 50% of Navy group to develop score  $\geq 4$  = 1.16 weeks.  
 Time for 50% of Marine group to develop score  $\geq 4$  = 3.0 weeks.

FIG. 3-D. Febrile respiratory episodes reported to sick call. Rates per 1000 plotted by weeks on board.

TABLE 5-D. Eaton agent CF serology (4-fold rises) in surveyed San Diego recruits, 1963

	Total tested	1-4 weeks	5-9 weeks	Total (1-9)
Navy	250	14.4%	7.2%	21.6%
Marine	213	2.8%	10.3%	13.1%

TABLE 6-D. ASO serology (2-tube rises) in surveyed San Diego recruits, 1963.

	Total tested	1-4 weeks	5-9 weeks	Total (1-9)
Navy	240	13.3%	11.3%	24.2%
Marine	213	7.5%	11.7%	19.2%

TABLE 7-D. Adenovirus CF serology (4-fold rises) in surveyed San Diego recruits, 1963.

	Total tested	1-4 weeks	5-9 weeks	Total (1-9)
Navy	250	38.0%	46.0%	76.4%
Marine	213	6.1%	30.0%	35.7%

Table 7-D shows adenovirus serology findings. Here we find a rather marked difference between the two population groups. Notice, there was about a 6-fold higher conversion rate in the first 4 weeks of training in naval personnel. When we get toward the end of training, there is still an appreciable difference; about a 2-fold higher conversion rate occurred in the Navy.

TABLE 8-D. Prevalence of pulmonary infiltrates in non-hospitalized Navy and Marine recruits, same military age (30 days) San Diego, California, 1963

	Marine recruits		Navy recruits	
	Number	%	Number	%
With infiltrate	4	1.1	19	4.9
Without infiltrate	<u>344</u>		<u>370</u>	
Total	348		389	

Table 8-D shows the results of X-ray surveys in which entire Marine platoons and Navy companies were marched to sick bay for lung X-ray at about the end of the first 30 days of training. You will notice that in the Marine recruits we found roughly 1.1% of so-called walking pneumonias, or at least walking infiltrates, and about 5% in the Navy. There is no question that there was more severe illness in the Navy. Also, contrary to what we expected to find, the Navy recruits were less apt to report their illness than were the Marine recruits.

What is the difference between these two populations? They come from the same part of the country, they are the same age group, same

sex. Probably a big factor is the population size; the Navy population being about three times larger than the Marine population. This results in less crowding in the Marines. I think another very important factor is that when the Marines arrive at San Diego from civilian life, they are put into platoons almost immediately and these platoons are kept separated, very little intermingling between the different platoons. Not so with the Navy. The Navy forms its companies up to 3 days later and the attempt to keep the companies segregated from each other is not nearly so apparent. The Marines have three independent training battalions. Input into these battalions is rotated on a weekly basis. That is, all recruits arriving in the same week are put into one battalion and the next week's arrivals in the next, and so on. This results in an intermittent input and tends to break the chain of transmission from the sick to the well. There is a continuous input into the same battalion in the Navy. The Marines have more of their training outdoors than the Navy recruits, because the Navy recruits have to take much more class work. However, in the 1st week or two of training, there is about the same time percentage spent indoors or in classroom work in both populations, and by the 2nd week a higher incidence has occurred in the Navy. The Marine platoons are roughly the same as the Navy company but they split these platoon into squads. There are usually about 20-25 men/squad and these squads are housed separately. Therefore, the size of the aggregation of susceptibles is much less for the Marines than for the Navy. All of these factors are, I am sure, important, and I think it just illustrates the point that the environment must be considered right along with the parasite and the host.

Acker: As I understand it, substantial bearing on this problem might have been due to the schedule and immunizing injections for these recruits; that was a very interesting point.

L. Miller: This is so. Our study on this, which was done at Great Lakes, was set up to run 1-1/2 yr during which approximately half the recruits were put on a routine immunization schedule where they get about 21 different antigens; 9 ml of vaccine of one kind or another in the 1st half of training when they have the maximum amount of illness. The other 50% received immunizations other than respiratory disease vaccine, like the adenovirus vaccine and influenza vaccine, in the last half of training when there is much less ARD normally occurring. In a population of over 50,000 studied, we found that admissions for

pneumonia were about 20% less in recruits on the delayed schedule; also, the ARD incidence was about 20% less -- that is, reported illness. When we did X-ray surveys, we found about the same reduction in the prevalence of pulmonary infiltrates. I'd like to encourage people to work on this! I think Dr. Schulman may be doing something on this at the present. We set up the hypothesis that the endotoxin-like properties in some of these vaccines, particularly typhoid vaccine, have a biphasic effect on susceptibility: that in the first few hours, perhaps up to 12 hr after immunization, there is an increased non-specific type susceptibility, followed by an increased resistance. When you give the immunizations at a time when illness is incubating, with vaccine against which there is little if any challenge, the epidemic respiratory disease problem is accentuated.

VFTF: Is there any difference between the scheduling of Marines and Navy recruits regarding immunization?

L. Miller: No, it's about the same in San Diego.

Shinefield: May I ask one question relative to the incidence of bacterial pneumonia in the cases with ARD? Do you have any authoritative facts illustrating the relationship between bacterial pneumonia and those individuals that had ARD?

L. Miller: No. I would say 80% of the recruits, and I talk about the recruit population, have ARD of a mild nature, primarily. In studies at Great Lakes it has been difficult to find recruits that have pneumonia, bacterial pneumonia, that did not have preceding ARD. We have very little bacterial pneumonia occurring at Great Lakes for several reasons. I think the most important one is the routine Bicillin prophylaxis given to all recruits who are not sensitive to penicillin. They get 1.2 million units of Bicillin shortly after they arrive or within the 1st couple of weeks after arrival. On one occasion we have seen this noticeably terminate a streptococcal pneumonia outbreak. The prophylaxis program was started after the outbreak had been underway for about 10 days and as soon as the program was started the epidemic terminated. If we hadn't used it, and since strep starts to become a problem in the fall and becomes more and more of a problem as we progress into the winter, we would have expected more streptococcal pneumonias. I think the fact that we used Bicillin is one important factor or reason we don't have very much bacterial pneumonia.

Madin: In relation to the amount of vaccine given, did you notice any systemic reaction such as kidney troubles, etc?

L. Miller: No, not that we ever became aware of. We had the immediate systemic-type reaction but no more than what would be expected. We did not run across the problem you mentioned. The typhoid vaccine was usually the worst one for immediate reactions. We did not run across the problem with any vaccine such as we ran across at NAMRU-1 when we were using the plague vaccine in which we got those severe delayed reactions after 24-48 hr. Insofar as kidney sequels, and so on, I don't think it occurred.

## EPIDEMIOLOGY OF MENINGOCOCCAL EPIDEMIC

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If this were to be a sermon, I would choose for my text Newton's Third Law of Motion: For every action, there is an equal and opposite reaction. In the field of infectious disease control, there are many examples to indicate that we may be moving towards this truth. In the postwar years we have seen the nearly miraculous effect of penicillin on the staphylococci and the equally startling development of wide-spread resistance of these organisms to the antibiotic. The one-time promise of DDT to completely eradicate mosquitoes from most of the world has been seriously threatened by the propagation of genetically resistant strains of mosquitoes and strains which instinctively avoid alighting on DDT-covered interior surfaces. Syphilis, which for a time was so rare that many graduates of medical schools in the 1950's had never seen a case of infectious syphilis during their training, has been vigorously resurgent for the past 6 yr.

Thus it is with meningococcal meningitis and meningococemia. During World War II, victory against these diseases at last seemed close at hand with the discovery that even a single 2 g dose of a sulfonamide could sterilize most persons with respect to the meningococcus. So effective have the sulfonamides been against this disease that, with the exception of a moderate rise in incidence during the Korean War period, there have been no major epidemics of the disease in the United States since 1944. During the 10 yr period 1953 through

1962, for example, fewer cases occurred than during the 2 yr period 1943-1944. It was possible to ignore the careful work on environmental control of the disease accomplished in this country and in England during World War I with the wonder drug at hand some 25 yr later. It was even possible to stop further research on the disease, since supplies of the sulfonamides were virtually unlimited, cheap, and extraordinarily effective.

This period of complacency has come to an abrupt end. Earlier this year, Millar et al.<sup>(1)</sup> reported the recovery of a Group B Neisseria meningitidis resistant to safe clinical levels of sulfonamides both in vitro and in vivo. This strain was largely responsible for a large outbreak of meningococcal infection and disease among naval recruits to be discussed in this paper. When knowledge of the resistant strain transpired, it was then shown that a concurrent outbreak of the disease at a military base several hundred miles distant was caused by a similarly resistant strain of meningococcus. Spread of the sulfonamide-resistant organism from naval and military populations to the civilian population of the United States has now been carefully documented in two instances and is suspected in a third case.

Because meningococcal disease is a disease of humans only, because the case fatality ratio still averages about 12% in this era of antibiotics, because the infection can spread so rapidly among crowded populations, and because a potential danger exists from the occurrence of sulfonamide-resistant strains of meningococci, it is urgent that the research on control of this disease which was terminated (for all intents and purposes) in the last years of World War II be resumed once more. Because the major means of spread of the infection are through direct contact and by short-range aerosols from the noses and mouths of infected individuals, it is appropriate to present some of the interesting features of the recent epidemic at San Diego before this Symposium.

#### DESCRIPTION OF THE EPIDEMIC

Between 1 January 1963 and 30 June 1963, a total of 34 cases of meningococcal disease, including three deaths, were reported in recruits at the U.S. Naval Training Center, San Diego, California. During the same period, by conservative estimate, an additional 10,000 recruits became healthy carriers of the meningococcus and were dispersed throughout the Navy upon completion of recruit training.

Follow with me, now, the various events that took place during this 6 month period and for several months afterwards. I have chosen a chronological rather than a categorical presentation of the facts, for I believe this will better relate the manifold findings of the epidemic.

First, let us become oriented by a brief description of the training center and the recruit training program. The Center is located about 20 min drive from downtown San Diego (Fig. 1). It is "home" for 8,000-12,000 recruits at any one time. In addition, nearly 8,000 other personnel are based there on a more permanent duty; these personnel comprise such groups as company commanders, medical and supply department personnel, instructors, students at advanced training schools, and so on.

#### U. S. NAVAL TRAINING CENTER, SAN DIEGO

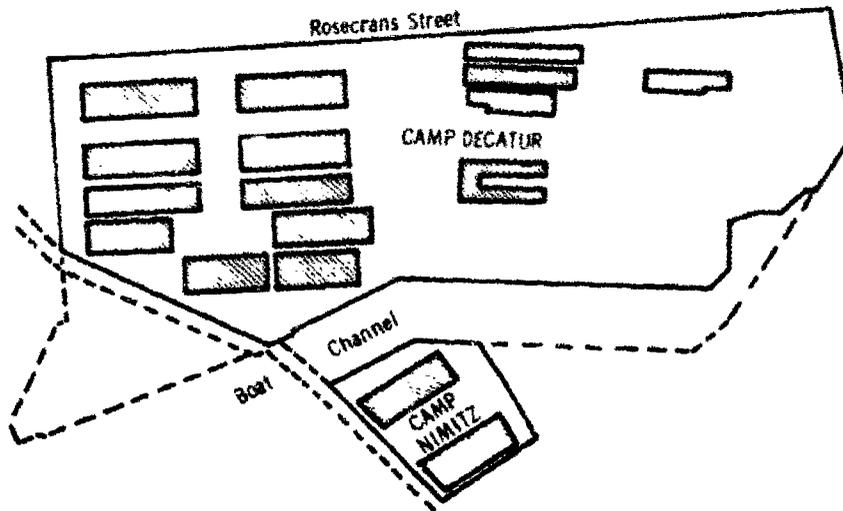


FIG. 1

Recruits come from all 50 United States; the majority come from the western half of the United States. Upon arrival at the Center, they remain in a general holding area for about 2 days and then form recruit companies of approximately 75-80 men each. During the first 3 weeks of the 9-week training course, companies are assigned to barracks in the Camp Nimitz area. Each company occupies one floor of one

wing in a two-story, "H"-shaped barracks. Mess facilities for the Camp Nimitz area consist of a single, large, open area serving an average of 2,500-4,000 persons/meal. Recruits in this first trimester of training are partially isolated from the remainder of the recruit population. Classes are frequently held at the main training center at Camp Decatur. This procedure permits some mixing of recruits in the early and advanced stages of training. After the initial 3 weeks, recruits move to Camp Decatur and occupy barrack spaces similar to those at Camp Nimitz; messing takes place in two mess halls separated by a common gallery. Throughout the training period, recruits come in contact only with specialized groups of permanently-based personnel who are responsible for their care and training. Liberty is granted only after completion of the 5th or 6th week of training. Upon completion of recruit training, recruits receive a 10-day leave and then move to permanent ship or shore station duty or to advanced training schools.

At the beginning of the epidemic -- which was arbitrarily defined later in its progress -- certain significant conditions existed:

A. Training was intensive, with the production of considerable fatigue among recruits. Until the outbreak was well-advanced, recruits averaged about 3-1/2 hr sleep each day. To add to the burden, personal laundry was done at the close of each day and each recruit participated, from time to time, in a 2 hr night watch to safeguard the laundry in barracks.

B. Barracks were greatly overcrowded. Before the end of April, each sleeping unit occupied about 25 sq ft, just 1/2 the minimum value prescribed by the Bureau of Medicine and Surgery of the Navy Department and but 1/3 the desirable value of 72 sq ft per sleeping unit.

C. Mouth-to-mouth contamination occurred in the use of drinking fountains. Each company area in a barracks had one drinking fountain. Because of the training schedule, all recruits tended to queue for water at the same time. A noticeable drop in water pressure occurred as a result and the jet of water from the fountain was reduced to the point where the recruit needed to place his lips on the orifice for his drink.

D. Routine immunizations against ten separate diseases were crammed into a tight schedule. Such crowding of inoculations has been demonstrated to predispose to respiratory infections<sup>(2)</sup>. In addition,

the febrile response from these inoculations occurred so often that dispensary physicians at first confused the febrile response of meningococcal disease with the inoculation reaction. Diagnosis of the disease was thus delayed occasionally.

E. The program for control of meningococcal disease was a post facto one. No program was in force until one or more cases occurred. Surveillance for subclinical infections was not considered necessary because mass sulfonamide prophylaxis was always available for control of high carrier prevalence signaled by the occurrence of disease.

This, then, is the framework upon which the epidemic was built. Sporadic cases of meningococcal disease throughout the year were always expected, but caused no undue alarm. When several cases occurred within a short span of time, sulfonamide prophylaxis would be given to the company contacts of the cases or, less frequently, to the entire recruit population. This latter routine occurred as recently as June and July 1962, when 11 cases due to a Group C meningococcus developed. This outbreak was abruptly terminated, as usual, by sterilizing the throats of carriers with sulfadiazine.

In Fig. 2, the 1963 epidemic is shown in detail. Three sporadic cases occurred in January and February. During the 1st week of March, four cases, including one death, were reported, and sulfadiazine prophylaxis was administered to all recruits. The strain isolated from patients at this time was Group C. There followed a period of 17 days (11-27 March) when no additional cases were reported. On 28 March, a patient was admitted in whom Group B meningococci were isolated and, 5 days later, a second patient was admitted with a Group B infection. Mass sulfadiazine prophylaxis was again ordered -- this time not only for all recruits but also for the training cadre as well.

Additional cases were reported, however. It was felt at that time that, while the existence of sulfonamide resistance in the Group B meningococci was a remote possibility, the most likely explanation of the additional cases was a purely administrative one, namely, personnel either missed taking prophylaxis or deliberately avoided taking their drug. Therefore, on 16 April, indicated by the third arrow in Fig. 2, sulfadiazine prophylaxis was administered to all recruits again. This time (and subsequently in all prophylaxis programs), however, strict controls were enforced to insure that all recruits consumed their tablets at the times directed. The drug was stubbornly

CASES OF MENINGOCOCCAL DISEASE BY WEEK OF ONSET  
 U. S. NAVAL TRAINING CENTER SAN DIEGO, 1 JAN. - 30 JUNE 1963

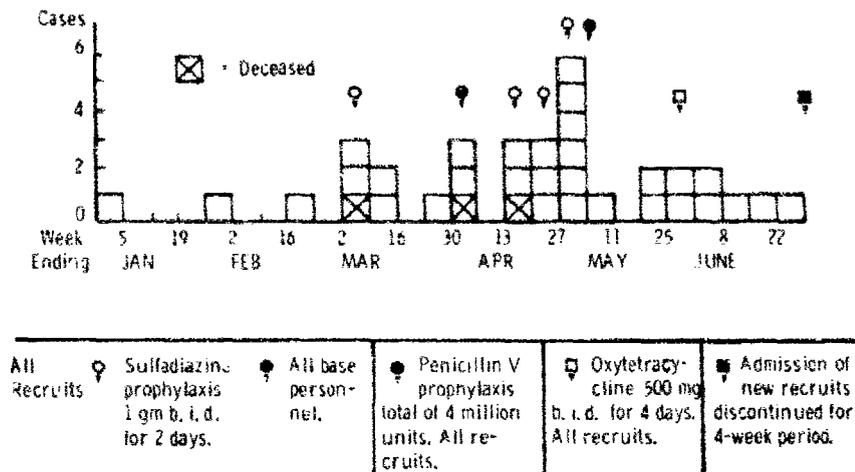


FIG. 2

readministered on 22 April and 1 May, the the disease just as stubbornly refused to die out. By this latter date, Group B meningococci were, and remained, the predominant group cultured from the throats of carriers and patients.

The details of establishing the proof of sulfadiazine-resistant meningococci have been presented in another paper<sup>(1)</sup> and need no further comment. To indicate the magnitude of the problem, however, it should be stated that administration of sulfadiazine in a regimen that would normally prevent culture of meningococci 4 hr after taking the first dose failed to yield a significant reduction in a carrier ratio of nearly 60%!

This was the first of two major crises to be encountered. The automatic tendency, once it was known that sulfonamides could not affect the carrier state, was to seek other antibiotics as substitutes in mass prophylaxis. Certain environmental changes were also made at this time, including:

- A. Changes in training to insure 7-8 hr sleep each night.
- B. Alteration of the immunization schedule to space inoculations as evenly as possible throughout the 9-week training period.

C. Reopening of unused barracks to increase floor area per sleeping unit.

D. Discontinuing barracks laundering and the night laundry watch.

E. Observation and culturing of throats of recruits reporting to the dispensary with febrile illnesses.

The search for another antibiotic as effective as sulfadiazine led to use of oral penicillin G, oral penicillin V, sulfamethoxy-pyridazine, oxytetracycline, and finally, in studies at the Naval Training Center, Great Lakes, Illinois, to Ampicillin (P-50), still an experimental drug at the time of study. None of these accomplished the elimination of the carrier state with the efficiency had in the past, though in vitro studies indicated that the meningococci tested were very sensitive to the drugs used. Figure 3 indicates the general experience with several drugs. Oxytetracycline showed the most promise, but within 4 days of discontinuing the drug, prevalence of meningococcal carriers had returned to pretreatment level. Use of these antibiotics among recruits did have the effect of reducing the frequency of clinical cases, but they were not administered on a long-term basis because of troublesome side effects and because we were convinced that once the "antibiotic lid" was lifted, the "disease pot" would boil over again.

Our narrative has now progressed to June 1963 and before presenting the happy ending to the story, I would like to summarize the clinical material on the 34 patients with meningococcal disease. Table 1 illustrates the diagnostic classification of cases, together with the evidence on which diagnosis was based. Note that a total of 27 of the 34 cases were confirmed by culture or spinal fluid smear. The initial signs, symptoms, and laboratory findings are presented in Table 2 and as may be seen are not at all unusual for the disease. The three deaths that occurred were all fulminating cases who sought medical attention when no therapy could save them and who died within 24 hr of admission. The remaining 31 patients showed a good response to treatment and have had no evidence of permanent neurological sequelae. Treatment consisted of intravenous sulfonamides and intravenous infusion of 16-24 mega-units of aqueous penicillin daily. Because of the use of penicillin in addition to intravenous sulfonamides, it is impossible to answer the tantalizing question of whether the response of

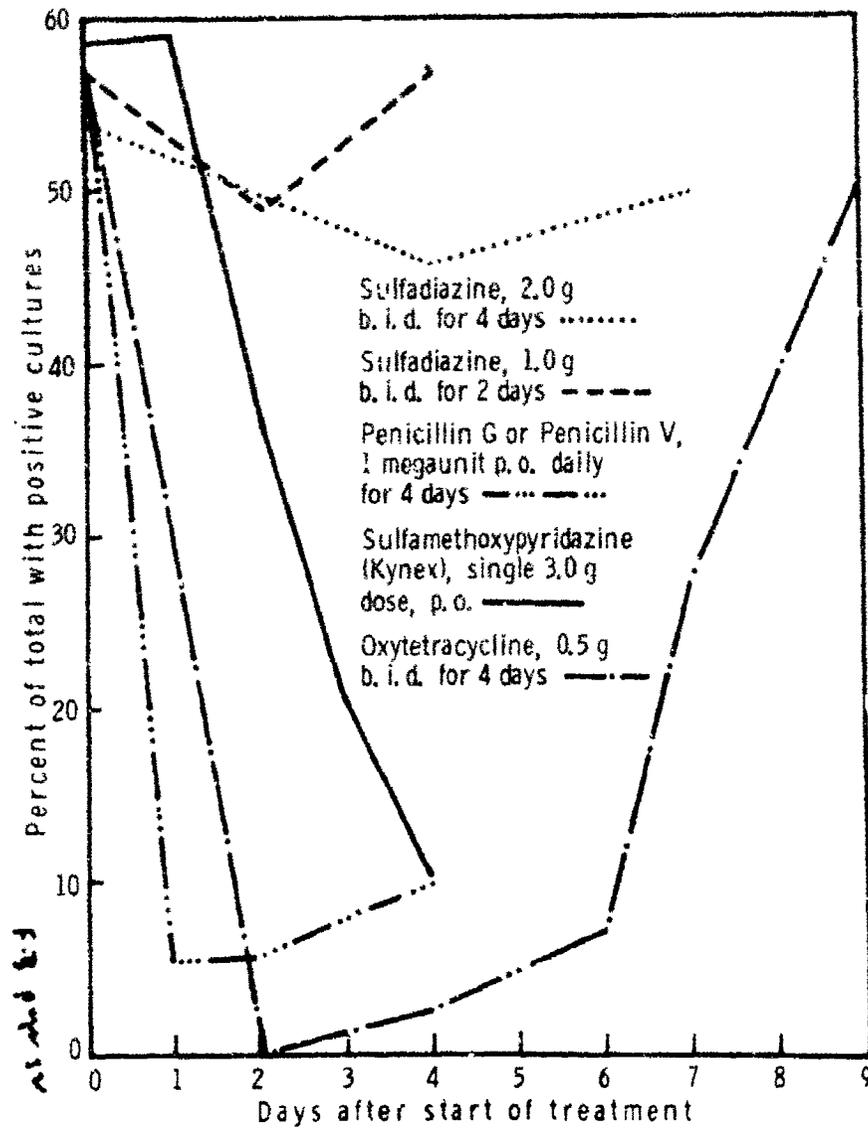


FIG. 3. Effectiveness of various drugs on eradication of meningococci from carriers. U. S. Naval Training Center, San Diego, 1967.

TABLE 1. Confirmation of diagnosis cases of meningococcal disease.  
Naval Training Center, San Diego. 1963.

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Total of 34 cases officially reported by the U. S. Naval Hospital,  
San Diego, from 1 January through 30 June 1963.

of these, 32 records were analyzed.  
Diagnosis was:

Meningococcal meningitis in - - - - -	25
Meningococemia in - - - - -	<u>7</u>
Total - - - - -	32

---

Of the 25 cases of meningococcal meningitis:

Diagnosis confirmed by positive CSF culture or pleocytosis in CSF and positive blood culture in - - - - -	19
Diagnosis confirmed by purulent CSF and positive CSF gram stain, although cultures negative in - - - - -	2
Diagnosis made on basis of purulent CSF in the presence of an epidemic -- all cultures and gram stains being negative in - - - - -	<u>4*</u>
Total - - - - -	25

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Of the 7 cases of meningococemia:

Diagnosis confirmed by positive blood cultures in the absence of stigmata of meningitis in - - - - -	6
Diagnosis made on basis of clinical findings only (fever, petechial rash) in presence of an epidemic without confirmatory laboratory findings in - - - - -	<u>1</u>
Total - - - - -	7

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\* One of these patients was already on antibiotics at the time of culturing.

TABLE 2. Clinical summary cases of meningococcal disease.  
Naval Training Center, San Diego. 1963.

	No. on whom data available	No. positive	% positive
<u>Signs and symptoms:</u>			
Temperature $\geq$ 101 F	21	19	91
Stiff neck	21	15	71
Petechiae	21	13	62
Headache	21	9	43
Abnormal state of consciousness (agitated, "delerious", semicomatose)	21	6	29
<u>Laboratory findings:</u>			
Peripheral WBC $\geq$ 15,000	15	12	80
CSF-WBC $\geq$ 1,500 and $\geq$ 90% polys	8	6	75
CSF-WBC $\leq$ 500	19	11	58
CSF-WBC $\geq$ 1,500, differential unknown	19	9	47
<u>Miscellaneous:</u>			
History of sulfonamide prophylaxis within 3 days of onset	21	0	43
<u>Other laboratory findings:</u>			
	<u>Mean</u>	<u>Median</u>	<u>Range</u>
CSF sugar (11 cases)	69	57	10-171
CSF protein (13 cases)	140	52	25-244
<u>Mortality:</u>			
	<u>Total Cases</u>	<u>Deaths</u>	<u>Case fatality Ratio</u>
	32	3	8.8%

meningococci to sulfonamides would be more favorable in clinically ill patients than in carriers. In view of a case fatality rate of 9%, however, no one dare assume the risk of administering sulfonamides alone.

When clinical cases were analyzed by length of residence at the Naval Training Center (environmental age), the findings were interesting. There was a bimodal distribution of cases (Table 3), though the majority of cases occurred during the first 4 weeks of training. The experience of past years has been for cases to occur at an early environmental age; with the use of a number of mass prophylaxis programs, however, some recruits destined to become patients had a postponement of their illness until later in training.

TABLE 3. Cases of meningococcal disease by environmental age at time of onset. Naval Training Center, San Diego, 1963.

Week of training at time of onset	No. of cases	% of total	Cumulative No. of cases	Cumulative % of total
1	6	17.6	6	17.6
2	6	17.6	12	35.2
3	7	20.6	19	55.8
4	8	23.6	27	79.4
5	1	2.9	28	82.3
6	5	14.7	33	97.0
7	1	2.9	34	100.0
8	0	0	34	100.0
9	0	0	34	100.0
Total	34	99.9	34	100.0

Resuming the chronology of the epidemic once more, in mid-June the drastic step of stopping the flow of recruits to the Naval Training Center, San Diego, for a period of approximately 5 weeks was ordered. This was possible because, at the anticipated rate of input of recruits into the Navy each week, they could be assigned to the Naval Training Center, Great Lakes, for this period of time without overtaxing the latter's capacity. Those of you with military experience can appreciate the magnitude of this step -- the first such occasion in this generation to my recollection. The logistics involved were enormous.

All recruiting stations had to be notified of the shift and had to prepare themselves for a major public relations problem. Large sums of appropriated funds were shifted from one command to another. Politicians and their constituents required explanations. Company commanders had to be sent from San Diego to Great Lakes, a step which later proved exceptionally ill-advised.

Fortunately, the effect of this step on control of the epidemic was as predicted. No cases of confirmed meningococcal disease have been reported since 28 June. After 3 weeks, Camp Nimitz was empty and, at the end of the 5th week, the recruit population had fallen to the amazing low of 3,600 persons. This decrease in recruit population made it possible to decrease the size of each recruit company and increase the living space per man to above the minimum requirement of 50 sq ft per man.

Concomitantly, a program to reduce frequency of contacts between early recruits and more advanced recruits or permanently-based personnel (among whom were relatively chronic carriers) was planned and subsequently effected. The major feature of the program was the requirement of wearing blue baseball hats during the first 3 weeks of recruit training and to warn all other naval personnel (who wore the traditional white hats) to avoid contact whenever possible with persons in the baseball caps.

Having reduced the carrier ratio by these means to an acceptable normal range (by the end of August 1963, the prevalence in recruits was 1% and in permanently-based personnel, 5-8%) and with it stopped the occurrence of cases for the time being. A proper program of prevention of infection was then instituted. Normally, between 2 and 5% of the newly arriving recruits can be expected to carry meningococci. This range is also that of the United States civilian population at the present time. To detect sharp and potentially dangerous rises in meningococcal prevalence in recruits, a bi-weekly cultural surveillance is maintained, using randomly selected recruits or recruit companies as subjects. Because chronic carriers are known to exist in the permanently-based personnel and because it is not practical to eliminate this endemic focus of infection, unless there is threat of an epidemic, surveillance has been extended to this group. The permanently-based personnel are divided into primary contact sources, which have frequent access to recruits, and secondary contact sources, which have

less frequent or less close-range contact with recruits. The first group consists of such persons as drill commanders, medical and dental officers, public health nurses, barbers, cooks, etc. The second group comprises chaplains, clothing issue and supply personnel, Red Cross workers, recruit evaluation personnel, etc.

As permanently-based personnel carriers are detected by sampling, they are given antibiotic treatment. If this fails to clear infection, it is attempted to shift them to jobs on the base which will not involve contact with the recruits.

What approach will be necessary for control of potentially dangerous carrier levels will be determined by the response of the prevailing strain to antibiotics. To date, sulfonamide-sensitive strains still dominate the meningococci in this country. Whether this fortuitous state in which sulfonamides can be used successfully in most outbreaks will remain for long cannot be guessed. What remains to be accomplished is preparation, in the form of epidemiological research, for environmental control of future outbreaks.

#### DISCUSSION

It is a strange thing that so many infections which produce no illness whatsoever in the vast majority of hosts should cause reactions with such serious prognosis when they do in fact become manifest. In this epidemic, it seems ironic that 10,000 or more persons remain perfectly healthy during their infection with meningococci and yet, in 9% of the clinical cases, death should result.

It should be apparent to everyone that the epidemic referred to was that of subclinical infections in the carrier group, not that of the unfortunate by-product of clinical cases. For this reason, the attack of medicine on the disease must be aimed primarily at the carrier state if incidence of disease is to be reduced.

Physicians enjoy summing up such behavior as due to differences in infected individuals, differences in virulence of the organism, differences in host responses to the organism, and various combinations of these factors. While this comprehensive statement is quite true, it is quite useless. It is an elegant way of saying, "We don't know why some people become ill and why some remain well during infection."

Much attention has been devoted to the study of virulence of respiratory pathogens -- the organisms can be removed from the patient and placed on the laboratory table for critical and leisurely study. What has been largely ignored is the natural site of initial infection: the epithelium of the nose and throat. We know, from Clover's classical report on World War I meningococcal infections<sup>(3)</sup> that the carrier state may exist for months or years in persons with anatomical abnormalities in the nasopharynx, most often deviated nasal septa and hypertrophic turbinates. We know that the meningococcus settles on columnar epithelium rather than squamous epithelium such as that found in the mouth proper. In this sense, it behaves in the same manner as its Neisserial brother, the gonococcus. Does it likewise invade the submucosa during the course of infection? If so, do the factors which determine localization or dissemination of the infection operate at this level of the mucosa? Is drying of the columnar epithelium, with resultant loss of ciliary action, a contributing factor in determining disease or carrier state? What, indeed, permits the fragile meningococcus to alter its commensal position atop the mucosa and occasionally seek the bloodstream or the meninges?

In the matter of virulence of the organism, we may ask what differences there are between the saprophytes of the Neisseria such as N. catarrhalis and N. flava and the pathogens N. meningitidis and N. gonorrhoeae. We know exceptionally little of the intermediary metabolism of this group of organisms and because of this, have not pursued the development of agents for poisoning Neisserial enzyme systems or blocking their chemical pathways (though we may assume that some antibiotics probably act in this manner).

The importance of the antigen-antibody response in meningococcal disease is just being realized. Human antibodies against the meningococcus have been known for about 50 yr and form the basis for dividing the meningococci into groups or types. We can detect antibodies in patients about a week or so after disease is apparent. Antibodies are also detected in carriers and, at San Diego, certain companies of recruits have been selected at random for baseline determination of antibody level and weekly titer determination during their entire training period. Unfortunately, these data are not yet ready for presentation.

One of the intriguing findings of chronic meningococcemia, reported by Benoit<sup>(4)</sup> in his studies at the U.S. Naval Hospital, Oakland,

California, was the complete failure of the serum to agglutinate organisms cultured from the same individual with meningococcosis. He suggested a hypothesis of bacteriocidal and bacteriostatic antibody responses to explain variations in the manifestations of meningococcal infection; this schema certainly deserves investigation.

Moving away now from the question, "Why the carrier state of meningococcal infection?" I would like to devote the remainder of my discussion to the actual spread of such infections among a defined population.

One of the oldest observations in epidemiology is the interplay between those infected with a communicable disease (cases), those subject to attack from the disease (susceptibles), and those who have protection against the disease (immunes). A number of mathematical models have been created to help understand this interplay, and the solutions to the models may be calculated directly or by empirical observation using Monte Carlo methods. A solution is most often represented as a graphic plot of number of cases at each interval of time elapsed (generation). In most models, the infection spreads slowly at first, suddenly explodes, and then, as the ratio of immunes to susceptibles increases, it dies rapidly. In a closed population, where no one enters the population and no one leaves except by death, the epidemic will eventually die out as susceptibles are exhausted or when contact between cases and susceptibles is so infrequent that it does not occur during the period of communicability of the disease.

On the other hand, a mathematical epidemic will tend to perpetuate itself if new susceptibles are continuously introduced into the population. Under these circumstances, the ratio of immunes to susceptibles remains reasonably constant and the frequency of contact between cases and susceptibles remains suitably high. Whether the disease will increase to epidemic proportions or will remain at low endemicity will depend on a number of parameters in the model, especially the proportions of cases and susceptibles in the population, the frequency of case-susceptible contacts, and the success of transmission of the organism from case to susceptible during the contact period.

If we now attempt to relate our real epidemic at San Diego to our mathematical model, it is found that the following similarities exist:

- A. Infectious cases are present in the form of acute and chronic

carriers of meningococci.

B. Immunes are found in permanently-based and advanced recruit populations.

C. Susceptibles are continuously added to the population. Each week new recruits arrive, most of whom are susceptible to the meningococcus.

D. Frequent contact occurs between carrier cases, susceptibles and immune personnel.

E. A nearly constant ratio exists between immunes and susceptibles. The ratio in permanently-based personnel is probably constant throughout the years. In the recruit population, recruit immunes graduate each week and are replaced by roughly an equal number of susceptible new recruits.

We do not have information at hand concerning the success of transfer of the organisms from person to person. It is a reasonable assumption that if transfer is relatively unsuccessful, prevalence of infection will be low and that if transfer is usually successful, prevalence will be high.

If one looks for a convenient model for the study of speed of spread of infections, one comes upon a most interesting analogue which behaves with mathematical niceness. That is the phenomenon of nuclear fission in heavy elements. Consider a subcritical mass of uranium<sup>235</sup>. Thermal neutrons collide with atomic nuclei and occasionally cause nuclear fission, just as our case-susceptible collisions occasionally cause infection or clinical disease. As critical mass is approached, fissions occur at a more rapid rate and finally, as critical mass is reached, fissions occur with infinite frequency.

In our epidemic, if we consider population density as mass, we note that infections occur more frequently as population density increases and, for each strain of organisms and each set of environmental and host conditions, infection spreads with violent rapidity as the critical population density is reached.

Glover<sup>(3)</sup>, in his World War I studies, was so impressed with the relationship between population density and prevalence of infections that he defined the "critical mass" for his populations. He stated that when the prevalence of carriers was 2% of the population, a

further, rapid, and dangerous rise in prevalence would occur and that clinical cases of meningococcal disease would appear. He was able to document this phenomenon in his groups and was able to predict rather accurately the occurrence of cases in other confined groups by following the rise of carrier ratios. Since then, other workers (5-9) have shown that high carrier prevalence may not result in the occurrence of cases and that a quantitative and fixed ratio of carriers in a population to cases may not exist. It is not unlikely that the rise from a normal prevalence of 5% to one of 60% (usually dangerous in our experience) occurs so rapidly that any measurement of prevalence taken during this period of rapid spread is subject to an enormous range of measurement error.

All workers agree that high population density -- overcrowding, in plainer language -- is an important determinant in the spread of meningococcal infections. Again among English troops in the First World War, it was possible to show the relationship between prevalence of infection and distance between beds in barracks. When the beds were 2 or more ft apart, prevalence of infections remained about 5%; if the distance was reduced to 1 ft, 9 inches, prevalence increased to approximately 10%; when distance was further reduced to 1 ft, 6 inches, prevalence jumped to 30%. Conversely, in barracks having an initially high prevalence because of overcrowded conditions and other factors, prevalence could be reduced substantially by spreading beds to the required 2 ft interbed distance.

We did not perform such beautifully quantitative studies at San Diego, but all our evidence is in agreement with the above observations. The barracks were overcrowded. Buildings at San Diego have accreted during three wars and some have become so aged that they cannot be used. To maintain the size of today's Navy, the Naval Training Center must process so many recruits each year and each recruit must receive a certain minimum number of weeks of training. These requirements generally fix the lower limit of the recruit population at San Diego and necessitate crowding of barracks beyond the standards recommended by the Medical Department of the Navy. With only 25 ft of berthing space per man, I can assure you that men are overcrowded.

What would have occurred at the Naval Training Center, San Diego, if each recruit had had 50 sq ft of living space? Possibly no epidemic, though sporadic cases would have occurred. This is not an

unfounded speculation. Adjacent to the Naval Training Center, and separated from it only by a chain-anchor fence is the Marine Corps Recruit Depot, which performs approximately the same functions as the Training Center in the Navy. The population of the Marine Corps Recruit Depot is normally somewhat smaller than at the Training Center, though it is still numbered in the thousands of recruits.

During the period in which 34 cases occurred in naval recruits, only four confirmed or suspected cases occurred among this group of Marine recruits. Differences in fatigue between the two populations cannot account for difference in disease incidence, for if anything, the Marine recruits work harder during training than Navy recruits. The major differences between the two populations which are significant were (1) the individual Marine recruit platoon of about 60 men is completely isolated from other platoons, (2) the Marine platoon is quartered in smaller barracks (not four to a building as with Naval recruit companies), and (3) each sleeping unit occupies approximately 50 sq ft. Significant also is the average prevalence of meningococcal infections in Marine recruits of 1-5% at a time when prevalence among Navy recruits was about 50%.

Another piece of evidence to illustrate the influence of overcrowding on spread of infection resulted from the decision to divert new recruits from San Diego to the Naval Training Center, Great Lakes, for a 5-week period. This latter Center had had an outbreak of eight cases of meningococcal disease during March 1963, but had controlled further spread by administration of mass sulfadiazine prophylaxis. Sulfadiazine was also given all new arrivals for a 2-day period to prevent re-introduction of meningococci into the Center. Following this, cultures taken at intervals on small samples of recruits indicated a normal or slightly below normal prevalence of carriers.

As the recruit population at San Diego decreased to its low of 2,600, that of Great Lakes increased to over 18,000 well above the usual population of the base. This rise in population density was expected, but because of the low prevalence of meningococcal carriers, the associated risk of a meningococcal outbreak was considered low. When it was decided to transfer 80 recruit company commanders from San Diego to Great Lakes because of the swollen recruit population, great care was taken to sterilize them with respect to sulfonamide-resistant meningococci or, if this could not be done, to keep them from recruits

until their infections cleared. What was not realized at the time was that the San Diego sulfonamide-resistant strain was already present at Great Lakes, among recruit graduates of San Diego who had been transferred to Great Lakes for advanced training or for permanent duty.

Near the end of July 1963, when the moratorium at San Diego had nearly ended, it was reported that 20% of a small sample of Great Lakes recruits had positive cultures for meningococci; most of the isolates were sulfonamide-resistant. Within 2 weeks, nearly a dozen suspected cases of meningococcal disease were reported, of whom about half were subsequently confirmed. This time, we did not depend on antibiotic prophylaxis for control. Instead, the recruit population was rapidly reduced by early graduation of several thousand recruits and by diverting some, but not all, scheduled arrivals to San Diego. No additional cases have been reported for the past month from either Training Center. After the experience earlier in the year, I am too cautious to state that the danger of a large epidemic has passed.

While I lack specific experimental data to confirm it, I believe that the explanation of the effect of overcrowding on this infection is not difficult. Because the meningococcus is spread through droplets sprayed from the nose and mouth (we can ignore direct contact spread as being minor under most conditions) and because the meningococcus is exquisitely sensitive to drying and changes in temperature, the infectious aerosol must be inhaled at relatively short range and in a relatively short time after generation if infection is to occur. People must be crowded and must have frequent close contact with one another for these conditions to exist. When crowding occurs in sleeping quarters conditions for successful aerosol infection are even more favorable, because ventilation may be minimal and because most people sleep with their mouths open thus by-passing the filtering mechanism of the nose.

Here is an excellent model for workers in experimental aerosol infections. The space and time relationships mentioned above have not been observed yet under rigidly controlled conditions. Decay curves for neisseria aerosols have not been computed. The range and maximum effective size of aerosol droplets have not been determined. The minimal infectious dose of meningococci is unknown.

Since it will be necessary to use human volunteers in these studies eventually, the microbiologists can contribute to this work

by developing an attenuated and completely nonpathogenic strain of meningococci for use in aerosols. Perhaps a saprophytic Neisseria species rare or alien to the human throat might be substituted for the time being.

I hope that the headaches suffered by all of us that have been involved in this epidemic will have been worthwhile by demonstrating the value and urgent need for general experimentation on meningococcal infections.

In closing, I would like to acknowledge those who did all the work: U.S. Navy Preventive Medicine Unit Number Five, San Diego; U.S. Naval Medical Research Unit Number Four, Great Lakes; U.S. Naval Hospital, San Diego; the Command and Medical staff of the Naval Training Center, San Diego; the Communicable Disease Center, U.S. Public Health Service, Atlanta, Georgia, and Dr. Harry A. Feldman, Upstate Medical Center, Syracuse, New York. For those who may be interested in the laboratory methods used by them in their study, I have added an appendix to the manuscript submitted today.

#### APPENDIX LABORATORY METHODS

Pharyngeal cultures were obtained using sterile cotton swabs on straight wooden applicator sticks. These were then streaked immediately on either chocolate agar or Mueller-Hinton agar plates. Neither para-aminobenzoic acid or penicillinase were routinely added to culture-media. The plates were then incubated in a candle jar for 24 hr at 37 C. Suspicious colonies (usually three to four per plate) were then picked and subcultured. Isolates were then identified and grouped by slide agglutination test, employing group specific rabbit antiserum provided by the Communicable Disease Center, U.S. Public Health Service. Fermentation tests were not performed in routine identification, but were done as a check from time to time; such tests confirmed the identification of N. meningitidis in all cases. While some criticism of this method may be deserved, it should be realized that three trained laboratory technicians handled over 4,000 specimens during the 3-month period of intensive study and could not handle the additional burden of routine carbohydrate fermentation tests.

Studies of in vitro drug sensitivity were performed by a plate dilution method in which Mueller-Hinton agar was used to minimize the

effect of sulfonamide inhibitor. Serial dilutions of the test drug were prepared and mixed with the medium before gel state occurred. Final concentration was expressed as milligrams or units of drug per unit volume of medium. Plates were inoculated by streaking out a single loopful of uniform size, drawn from a translucent nutrient broth suspension of the organism. The complete inhibitory concentration (CIC) was defined as that concentration of drug which completely inhibited growth after incubation for 24 hr. Although no attempt was made to quantitate the size of inoculum to a finer degree, the identical technique was employed in processing multiple specimens from the Naval Training Centers at San Diego, California and Great Lakes, Illinois; the results obtained were consistent within each recruit population and demonstrated a marked difference in sulfadiazine sensitivity between the two populations. Representative isolates of meningococci were sent to four additional laboratories in other parts of the United States; results from these laboratories were in agreement with the complete inhibitory concentrations obtained locally in San Diego.

Blood sulfonamide levels were determined by the method of Bratton and Marshall, corrected for sulfadiazine.

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## PATTERNS OF ADENOVIRUS INFECTIONS IN MARINE CORPS PERSONNEL\*

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During continuous surveillance of respiratory disease in Marine Corps personnel, a pattern of adenovirus infection and associated illness emerged which was significantly different from that which has been reported for other military populations. In previous studies adenovirus infection in the military was found to be an important cause of respiratory disease throughout the year with peaks of activity occurring during the fall and winter months<sup>(1,2,3)</sup>.

During a 4-yr period, four yearly, sharply demarcated, winter epidemics were observed in advanced Marine recruit trainees and "seasoned" personnel. Adenovirus infection occurred infrequently or not at all during the 6 month interepidemic periods. Significantly lower infection rates were observed for recruits during their first 11 weeks of training at Parris Island, S. C. than during subsequent advanced recruit training at Camp Lejeune, N. C. The almost complete absence of adenovirus infections during the interepidemic periods presented an unusual opportunity to investigate the possible mechanism responsible for the initiation and cessation of the outbreaks.

### Location and Composition of the Study Population

The Marine Corps Recruit Depot, Parris Island, S. C. (PI) has been previously described<sup>(4)</sup>. Several major differences exist between the training program at PI and other Army and Navy recruit centers.

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\* Based on a paper to be published in the Am. J. Hyg., 1964.

Marine recruit training, which lasts 11-12 weeks, is 3-4 weeks longer in duration than that of Army and Navy recruits. In addition, Marine recruits are effectively isolated from the non-recruit military and civilian communities. The training program also greatly restricts contact between the various recruit platoons (75-man training units).

Camp Lejeune, N.C. is a large Marine Corps base with a military population approaching 30,000. The component organizations and their major functions have been previously described<sup>(5,6,7)</sup>. The Infantry Training Regiment (ITR) is part of this complex and merits special attention. After the completion of PI training, and without any leave period, the men are sent directly to ITR where they remain for 30 days and receive an intensive course in infantry tactics. The number of men in training varies greatly from just over 1000 to more than 5000. The normal Marine Corps enlistment pattern results in the population at PI reaching its highest levels in the early fall which subsequently leads to maximum troop strength at ITR in December-January.

"Seasoned" personnel were selected for study from Marines of Force Troops (FT), Fleet Marine Force, Atlantic, which is located at Camp Lejeune, N. C. This organization is composed of approximately 5000 members divided into seven units. The FT environment, although military, is quite different from that of ITR. FT personnel represent a more dispersed population. Each unit has its own housing, messing and medical facilities. Their military job specialities also tend to aid in reducing constant close associations between individuals of different units.

The methods used in the selection of patients, collection of specimens and subsequent laboratory procedures have been previously described<sup>(5,6,8)</sup>.

#### Respiratory Disease Morbidity at Camp Lejeune and Parris Island

Distinct waves of respiratory illness were observed at Camp Lejeune during a 42-month period (November 1959-April 1963). A comparison of the total respiratory illness rates per 1000 men per month among recruits at PI, advanced trainees at ITR and permanent personnel at FT revealed that the "seasoned" personnel experienced relatively low respiratory illness attack rates throughout most of the year (10-375). The recruits at PI experienced considerably less respiratory illness during their 11-12 weeks of training (50-375) than was

observed among the same men during a subsequent 30-day interval at ITR (120-1200). Although the magnitude of the rates differed among the three groups, similarities were observed in the temporal relationship of peak periods of reported illness. It was of interest that the advanced recruits at ITR experienced a sharp outbreak of respiratory disease each winter, starting in January and lasting through March or April. Febrile illness rates (100 F or greater orally) correlated well with the high January-April incidence of illness at ITR.

In an attempt to define factors that might be related to the sharply limited yearly epidemic of adenovirus at ITR, continuous indoor and outdoor temperature and humidity recordings were obtained beginning in August 1961. A suggestive correlation between low indoor humidity and high adenovirus illness rates was noted.

#### Epidemiology of Adenovirus Infection at Camp Lejeune

Adenovirus types 4 and 7 were recovered predominantly during the January-June periods of 1960, 1961, 1962 and 1963. With the exception of 25 strains of adenovirus type 7 recovered in 1960, five strains in 1961 and two strains in 1963, type 4 was the predominant serotype recovered during the epidemic periods (1172 strains recovered). Adenovirus activity was not exclusively limited to the epidemic periods. For instance, nine strains of adenovirus type 4 were recovered in the July-December periods of 1960-1962. The rate of isolation of adenovirus from the ITR group was always higher than from FT personnel.

The isolation rate of adenovirus from patients with respiratory illness was always significantly higher than from a group of patients with non-respiratory complaints.

In other military studies, seasoned personnel have had low adenovirus infection rates<sup>(9)</sup>. In contrast, the FT personnel with respiratory illness studied at Camp Lejeune had a relatively high incidence of adenovirus infection. In view of this finding and the pattern of sharply circumscribed yearly adenovirus epidemics at ITR, it was of interest to determine whether length of service in the Marine Corps correlated with adenovirus infection in FT personnel. Time of entry into the Marine Corps was used as a basis for this comparison and a significant association existed between entry into service during an interepidemic period and subsequent susceptibility to adenovirus infection during the following year's epidemic. Thus, recruits who

trained during an interepidemic period were at high risk with regard to adenovirus infection during the subsequent epidemic.

#### Epidemiology of Adenovirus Infection at Parris Island

Previous studies of primary atypical pneumonia among recruits at PI revealed that adenovirus activity was at a low level during many months of the year<sup>(4,10)</sup>. In order to more fully understand the epidemics at IIR, observations and sampling were conducted at both ITR and PI simultaneously. Surveillance was carried out at PI during the winter respiratory disease seasons of 1960, 1961, 1962 and 1963.

Adenovirus infection was associated with a significant proportion of that febrile respiratory disease and pneumonia that occurred at PI during the late winter and spring of 1960 and 1962. Since the incidence of febrile respiratory disease at PI remained low throughout the year, the total amount of adenovirus-associated illness remained low throughout the year. Epidemics of adenovirus illness of the type seen at ITR were not observed at PI. Recruits in all weeks of training were affected with no individual training unit (75-man platoon) experiencing a disproportionate amount of adenovirus infection.

Adenovirus infection, as evidenced by virus isolation or antibody rise, was rarely detected in the July-December sampling periods. These findings were consistent with those observed at ITR.

#### Clinical Features

Forsyth<sup>(11)</sup> recently compared the symptoms reported both by ITR patients from whom adenovirus, rhinovirus, or Coxsackie A-21 virus was isolated and a matched group of men from whom no agent was recovered. These results indicated that adenovirus was associated with a more severe respiratory illness than usually reported by patients either infected with rhinoviruses, Coxsackie A-21 virus, or who were virus negative. The character of the adenovirus illness patterns observed at Camp Lejeune were similar to those reported in other military populations. Respiratory symptoms were reported more often than were those of a systemic or gastrointestinal nature.

Of 1,200 ITR Marines with respiratory disease in the January-April 1962 outbreak, X-Ray examination revealed infiltrates in approximately 10% of the group. A comprehensive laboratory study of 40 cases of pneumonia with matched upper respiratory and non-respiratory control patients will be presented separately<sup>(12)</sup>.

#### Other Agents

Other respiratory viruses and Micrococcus pneumoniae were isolated during the periods of adenovirus prevalence. Except in 1961, rhinoviruses were usually isolated during the early and late stages of the outbreaks but not during the height of the outbreak. The other agents were isolated sporadically at a low level throughout the entire course of the epidemic. In 1961 respiratory syncytial virus and rhinoviruses appeared to be responsible for as much afebrile respiratory illness as adenovirus<sup>(8,13)</sup>.

The pattern of adenovirus infection observed in Marine Corps personnel in this study was substantially different from that previously reported for other military populations<sup>(9,14,15)</sup>. In Army and Navy training centers, adenovirus infection was found to occur predominantly during the first 6 weeks of recruit training<sup>(9,14,15)</sup>. Adenovirus disease occurred in these populations throughout the year with seasonal increases during the fall or winter months. This pattern was thought to be characteristic of military adenovirus infections. However, the present study revealed at least three other patterns of adenovirus disease in military personnel. The level of adenoviruses were present and responsible for a considerable proportion of those respiratory illnesses which occurred during the winter months. The same men, as advanced recruits at ITR, Camp Lejeune, exhibited a different pattern; men in this population were subject to extensive sharp seasonal epidemics of adenovirus respiratory illness and were relatively free of the disease during the remaining months of the year. A third pattern was exhibited by seasoned Marine personnel who were susceptible to adenovirus infection and illness during the yearly epidemic if they had undergone recruit training in the past year during an interepidemic period.

It is of interest to speculate as to the reasons for these different patterns of adenovirus disease. There were major differences in training and housing among these groups. At PI men in one recruit platoon had relatively little close contact with men in other platoons and the recruits were not allowed any time away from their platoons during their entire stay at PI. The training platoon represented the epidemiologic unit in this population. The observation that adenovirus infection did not spread rapidly within this group suggest that adenovirus requires a population larger than 75 men to generate a rapidly

spreading extensive epidemic.

The methods of housing, messing and training at ITR were more like those of the Army and Navy recruit centers where there is a higher rate of adenovirus infection than at PI. At ITR the training unit was 240 men and the trainees had freedom of movement in off-duty hours; i.e., movies, post exchange, and recreation centers. However, since the methods of training remained constant throughout the year, factors other than training conditions must be responsible for the sharp seasonal extensive epidemics of adenovirus infection.

The PI personnel had all the privileges of permanent troops. Many of these men were married and lived out of the barracks in the community. They mixed freely during the entire day and come in contact with other groups during routine leave and liberty.

In addition to differences in training, PI is also in a different geographical location from ITR. PI is 300 miles south of Camp Lejeune. At the present time it is not possible to evaluate the contribution of different climates to the different patterns observed at PI and ITR. It is of interest, however, that the Fort Bragg respiratory disease experience reported by the Commission of Acute Respiratory Disease was similar to that found at ITR<sup>(11)</sup>. Fort Bragg is 75 miles west of Camp Lejeune and has a similar climate. Whether the apparent similarities in the epidemiology of adenovirus infection in the two populations can be attributed to the same geographical and climatological conditions cannot be answered at the present time.

The reasons for the seasonal epidemics of adenovirus illness at ITR merit special consideration. The inability to detect adenovirus type 4 in our population during most of the July-December periods may merely reflect inadequate sampling during periods of low-level virus activity. This was the case in the fall of 1960 during an outbreak of Coxsackie A-21 virus when the number of patients sampled was increased above the normal rate<sup>(6,7)</sup>. During this time period eight strains of adenovirus type 4 virus were recovered. Again in the Fall of 1961 one strain was recovered during a period of increased sampling. Therefore, it seems probable that adenovirus type 4 was present in the camp at a low level during the entire year and that some as yet undetermined environmental factor was responsible for epidemic occurrence of infection and illness during the January-April period.

Since adenovirus infection was not extensive at PI, it is unlikely that an exhaustion of susceptibles at this base was responsible for the termination of the outbreak at Camp Lejeune. The results obtained in longitudinal company studies in 1962 are consistent with this hypothesis since the proportion of susceptible individuals who entered advanced training at Camp Lejeune remained constant throughout the epidemic and during its termination<sup>(17)</sup>.

It was observed that the indoor relative humidity (RH) decreased markedly approximately 1 month prior to the onset of the epidemics in 1962 and 1963. A forced dry air heating system was used in the ITR barracks and the difference between the RH outdoors and indoors was most apparent when the barracks were heated (November-April). It is possible that the lowered indoor RH may have played a role in facilitating virus spread. Hemmes et al.<sup>(18)</sup> have suggested that the indoor RH may be of importance in the usual winter occurrence of influenza virus morbidity. Stability of influenza aerosols under controlled conditions of temperature and RH has been studied by Hemmes et al.<sup>(19)</sup> and Harper<sup>(20)</sup>. Their findings indicated that survival of influenza virus was greatly reduced when the RH exceeded 50%. Virus survival was prolonged at lower temperatures. No data concerning the stability of adenovirus type 4 aerosols are available. However, Buckland<sup>(21)</sup> found that adenovirus types 1 and 3 survived best at high RH. It may be that the effect of the RH and temperature on the host defense mechanisms may be more important in determining the seasonal occurrence of epidemics than the effect of these factors on virus survival in vitro. The correlation of the low indoor humidity with the course of the adenovirus epidemics at ITR remains to be determined in subsequent studies. If the relationship is significant, an approach toward limiting the spread of this agent can be realistically provided.

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#### DISCUSSION

Schulman: What about the seeming discrepancies regarding seasonal variations of adenovirus that different people report. In some instances, if you look at it in terms of rise in antibody titer, they report that there is not that much seasonal variation; the in toto incidence is the same all year around, but the ratio of febrile cases is much higher in the Winter than it is in the Summer.

In the figures you show it would seem, in your study groups at least, the over-all incidence of infection per se was much lower.

Bloom: That's correct. Our studies have been considerably different from what has been reported in studies of adenovirus type 4 at Fort Ord, Fort Dix and Great Lakes. This is why we believe it was worth the effort to do the longitudinal study in an attempt to quantify these differences. As far as febrile illnesses go, 85% of all febrile illnesses sampled during these epidemic periods were associated with pneumonia, about 10% of all men admitted for respiratory disease were found to have an infiltrate and these cases were almost invariably associated with adenovirus type 4.

VFIF\*: Did you find any association between these recruits that had the adenovirus disease and any bacterial diseases.

Bloom: There was practically no bacterial pneumonia among recruits at Parris Island or among the young military people at Camp Lejeune. The rate of bacterial pneumonia is very low for almost all military recruit populations.

VFIF: You remarked that the last recruit groups who went through the longitudinal study had a very high incidence of what we might call common colds. Do you think that the reduced rate of adenovirus infection might therefore, in this particular study, have been a fortuitous thing representing viral interference?

Bloom: I don't believe so, because in the cross-section studies, when the 5,000 advanced recruits returned from Christmas leave, we were able to isolate all of the so-called newer respiratory viruses. Adenovirus type 4 then began to replace the rhinoviruses quite rapidly.

Capt. Miller: Exclusion of rhinoviruses by adenoviruses in this relatively select group of recruits when in training, right in the beginning, middle and end of the training is interesting. We had this high instance of adenovirus all through this period of the year. We also had serologic evidence with 2060 (ECHO 28) virus and this ran about 15-20% rhinovirus into the year. We did not go so far as to see what happened in the individual recruits, that is, whether we had an adenovirus rise or not.

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\* VFIF = Voice from the floor.

Bloom: In some of the studies reported from Great Lakes, there is evidence of multiple infections but of a higher order of magnitude than ours have been. We had very little evidence of multiplicity of infection. Eaton agent pneumonia appeared throughout our adenovirus epidemic. One of the problems is to be able to select out the treatable Eaton pneumonias from the adenovirus pneumonias which are "untreatable". Eaton has the capacity of not being replaced by adenovirus. As far as the rhinoviruses go, the last longitudinal study group yielded primarily RGF virus and we also have evidence of ECRO 28 in the population appearing at the end of the adenovirus outbreak.

## THE PATHOLOGY OF CHRONIC BRONCHITIS

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The understanding of the pathology of chronic bronchitis has been limited by the inferior quality of microscopic slides of bronchi prepared in the routine manner in most laboratories. Formalin fixation and paraffin embedding introduce many artifacts because of the marked shrinkage and the partial loss of edema and mucus. A modified method for the preparation of microscopic slides of bronchi was used in our laboratory. Bronchial tissue prepared by this method showed minimal shrinkage, and mucus and edema were appropriately retained within the bronchial lumens and walls.

It is the purpose of this paper to report the pathologic findings of the bronchi in 15 cases of chronic bronchitis. In each case a lung was available from an autopsy or surgical pneumonectomy.

### METHODS AND MATERIALS

The diagnosis of chronic bronchitis had been established clinically in each of 15 cases. This diagnosis was based upon the combination of cough, sputum and heavy cigarette smoking for at least 2 yr. Twelve of the cases died of other causes and were autopsied. Three cases had a lung removed because of bronchogenic carcinoma. All cases were free of terminal suppurative bronchitis, pneumonia or aspiration.

Segments of the bronchi were removed with a scalpel from various levels of the bronchial tree of each case and placed in Carnoy's



FIG. 1. A microscopic cross-section of a segmental bronchus showing the hypertrophied mucous glands (arrows), the dilated gland ducts and the mucopurulent debris in the bronchial lumen.  
H and E. X 16

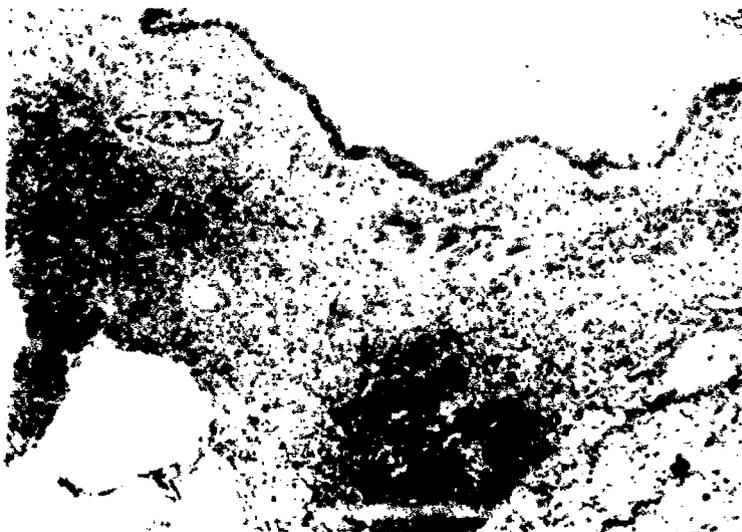


FIG. 2. Chronic inflammation involving the wall of a medium sized bronchus. There is an intense infiltration of the wall by chronic inflammatory cells.  
H and E. X 80

fixative After 24 hr these tissues were placed in several changes of absolute ethyl alcohol and embedded in nitrocellulose. The tissue was sectioned at 6  $\mu$  and stained variously with H and E, Mallory's trichrome, Verhoeff elastic-van Gieson, and PAS stains.

#### OBSERVATIONS

All 15 cases of clinically diagnosed-chronic bronchitis showed inflammatory changes involving the bronchial wall. Most noteworthy was the edema which permeated the entire bronchial wall, causing it to be considerably thickened (Fig. 1). Distended lymph vessels accompanied the edema. The thickened mucosa produced narrowing of the bronchial lumen. Frequently the respiratory epithelial layer was partially detached from its basement membrane by small localized vesicles of edema fluid. Edema fluid was also commonly seen mixed with mucus within the lumens of the bronchi.

Chronic inflammatory cells consisting of lymphocytes and plasma cells were always present in the walls of the bronchi (Fig. 2). Some of the cases possessed more inflammatory cells than others and generally correlated with a greater degree of edema, thus indicating a more severe degree of inflammation. The plasma cells often possessed bright red granules within their cytoplasm. These granules, known as "grape cells" or "Russell-Fuchs bodies", were also observed in plasma cells within the peribronchial lymph nodules.

The mucous glands of the bronchi were markedly hypertrophied and hyperplastic, as described by Reid<sup>(1)</sup>, in five of the cases and moderately so in the others. The gland acini were filled with mucus, and there was streaming of the mucus within dilated gland ducts (Fig. 3). This mucus extended from the ducts into the bronchial lumens. The respiratory epithelium contained increased numbers of distended goblet cells at all levels of the bronchial tree including the terminal and respiratory bronchioles which normally do not possess these cells. The walls of the goblet cells bulged with mucus onto the mucosal surface. Mucus within the bronchial lumens contained moderate numbers of acute and chronic inflammatory cells as well as clumps of detached epithelial cells. Bubbles of air could frequently be seen as round empty spaces within the mucus. This mucus produced a partial to complete obstruction in many of the smaller bronchi.

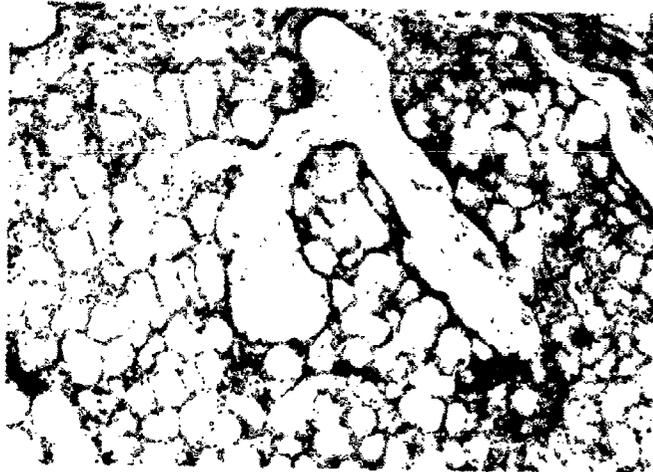


FIG. 3. Hyperplasia and hypertrophy of the mucous glands. Note the streaming appearance of the mucus within the gland ducts. H and E. X 80

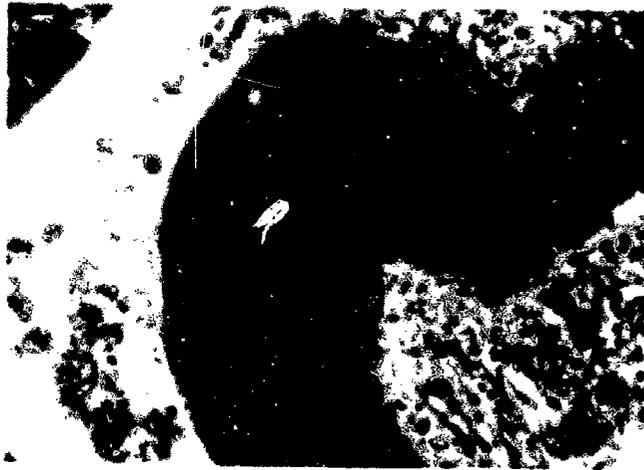


FIG. 4. Squamous metaplasia of the respiratory epithelium. There is atypism of the nuclei and numerous mitoses. H and E. X 250

Alteration of the respiratory epithelium was a frequent finding in all of the cases. The most commonly observed abnormality was a focal detachment of the epithelium from the basement membrane. This was usually accompanied by microscopic accumulations of edema fluid between the cells and the membrane. Occasionally there were ulcerations of the mucosa with replacement of the epithelium by fibrinous exudate overlying a zone of granulation tissue. A very common observation was stratification and squamous metaplasia of the respiratory epithelium. The stratification consisted of two or more layers of flattened epithelial cells devoid of cilia or mucous goblets. The metaplasia consisted of replacement of the respiratory epithelium by two or more layers of squamous cells showing keratin formation. Atypism of the nuclei of these cells was occasionally seen and usually associated with increased mitoses (Fig. 4). Carcinoma was not observed in any of the cases although two sites of borderline atypism were noted.

The basement membrane beneath the respiratory epithelium was thickened in all cases. This thickening possessed a hyaline appearance and the thickness varied within the same bronchus. Acute and chronic inflammatory cells were sometimes observed within the hyalinized membrane indicating a migration of these cells from the mucosa into the bronchial lumen.

#### COMMENT

The histologic observations of the bronchi of 15 autopsy and surgical cases with a clinical history of chronic bronchitis showed significant degrees of chronic inflammation involving the bronchial wall. This inflammation was manifested by changes in the bronchi consisting of edema, increased mucus production, inflammatory cell infiltration and alterations in the respiratory epithelium. The edema caused thickening of the mucosa with resultant reduction in the size of the bronchial lumen. The small bronchi were frequently partially or completely obstructed by a mixture of mucus and edema fluid within their lumens.

The small erosions and detachments of the respiratory epithelium indicated the destructive character of chronic bronchitis. The alterations of the respiratory epithelium to a squamous cell pattern apparently had resulted from repeated insults to the mucosa. The excess mucus production by the hypertrophic glands and increased goblet cells was probably a response to irritation.

It seems reasonable to propose that the inflammatory reaction involving the bronchial wall results in serious damage, some of which is permanent, to the bronchi. These damaged airways have less resistance to invasion by bacteria, and further inflammation results from the invasion by pathogenic organisms. The initial insult could likely result from repeated and prolonged attacks by viruses or exogenous chemical irritants.

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## EFFECTS OF NOXIOUS GASES ON RESISTANCE TO RESPIRATORY INFECTION\*

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Various factors not related to the natural development of the host, such as age, sex, or heredity, can have a profound effect on native or acquired resistance to infection. These factors may be ordinary environmental influences encountered by everyone on a daily basis, or they can be more extraordinary ones to which only a fraction of the population is exposed. In the last group one can include noxious gases in community air pollution or those present under certain circumstances as an industrial hazard.

Through their effect upon the physiology of the host, environmental factors may alter the host-parasite relationship resulting, in many instances, in lowering the resistance to infection. Thus they may interfere with normal defense mechanisms through destruction of leukocytes or inhibition of antibody production. They also may produce changes in the physical defense mechanisms of the body such as ciliary movement or mucus excretion in the respiratory system. The degree of damage produced is usually closely related to the duration of exposure and concentration of gas to which the host is exposed.

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\* This study was supported by funds provided by the U.S. Public Health Service, Division of Air Pollution, under Contract No. SApH 73297.

The effects of noxious or irritating gases on health frequently occur as the result of contact between the gases and membranous surfaces of the respiratory system; the latter serve as defense mechanisms against respiratory infections. Protective action takes place in the tracheobronchial tree through the production of mucus and through ciliary movement. An irritant gas reaching the epithelium of the trachea or the bronchi can paralyze the cilia or cause destruction of the surface layers of the epithelial lining. Damage to the bronchial epithelium cannot always be detected, especially if the concentrations of the irritating gases are relatively low. However, damage may become apparent when the subject is exposed to a secondary stress, such as respiratory challenge with infectious microorganisms.

Effects of atmospheric pollutants on resistance to infection have been studied to a very limited degree. Two basic approaches were used, namely, epidemiological surveys and animal experimentation.

The Kettering Laboratory of Applied Physiology, University of Cincinnati<sup>(1)</sup>, reported on an epidemiological survey of workers exposed to sulphur dioxide. The incidence of colds was not significantly different from that of the control group. However, the duration of colds was extended in the exposed group. Other significant effects were a higher incidence of nasopharyngitis, alteration in sense of smell and taste, higher incidence of abnormal urinary acidity, tendency toward increased fatigue, shortness of breath on exertion, and increased sensitivity to other irritants.

Mills<sup>(2)</sup> found a highly significant relationship between the incidence of soot and respiratory disease death rates in various districts of Cincinnati and Pittsburgh. Pneumonia and tuberculosis were much more prevalent among people living in the most populated areas of these urban communities.

More recently Dohan<sup>(3)</sup> reported on an epidemiological study among workers in various RCA plants. He was able to correlate the incidence and extent of sulfur dioxide pollution with the absenteeism lasting more than 7 calendar days due to upper respiratory diseases.

Other epidemiological surveys, on the other hand, had failed to support the view that coal dust is a predisposing factor to pneumonia. For example, Kibby<sup>(4)</sup> found that mortality from pneumonia was lower among coal miners than among surface workers. In addition, necropsies

have yielded conflicting results on the relation of coal dust to pneumonia.

Laboratory studies in this area are rather sparse. In the past they concentrated more on the effects of particulate air pollutants than gaseous pollutants. Vintinner<sup>(5)</sup> studied the effects of aluminum dust on susceptibility to lobar pneumonia produced by intrabronchial inoculation of Type I pneumococci suspended in mucin. He concluded that rats exposed to this type of dust were not more susceptible, in fact, their resistance appeared to increase. Similar results were reported by Vintinner and Baetjer<sup>(6)</sup> for rats exposed to coal dust or smoke produced by the combustion of coal.

Experimental. The methodology used for exposure of animals to ozone and nitrogen dioxide and for respiratory challenge with the infectious organism Klebsiella pneumoniae was described in detail in previous publications<sup>(7-9)</sup>. The concentration of ozone was determined by titration with aluminum chloride-buffered potassium iodide. The concentration of nitrogen dioxide was determined by the method described by Saltzman<sup>(10)</sup>.

Male and female Swiss albino Webster strain mice, weighing approximately 26 g, were used. The animals were selected at random and exposed to the experimental conditions in groups of ten. Death of animals after exposure was recorded twice a day for 14 days. Gross autopsies were performed on all dying animals, and their hearts were assayed for microbial content. Animals surviving the 14-day observation period were sacrificed, autopsied, and the hearts assayed as above.

Stock culture of K. pneumoniae was isolated from the heart of a mouse killed by intraperitoneal injection of this organism. Mass cultures were grown on Difco blood agar base and harvested in sterile water. Vials containing 1 ml aliquots of the harvested culture were frozen at -70 C and stored at -10 C. Concentration of the organisms in each vial was approximately  $10^{10}$ /ml. For aerosolization, the frozen material was thawed and regrown on blood agar. The growth was harvested with sterile water and brought to a concentration of approximately  $10^5$  organisms/ml with sterile water. This material, when disseminated, usually produced 30-50% mortality in mice. The estimated inhaled LD<sub>50</sub> dose was between 500-1000 organisms.

Mice in groups of not more than three were placed in small, specially designed cages, which were introduced into the aerosol chamber through an air lock. Mice were exposed to a dynamic aerosol for 10 min. After challenge, aerosol production was interrupted and animals were air-washed for 15-30 min. They were then removed and placed in cages protected by ultraviolet light. Such experiments were repeated until 50 mice had been exposed to a given experimental condition.

For quantitative assay, the aerosol was collected in an all-glass impinger containing 20 ml of gelatin phosphate solution. The content was plated using conventional serial dilution techniques.

Results. Two basic experimental conditions were used. In one, the mice were exposed to the pollutants first and then challenged at various time intervals with the infectious agent. In other groups of experiments, the infectious challenge preceded exposure to gaseous pollutants.

The next group of figures shows some of the results obtained during the studies. Some of the data were reported in publications mentioned previously. Table 1 shows the effect of pre-exposure to ozone, at various concentrations and for various durations, on resistance to K. pneumoniae infection. The time interval between the exposure and the challenge was 1 hr. Increases in mortality rates were significant at each experimental point. It is interesting to note that in hamsters no deaths were produced by infectious challenge alone. However, upon pre-exposure to ozone up to 100% mortality occurred. Table 2 shows similar data obtained upon respiratory challenge with Streptococcus sp. Although smaller numbers of mice were involved in those experiments, the same trend of increased susceptibility as with K. pneumoniae can be observed.

Table 3 shows the effects of time interval between exposure to ozone and the infectious challenge. Ozone concentration was kept constant at 4 ppm and the exposure time was 3 hr. It can be seen that the effect of ozone persists for less than 19 hr when mice are exposed to this gas prior to the infectious challenge. However, when infected animals are exposed to ozone, the reduced resistance is apparent for at least 27 hr and probably for even longer periods of time.

TABLE 1. Effect of pre-exposure to ozone on resistance to *Klebsiella pneumoniae* infection

O <sub>3</sub> Exposure	Mortality, %		Mortality Increase, %	P <
	Control	Ozone		
<b>Mouse</b>				
4.4 ppm/3 hr	36	88	144	0.001
1.3 ppm/3 hr	42	68	62	0.001
0.8 ppm/100 hr	23	62	169	0.001
0.8 ppm/4 hr/5 d/2 w	21	30	138	0.001
<b>Hamster</b>				
4.4 ppm/3 hr	0	100	100	0.001
1.3 ppm/3 hr	0	75	75	0.01
0.8 ppm/100 hr	0	33	33	0.005

TABLE 2. Effect of pre-exposure to ozone on resistance of mice to *Streptococcus* infection

O <sub>3</sub> Exposure	Mortality, %		Mortality Increase, %
	Control	Ozone	
4.4 ppm/3 hr	42	76	81
1.3 ppm/3 hr	42	58	38
0.8 ppm/100 hr	57	78	37
0.8 ppm/4 hr/5 d/2 w	25	52	68

TABLE 3. Effect of time interval between exposure to ozone and infectious challenge on resistance of mice (4 ppm O<sub>3</sub> for 3 hr)

Time Between O <sub>3</sub> Exposure and Challenge, Hr	Mortality, %		Mortality Increase, %	P <
	Control	Ozone		
1	37	78	111	0.05
3	38	92	142	0.05
6	38	67	76	0.05
19	38	45	18	
27	38	42	11	
<b>Time Between Challenge and O<sub>3</sub> Exposure, Hr</b>				
3	43	80	86	0.05
6	40	73	83	0.05
27	43	67	56	0.05

The next table (Table 4) summarizes the data on the effects of a 2-hr exposure to various concentrations of nitrogen dioxide on resistance. The exposure to the pollutant occurred 1 hr before the infectious challenge. It can be seen that nitrogen dioxide has an effect similar to ozone. Concentrations equal to, or higher than, 3.5 ppm reduced the resistance of mice to the infection. The persistence of the effect produced by nitrogen dioxide was studied next. At effective concentrations (< 3.5 ppm) the reduced resistance was not noticed when the time interval between the exposure to the gas and the infectious challenge was more than 24 hr. However, when infected mice were exposed to nitrogen dioxide, significantly higher mortalities were observed even after 72 hr.

TABLE 4. Effect of a 2-hr pre-exposure to nitrogen dioxide on resistance to Klebsiella pneumoniae infection in mice

NO <sub>2</sub> , ppm	Mortality, %		Mortality Increase, %	P <
	Control	Nitrogen Dioxide		
25.0	40	92	124	0.05
15.0	33	88	167	0.05
10.0	48	98	104	0.05
5.0	45	94	109	0.05
3.5	44	98	123	0.05
2.5	34	40	18	
1.5	47	57	21	

At necropsy, exudate was found in the pleural cavities of most of the infected mice that died during the 14-day observation period. The lungs of these mice were consolidated and frequently showed white plaques resembling agar colonies of K. pneumoniae. Bacteriology of surviving mice sacrificed after the 14-day holding period was negative.

It is apparent from the experimental data that ozone and nitrogen dioxide increase the susceptibility of laboratory mice to respiratory infection caused by inhalation of K. pneumoniae. The damage produced by these gases at the exposure levels studied is not permanent and recovery takes place within less than 24 hr.

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#### DISCUSSION

Schulman: Have you considered the possibility of using other parameters to measure this potentiation; have you considered seeing whether the infective dose was changed rather than the absolute mortality in the two different groups, and have you considered actually counting the numbers of organisms present in the lungs of the infected animals at intervals with and without exposure to the noxious gases? The reason I ask is that I think we can all agree that mortality is really one of the grossest indications we can have of susceptibility to infection, and I think you may be missing much more important and subtle differences by using mortality as your only parameter of susceptibility.

Ehrlich: I would like to make two comments. One: in all experiments the animals exposed to various NO<sub>2</sub> or ozone conditions, and control animals not exposed to the pollutants, were challenged simultaneously with the infectious aerosol. Thus, all animals at least theoretically received the same dose, and I believe direct comparison can be made. Two: some of the experimental work underway in our laboratories pertains directly to your comment. We are studying the actual distribution of the organisms in the lungs, immediately after, and at various time intervals after, the infectious challenge. Control

mice as well as mice exposed to the pollutants are included in the experiments. We are also looking at the ciliary movement and mucus excretions to study more thoroughly the mechanisms involved in the phenomenon reported here.

Morton: Did you ever expose the animals to the contaminant gases and to the challenge organisms simultaneously?

Ehrlich: No. There are problems relating to possible effects of some of the gases on the organisms.

Morton: I am interested in Dr. Schulman's question, because it did not occur to me when you were speaking that probably the difference between exposure to gas, before and after challenge, might simply stay there, as you know, for a long time.

Silver: What sort of dose response slope did you get for this type of infection? If you got a very steep slope, you might expect to show quite rapid response with quite small increases in the dose.

Ehrlich: The dose response curve is not at all steep as far as the *K. pneumoniae* is concerned.

Silver: This is actually the answer to the first question. You are just as well off using this sort of parameter as you would be if you measured the after-effect of response that occurred.

Ehrlich: I don't agree with that.

L. Miller: In our recruit population of many thousands of men, we had a higher incidence of respiratory disease in recruits that came from cities than in those that came from the rural areas. And when we got to the incidence of pneumoniae, there was a fairly large difference. I just wonder if there might be some prolonged effects -- that if you were to do some prolonged exposures, you would have found some residual effects which made animals more susceptible.

Ehrlich: We are conducting some experimental work on exposures to low concentrations -- approximately 0.5 ppm -- of NO<sub>2</sub> for prolonged periods of time up to 12 months. The data available, up-to-date, indicates that 3 months exposure to this concentration of NO<sub>2</sub> results in increased susceptibility, again as measured by mortality rates and survival time.

Middlebrook: I note that physicists and mineralogists, who studied air naturally polluted with particles of SO<sub>2</sub> and ozone, have observed that a high proportion of the gas is absorbed to the particles and is almost always present, at the same time, in the same volume of air. For this reason I wondered if you planned to use charcoals exposed to the gases as agents for investigation of the effect upon susceptibility to infection.

Ehrlich: We have no immediate plans in this area, but I know of this work, especially as far as SO<sub>2</sub> is concerned.

## A MATHEMATICAL CRITIQUE FOR SURGICAL WOUND INFECTION BY AIRBORNE BACTERIAL PARTICLES

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I want to present some facts and fancies concerning a phenomenon which meets almost none of the classical requirements of a problem in aerobiology. Surgical wound infection initiated by airborne particles containing Staphylococcus aureus, if a reality, is most likely caused by relatively large particles. Traditionally these particles have not been considered as airborne, and are generally excluded from consideration in Aerobiology.

In due deference to the state of mind which rejects the importance of larger particles in the air, I must confess that in our own work we entered the field of hospital ventilation with the bias that only particles of less than 10  $\mu$  diameter were worthy of study. We are indebted to our British colleagues, notably Drs. Lidwell and Blowers<sup>(1)</sup>, for pointing out that airborne microbial particles in operating suites were often much greater than 10  $\mu$  in diameter, and especially that staphylococci (staph) are most frequently found in particles nearly 20  $\mu$  diameter.

Subsequently we have assembled further data which support the British observations. Some of these are shown in Table 1. For those of you who are in any small measure disciples of the late William Firth Wells, it is of interest that his earlier observations compare favorably with more recent values. The United States values are averages of a number of current observations, arbitrarily arranged in three groups. You will notice that only for the case of good practice, which unfortunately is relatively rare, is the settling mean diameter as small as 10  $\mu$ .

TABLE 1. Apparent mean diameters of naturally occurring microbial aerosols emanating from personnel

Source of information	Settling velocity, fpm	Equivalent settling diameter, $\mu$
Wells* : Boston	2.04	18
Pittsburgh	1.56	16
	1.32	15
Iowa City	1.59	16
	0.83	12
	1.47	16
Noble† : England	1.2	14
U.S. ◊ : Poor practice	2.4	20
Average practice	1.0	13
Good practice	0.6	10

\* Wells, W. F. 1955. Airborne Contagion and Air Hygiene. Harvard University Press, Cambridge.

† Noble, W. C., O. M. Lidwell, and D. Kingston. 1963 The size distribution of airborne particles carrying microorganisms. J. Hyg. Camb. 61:385.

◊ From various published and unpublished data: Compiled on the basis of three levels of air contamination.

It has often been shown that such large airborne particles do not necessarily arise from secondary reservoirs. The results of some of our own studies illustrate this (Table 2). These observations were made in our simulated operating room, or with a glove box, and are illustrative only, indicating that fairly large airborne microbial particles can emanate from people in the absence of secondary reservoirs such as dust.

TABLE 2. Apparent mean diameters of naturally occurring microbial aerosols emanating from personnel

Conditions	Settling velocity, fpm	Equivalent settling diameter, $\mu$
Street clothes:		
Moderate activity, no talking	2.4	20
No activity, much talking	1.4	15
Tight surgical gowning,		
Moderate activity	1.0	13
Sneezing into box	0.21	6
Talking into box	0.12	4.5

Our simulated operating room<sup>(2)</sup> is a sheet metal room 12 x 15 x 10 ft high, in which various pieces of ventilation apparatus can readily be installed or removed. All studies have been of dynamic aerosols with continuous operation of ventilation and continuous generation of a standard bacterial aerosol which simulated those produced by people. Sampling was conducted at various locations and levels throughout the room. Particle sizes were estimated by determining the average rate of fall, and also from plots of the Andersen plate values, employing log-normal graph paper. We are indebted to Mr. Lee Kelly of Digway Proving Ground for providing values for the 50% efficiencies of the various stages of the sampler. Estimates of particle sizes greater than 9.8  $\mu$  (the 50% efficiency size of the first plate of the Andersen) were made from the upper portion of the plotted curve, which is an extrapolation. However, this extrapolation is justified because of the expected distribution of particle sizes produced by the atomizer employed. Furthermore, size estimates for larger particles were validated by the settling rate determinations made by comparing numbers settling per minute to the arial concentration.

In order to determine the distance of transport of larger particles, the atomizer was positioned at one end of the simulated operating room, giving a distance of travel of 16 ft to the other end of the room. It was found, in general, that the greater the velocity of the air currents, the greater the distribution of larger particles. To illustrate the transport capacity, two ventilation procedures producing low air velocities in the room have been selected: end-wall grille at low air flow, and perforated ceiling panels at higher air flows. Both of these produce air velocities in the room well below 50 ft/min. Table 3 shows that end-wall grille ventilation, which is by far the most commonly employed, efficiently transports particles as great as 20-30  $\mu$  diameter at least a distance of 12 ft. The perforated ceiling panels transport a much smaller, but significant fraction of these particles. Unfortunately, this ventilation practice is seldom applied in hospitals.

When we first realized that particles greater than 10  $\mu$  diameter were worthy of study, we were concerned as to the design of an aerosol chamber for investigating their behavior. For those interested, the distribution of counts within the simulated operating room, ventilated with an end-wall grille, is shown in Table 4. The distribution is

definitely not perfect, but is perhaps satisfactory for comparative studies. At higher flow rates or with the atomizer positioned overhead in the center of the room, the distribution is somewhat more uniform

TABLE 3. Transport of microbial particles for a 12 ft distance (4-16 ft) in simulated operating room, low turbulence ventilation

Ventilation procedure	Particle size, $\mu$	4 ft from source*	16 ft from source*	Particles transported, %
End-wall grille: 300 CFM; air currents parallel to long axis of room	$\leq 5$	48	45	94
	20-30	39	34	87
Perforated ceiling panels: 750 CFM, air currents downward from ceiling	$\leq 5$	20	17	85
	20-30	33	10	30

\* Numbers/unit vol.

TABLE 4. Distribution of heterogeneous aerosol ( $12 \mu$  CMD) in simulated operating room: end-wall grille, 300 CFM; atomizer at end of room beneath supply air inlet.

Distance from source, ft	Aerial concentration/ft <sup>3</sup>	Height above floor, ft	Aerial concentration/ft <sup>3</sup>
4	301	2	253
8	259	3.5	296
12	292	7	286
16	272		
Overall distribution:	Averages	281 $\pm$ 22	
	Individually	281 $\pm$ 68	

At this time staphylococci are of special interest, but the relative infrequency of occurrence of airborne staph makes exact quantitation of particle size difficult. However, long-term observations in Britain by Noble, Lidwell and others<sup>(1)</sup>, and in Australia by Rountree and Beard<sup>(3)</sup>, indicate that the most probable size of airborne particles containing staph is in the range 10-20  $\mu$  diameter.

One final fact: surgical wound infection due solely to aeri-ally transported pathogenic staph does occur. There are numerous reports in the literature which substantiate this, but none more definitive

then the one by Dr. Carl Walter<sup>(4)</sup>. In this instance there occurred two postoperative wound infections attributable only to a specific airborne staph; the particles which carried it originated from an individual who at no time had direct physical contact with the patient, or with anything related to the patient.

Thus far we have presented factual information regarding the origin and distribution of airborne microbial particles greater than 10  $\mu$  diameter, shown that it is probable that staph appear in the air in particles 10-20  $\mu$  diameter, and stated as fact that the aerial route has been implicated in surgical wound infection by staph. However, quantitative information as to the exact importance of airborne staph is very difficult to obtain -- and even to establish the probable risks turns us from fact to fancy.

Danger to the respiratory apparatus is accepted as a special province in the field of aerobiology. If there is a risk to the respiratory apparatus, considering airborne microbial particles greater than 10  $\mu$  diameter, it most probably involves the upper portions of the nasopharynx. Definitive data are lacking, but several authorities active in hospital-acquired staphylococcal infections have recorded the belief that colonization of the nasopharynx is a significant factor in the establishment of new carriers; other have suggested that colonization of the patient in this manner furnishes the source of material which subsequently infects the patient's own wound. Over 50% of the hospital personnel may be asymptomatic carriers of potentially pathogenic staphylococci. If they serve as reservoirs for the initiation of new carriers among themselves and among patients, then only the patient whose stay in the hospital is brief can hope to escape becoming infected or a carrier.

Reflecting on other evidence, I believe it is not rash to assume that nasopharyngeal colonization can be initiated by a single competent staphylococcal particle implanted at the right place at the right time\*. On this basis, we can make some conjectures as to the probability of a patient's being colonized during pre-operative stay in the hospital. The pre-surgical patient is rarely exposed to hospital personnel within his room for more than 1 hr/day; if 50% of the personnel

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\* See comments by Eichenwald and Shinefield in discussion following, which suggests the non-validity of this assumption.

are asymptomatic carriers, we can set the minimum condition for risk at 1 particle/10 ft<sup>3</sup> of air.

In this field of low-probability statistics, individual observations follow a Poisson distribution. For the exposure of the presurgical patient to the low concentrations of particles assumed above, employing the Poisson distribution, it is predicted that the relative frequency with which one will find samples of air of 10 ft<sup>3</sup> each which contain no particles is numerically  $e^{-x}$ , where  $x$  is the arithmetic mean. In this instance the mean has been assumed to be 1.0, and about 37% of the time ( $e^{-1}$  multiplied by 100) there will be no particles in a given volume of 10 ft<sup>3</sup> of air, and about 63% of the time we will obtain samples with one or more particles. In Table 5 is given the percent occurrence of samples with zero particles and the occurrence of samples with one or more particles, for arithmetic means of 1-5. These same relative frequencies of occurrence and non-occurrence apply to the risk of exposure of the patient; if 10 ft<sup>3</sup> of air is taken as the unit volume, where the average count is one particle per unit volume, about 37% of the time no risk will be incurred. The chance of occurrence of one or more particles per unit of air breathed or sampled increases rapidly as the average count increases; for an average count of three particles the risk is 95%, which amounts to statistical certainty.

TABLE 5. Low number particle counts; % observations with zero and with  $\geq 1.0$  counts; Poisson distribution

Average count/10 ft <sup>3</sup>	% observations	
	With zero count	With $\geq 1.0$ count
1.0	36.8	63.2
2.0	13.5	86.5
3.0	5.0	95.0
4.0	1.8	98.2
5.0	0.7	99.3

Carrying these conjectures one step further, we can estimate the risk of nasal colonization of the patient exposed to various aerial concentrations of infective particles. Table 6 shows these concentrations as mean numbers of particles per 100 ft<sup>3</sup> of air. Counts of this order for airborne staph have been reported; there is also indication

that the rates of colonization may be this great -- but truly definitive information is lacking. It can only be said that these values suggest that the risk of colonization of the pre-surgical patient by the arial route may pose a real threat.

TABLE 6. Conjectured risk of nasal colonization from airborne microbial particles

Average arial concentration, infective particles/100 ft <sup>3</sup> of air	% risk of nasal colonization
10	10
20	18
30	26
40	33
50	40
60	45
70	50
80	55
90	60
100	63

If we assume that a single infective particle can initiate the disease process, then arial transport and deposition is actually one of the most direct routes for the transmission of staph from personnel in the operating room to the surgical wound area. The individual occurrences follow a Poisson distribution, and the probabilities of occurrences and non-occurrence are indicated in Table 5. However, for the surgical wound, let us set our unit in terms of the settling of one particle on a wound area of 1/15 sq ft, exposed for 1 hr. These are not improbable values, and were chosen because this is the area and time of exposure of the usual settling plate sample in the operating room. It follows that if we have conditions under which infective particles settle out at the rate of 1.0 per wound area/hour, then about 63% of the time one or more particles will deposit on an exposed wound, or an exposed petri dish (Table 5). Please remember these are independent probabilities; a positive plate does not guarantee a positive wound, and vice versa.

More quantitative evidence was available for surgical wound infection than for nasal colonization. Computations based on the simple model, compared with reported rates of infection ranging from 2-10%, indicate that the simple model over-estimates the probability by about

80%. It may be that the infective particle deposits on a receptive area only 20% of the time, for example, an area that is partially dry or lacking full supply of blood; or perhaps the particle is competent only 20% of the time. The particle may have to contain more than one bacterium to be able to initiate infection; or it may be that only 20% of the patients are susceptible.

On the basis of a 20% incidence, and employing a 13  $\mu$  particle, which settles at 1.0 fpm, our model can be employed to indicate the relative risks of wound infection under conditions of various mean concentrations of infective particles per 100 ft<sup>3</sup> of air (Table 7). Although I will not strain your credulity further by attempting to document these data, a few observations can be made to substantiate the general approach. For example, in this country one group of operating rooms claiming to have a negligible staphylococcal wound infection rate reports a long-term sampling average of 1.5 coagulase positive staph per 100 ft<sup>3</sup> of air. The British experiences agree with the conjecture that between 12 and 23 infective particles per 100 ft<sup>3</sup> of air can yield an infection rate of about 10%, and a reduction of the overall bacterial load of the air in these same surgeries by a factor of ten reduced the wound infection rate to about 1%.

TABLE 7. Conjectured risk of wound infection from airborne microbial particles:  
Adjusted model for 13  $\mu$  diameter particles

Average aerial concentration, infective particles/100 ft <sup>3</sup> of air	Average number of particles settling on wound in 1 hr	Risk of infection, %
1.25	0.05	1
2.50	0.10	2
3.40	0.22	4
8.8	0.35	6
12.5	0.50	8
17.25	0.69	10
22.75	0.91	12
30	1.20	14
40	1.61	16
57	2.30	18
165	4.60	20

When dealing with low-probability events, we have the old, old problem of obtaining samples of sufficient size to demonstrate the

effect. To refresh our memories, Table 8 presents the confidence limits for the averages of samples of 100 or fewer cases, for true mean rates of occurrence of 10 and 20%. The distributions here are averages that follow a normal or Gaussian distribution. There are no negative occurrences, for real events, and the distribution of means is badly skewed toward zero -- that is, there will be an inordinate reporting of zero occurrences for small samples, even though the true mean rate of infection is 10 or even 20%. This is just to remind us that in dealing with low probability occurrences, the mean non-occurrence of an event does not prove that the true rate of occurrence is zero any more than individual non-occurrence in a Poisson distribution proves that the mean is zero.

TABLE 8. Confidence limits for small samples, % occurrence

Number of cases in sample	True mean rate of infection of:			
	10%		20%	
	Lower limit	Upper limit	Lower limit	Upper limit
10	0	40	0	60
20	0	30	0	45
40	0	22	2.5	38
60	0	20	6.7	34
80	1.2	19	7.5	32
100	2.0	18	9	31

To pursue this aspect of low-probability statistics one step further: there has been a great difference in the attitude toward the potential of airborne staphylococcal infection of surgical wounds in this country and in Great Britain. It is claimed that the incidence in this country is no greater than 1-2%; the British have confessed to as high as 10%. The information in Table 9 helps put these observations into proper perspective. Let us agree that the true rate of infection in the United States is actually only 1%. What chance have we of determining this by improving our procedures? If we assume a 1:1 cause and effect relationship and reduce the causation by 50%, we would have to collect 2000 cases before our probability was great enough to be determined with statistical confidence -- taking 5% probability as our acceptance level. For a 90% reduction in cause, the sample would

have to be 500 cases. It should be evident that if we are telling the truth in this country, no improvement in our procedures short of 90% is going to show a perceptible change in the infection rate. However, look closely at the situation for increases in causation. If our present practices are yielding a 1% infection rate, relaxation to permit a doubling of causation will not become evident in samples much smaller than 500, but a 4-fold increase in causation will become evident in samples smaller than 100. A 10-fold increase in causation could be readily distinguished -- and this latter seems to have been the relative position of the British surgeons before they improved their personnel and ventilation practices -- they claimed these changes brought the staph wound infection rate down from 10% to 1%.

TABLE 9. Probability of distinguishing a difference between a 1.0% rate of infection and rates of infection resulting from an increase or decrease in the cause of infection; a 1:1 cause-and-effect assumed.

Number of cases in sample	Probability, in %, of chance occurrence of a difference as great or greater than that observed for:			
	50% reduction in cause	90% reduction in cause	2-fold increase in cause	4-fold increase in cause
100	61.5	36.8	31.7	0.27
200	47.8	20.0	15.6	0.00
500	26.3	4.3	2.4	0.00
800	15.6	1.1	0.45	0.00
1,000	11.2	0.42	0.15	0.00
2,000	2.4	0.00	0.00	0.00

In conclusion, there exists a sufficient body of facts to prove that airborne particles greater than 10  $\mu$  in diameter containing infective material are shed by personnel, and that these particles can be transported throughout the room in which they originate. Furthermore, the probable size of the airborne particles containing pathogenic staph is between 10 and 20  $\mu$  diameter, and surgical wound infection initiated by airborne particles has been proven to occur.

It appears possible that larger airborne particles containing staph can play a significant role in nasopharyngeal colonization of new carriers. Conjectures as to the probability of risk of initiating new carriers and of surgical wound infection by airborne staph

indicate that such risks are very real. However, it appears that in the well-ordered or perhaps fortunate hospital, these events have a low probability of occurrence. As a result, attempts to quantitate these occurrences under these conditions will almost certainly be fruitless; only under conditions of much higher risk will these occurrences become evident.

#### ACKNOWLEDGMENTS

Investigations on the distribution of bacterial aerosols in simulated operating rooms were supported in part by funds from Grant OH-00019, Division of Occupational Health, Bureau of State Services, United States Public Health Service.

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#### DISCUSSION

VFTF\*: What type of ventilation practices seems to be most effective in reducing the numbers of large particles in surgery?

Kethley: The overhead ventilation seems most effective, I think. Some of you should be familiar with Dr. Blower's work, where he attempted to get a piston effect. Unfortunately the most successful piston effect obtained was one that brought in air warmer than the room. This practice has considerable drawbacks but the introduction of air from above with minimum turbulences does tend to push the

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\* VFTF = Voice from the floor.

larger particles directly out the exits.

Shinefield: Your calculations are based on the assumption that a "hit" means colonization. This may be true for a clean wound. We have evidence to indicate that this is not always the case with colonization of the nasal mucosa or the umbilical stump of the newborn with S. aureus. Previous colonization of these sites with S. aureus interferes with a "hit" by other coagulase positive staphylococci. Previous colonization with S. aureus probably represents only one of many host factors that are related to a "hit". These factors make it difficult to interpret the practical meaning of computations that have just been presented.

Kethley: The point is well taken. There was simply no information I could lay my hands on in the way of quantitation of the probability of colonization. As you noticed, I simply used the raw first model computation for wound infection -- I have a number of models -- this is the one I chose. There was a fair amount of quantitation available, still the model was wrong by a factor of 80%. I am sure if we had similar information for colonization we would find a similar error in any such model that we might project only for the purpose of having some numbers to kick around.

Eichenwald: To elaborate on Dr. Shinefield's comments -- this was primarily his work, but by using the newborn infant, where one does not have the problem of pre-colonization, it can be shown that the nasopharynx actively rejects colonization, and that it takes in the range of 500 organisms or more to produce colonization in more than half the infants while with the umbilical stump one can achieve a high colonization rate with as few as three or five staphylococci. The second point of great interest to me is this: those of us who have been interested in nursery epidemiology as compared to operating room epidemiology have found it is very difficult to agree on what the important factors are. We went from having extremely poor nurseries, where it is very easy to demonstrate airborne infection, to very good nurseries where it was very difficult to demonstrate airborne infection. I think what Prof. Kethley has shown through distribution curves is precisely what one would expect; at one end of the scale it is quite easy and at the other end of the scale it takes hundreds and hundreds of observations to show the same thing.

Middlebrook: I am worried about two points there. One, the concept of your infection efficiency; in your actual experiments relative

to a single bacterial cell of staphylococci -- does it also give rise to a colony and how often? Two, in view of the experimental observation that staphylococci on dust particles may initiate infection much more efficiently than staphylococci not on a piece of dust -- then I wonder what bearing this has on the experimental data you presented here. Didn't you actually have staphylococci on pieces of dust or did you nebulize them free of dust, in the air? What effect could this have on the relationship between numbers of organisms per particle, where only one colony has been detected? You might have had as many as 40 organisms in that particle. It seems to me that emphasis on these data is no less important than emphasis on the mathematical aspects describing where you are on a distribution curve of probability. Have you studied any of these matters in other experimental conditions available to you?

Kethley: As far as distribution of colonization from staphylococci is concerned, we encountered various test problems. Regarding the matter of infectivity of staph on a dust particle, I assume you are referring to the "irritant" theory -- that it requires staph to have some irritant material, the suture in the wound or something similar. We are actually carrying out studies along this line. Without going into any of the details of the problems of getting sufficient numbers to make any speculation, the matter of just plain probability of where a particle lands on a wound area seems more important. With particles riding on non-irritant materials we are getting infections at lower numbers than say with a plain suture. This is carrying infection by the airborne route. As to the growth of microorganisms and sampling of air, most of the data in the hospital are taken from solid-surface samplers. Certainly there are factors of error here as great as 10 because of widespread use of selective media. Such media will not demonstrate all the staphylococci, which makes it very difficult to get quantitation. The matter of organisms per particle: this is merely a matter of conjecture as I see it. The definition of what is a competent particle may concern the number of organisms. Again it is extremely difficult to get information on the occurrence of these particles, if you simply are not fortunate enough to have a shedder among your personnel. The number of bacteria per particle may be the factor in this 80% over-estimate. It may be that only the very large particles are carrying a sufficient number to initiate the action. At present we are working with one cell per particle as a model. It is actually

a tremendously time-consuming job. When you are working with animals you must identify the strain you go in with, then you must identify all of the strains in the animal, and if one of these strains that the animal carries turns out to be the same strain you went in with, well, then that animal is no good.

Goldberg: With respect to selective media in hospital studies, we have one test on polymixia, and staph was completely resistant to the use of up to 500 µg. But against an aged aerosol in a mixed humidity range you can demonstrate inhibition as low as 3-10 µg/ml. So you very definitely must evaluate selective media against a potentially injured or aged aerosol, and you can get far greater than a 10-fold change in your estimate.

Harper: What was the source of these dust particles in all of your studies? Do you think they are coming from the nose of the pharynx of carriers, or have you considered the skin carriers?

Kethley: I assume that the majority are from the skin carriers.

VFTF: I would like to hear what Dr. Eichenwald has to say regarding nurseries when it comes to skin- and nasal-generated transmission.

Eichenwald: In a regular nursery where one deals with a variety of different situations as, for instance, infant to infant transmission, I think it is very clear that infection may occur from organisms generated from the nose and is airborne, and that one is dealing with rather small particles. Then there is ample evidence that there are carriers who cannot be detected by routine methods of culture. A nurse can infect an infant while she herself, by the techniques we can employ, is not a carrier of staphylococci. She carries such small numbers that these cannot be picked up. Whether she carries them in her nasopharynx or on her skin or clothing. I don't know. I would guess that in the nursery situations, one deals both with the single organism, and under other circumstances with very large particles containing large numbers of organisms.

Shinefield: In attempting to demonstrate the relative role of airborne staphylococci infection, we reduced the number of S. aureus in the environment in a controlled observation by ultraviolet light and studied colonization rates. We also increased the number of S. aureus in a nursery environment by reducing air changes/hr. Although the numbers of S. aureus recovered from the environment could be altered by these techniques the rate of colonization of infants did not

appreciably change. This would indicate that the role of air transmission in colonization of the infant seems to be limited.

VFTE: I might relate an experience we had as to the role of airborne and skin-contamination where nurses and graduating students sit in on the surgical room of post-operative patients to learn techniques, and we just record breaks in technique. It is of interest that the breaks in technique were in direct proportion to the extent of academic achievement -- the professors were the ones spreading more organisms, at least so far as our measurements indicated, and right down the ladder; the resident, the intern and medical students and finally nurses. The nurses were the ones with the best techniques. These are things that would bear some qualitative measuring once they get out of the operating room.

VFTE: I would like to comment on the five-hospital, ultraviolet light evaluation study that has been completed by the National Academy of Sciences and National Research. This was an attempt to evaluate the use of full operating room ultraviolet irradiation to reduce the incidence of post-operative wound infection. You may be familiar with Dr. Hart's ultraviolet set-up at Duke University since around 1936. This was an attempt to do a double-blind study, as it were, of five hospitals over roughly a 4-yr period. A total of something like 15,000 surgical procedures were performed, and the substance of the study was that with intense ultraviolet irradiation, bacteria settling on settling plates were definitely reduced, anywhere from 40-60% between the control and the ultraviolet lighted room, but the over-all wound infection rate was not changed at all. There was a slight decrease in post-operative infections in the refined clean surgical procedures, something like 3.8-2.6%, but in all other categories of surgical operations there were no effects comparative to irradiation. In fact, in the contaminated operations (grossly contaminated by cutting across abscessed areas and across bowels) there was an increase in post-operative wound infection rate in the UV irradiated rooms over the control rooms. Not a statistical increase, but it was a noted true increase throughout all of the hospitals. I suppose the studies of Dr. Mortimer in Cleveland should also be mentioned, where in some instances the infant evidently was not the disseminator of an unusual parasite or staphylococci, but where the nurse definitely transmitted the infection from newborn infant to newborn infant. As already mentioned, the definition of a disseminator is most important when talking about methods of staph

infection spread in hospitals.

VFIE: What was the incidence of wound infection the study presented?

VFIE: Over-all incidence?

VFIE: No; you mentioned two groups -- one with UV and one without.

VFIE: Something like 7.4-7.5% -- it was in that particular range.

Eichenwald: I think it should be pointed out, however, that Dr. Mortimer's data are considered by most people as invalid because, instead of carrying out a study for 24 hr/day, the controlled observations were only carried out for two of the three nursing shifts. Any reviewer would have rejected a paper on that basis. I don't think any conclusions can be drawn from these data and they should not be cited as an example.

Proc. First Intern. Symp. Aerobiol., Berkeley, Calif., 1963

## THE ROLE OF AEROBIOLOGY IN DENTISTRY

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Dentistry is performed in the mouths of humans by humans using instruments capable of disseminating contents of the oral cavity as well as organic and inorganic debris produced by dental procedures. The concept that most human infectious disease comes from other human beings<sup>(1)</sup> is one of particular interest and importance to all dentists. The dental operating room or clinic represents an environment utilized by two or more humans, who may be either sources or recipients of infectious microorganisms. Dental treatment combines humans with natural and/or artificial modal capabilities for the transmission of infectious disease agents.

The incidence of infectious disease resulting from personal contact, fomite, or aerosol exposure in a dental office, clinic or laboratory presently cannot be stated due to the infrequent and often obscure reporting of such cases in the literature. The professional, ethical, social, and medical-legal aspects of such reporting are, perhaps, significant deterrents. Nevertheless, there have been cases reported in the open literature or elsewhere where the health of the dental patient or operator has been adversely affected concomitant to receiving or providing dental health care.

Foley and Gutheim<sup>(2)</sup> in 1956 reported, "of 22 cases of serum hepatitis, 15 (or 68%) were due to exposure at the dentist's." It must be added that 3 of the 15 cases died as a result of their infection.

Salzman and Appleton<sup>(3)</sup> reported 31 dentists and 1 dental hygienist who contracted non-venereal syphilis while they were providing dental health services. For decades, syphilis has been known as a systemic disease which may be transmitted by dental procedures<sup>(4,5)</sup>. Hambrick et al.<sup>(6)</sup> reported the presence of primary herpes simplex lesions on the hands of medical personnel; he cites the case of a dentist who was infected after contact with an infected patient. Cuthbertson<sup>(7)</sup> reported that 11.2% of the dentists included in a survey of 858 dentists who died in the United States in 1952 died of sensory organ or nervous system diseases attributable to poliomyelitis. In 1952, the polio mortality rate for the general male population was 0.03%<sup>(7)</sup>. Cuthbertson speculated that poliomyelitis may have been an occupational hazard of dentists due to their intimacy with the respiratory tract of their patients. At the University of California Medical Center, San Francisco<sup>(8)</sup>, a review of student health records for the years 1957-1962 indicated that University of California dental students had 150-190 dispensary visits per 100 students per year for treatment of respiratory infection, whereas medical and pharmacy students of the same campus had 40-140 visits per 100 students per year. With the exception of 2 included years, the dental students had a higher rate of conversions to positive tuberculin skin test than did medical and pharmacy students. In one class, 42.8% of the dental students who were tuberculin negative at the beginning of their dental education converted to tuberculin positive when tested in their senior year. Thompson et al.<sup>(9)</sup> reported the occurrence of patient-to-patient transmission of viral hepatitis and upon further investigation of hospital records found three more cases of hepatitis where the probable modes of infection were dental procedures. California State Department of Public Health<sup>(10)</sup> records list separate instances of an oral surgeon and a dental assistant who contracted viral hepatitis while performing dental surgery for patients having this disease.

The dentist works directly with the hard and soft oral tissues of several patients each day<sup>(11,12)</sup>. In the mouth, he contacts saliva and debris, which have high microbial content<sup>(13,14)</sup> comprised of the common oral flora and any pathogenic organisms shed to the mouth in diseases involving the salivary glands, oropharyngeal lymphatic tissues and respiratory system, i.e., mumps, streptococcus infections and tuberculosis. Blood- or tissue-borne microorganisms can be shed to the

mouth as a result of the surgical trauma caused by dental instrumentation in addition to the "natural" shedding of organisms into the mouth. The significance of introducing infectious agents into an operating site can hardly be questioned, but the significance of the shedding of infectious agents from a dental surgical wound or operative site has been virtually unexplored.

The dentist is seldom required to treat a patient during the acute phase of an infectious disease. However, dental care may be provided for the asymptomatic patient who has a viremia or respiratory infection, with neither the patient nor the dentist being aware of the potential hazard of infection. There is increasing concern for the fact that several viral diseases have viremic phases in their pathogenesis<sup>(15)</sup>. Viremias may exist in the asymptomatic carrier<sup>(16)</sup> during the prodromal state, during frank clinical illness, and for prolonged periods of time after the abatement of symptoms.

Few people would choose to manipulate the contents of the oral cavity as a dentist usually does without the use of protective clothing or equipment, i.e., gloves, gowns, tongue blades and masks. Few aerobiologists would take a random sample of saliva, hemorrhage, and tissue debris of any description and atomize it 6-18" from his nose and mouth with an air syringe, rotary, or vibrating instrument without taking special precautions to prevent the inhalation of this material and the contamination of his person and environment. The dentist usually steps back when the patient sneezes or coughs, then bends over his patient -- air syringe in hand -- blows the operating area dry again and resumes his work. The association of oral cavity contents with aerosol production is not frequently recognized by the dentist as anything more than a nuisance requiring the operator to clean his glasses frequently while operating.

The thorough dental examination of an oral cavity with just a mouth mirror and explorer frequently includes the puncture or laceration of the gingiva. When a carious tooth is being prepared for a restoration, there are particles of sound tooth structure, decayed tooth debris, and frequently old filling material atomized by rotating cutting instruments and/or air and water sprays. Concomitantly, soft tissue laceration and trauma occur, resulting in hemorrhage and tissue fluid seepage at the operating site. The resultant slurry comprised

of saliva, tooth structure, decayed material, filling and base materials, hemorrhage and tissue fragments is often mixed and thinned with water and blown or flushed from the field by air, water, or mist sprays. If this material is evacuated from the mouth, the suction device often exhausts directly into the operating room air. If a reasonably dry field of operation is maintained by the use of a rubber dam or intra-oral suction device, the debris is routinely blown from the operating field by a blast of air. Besides the hazards of infection from viable particles, some indication of the possible effect of non-viable particles upon the health of the dentist has recently been published in the Journal of the American Dental Association wherein one dentist (who wished to remain anonymous) reported that he had contracted silicosis due to inhaling particles over many years while performing dental and technical procedures<sup>(17)</sup>. There also has been reported a case of mercury poisoning requiring hospitalization and treatment for a Navy dentist, an illness resulting from his inhaling silver amalgam dust generated by the air turbine handpiece during removal of old amalgam restorations from the teeth of patients<sup>(18)</sup>.

In oral surgery, high speed rotating cutting instruments are sometimes used to resect a tooth preparatory to its removal. Frequently, after reflecting the mucoperiosteum, high-speed cutting instruments are also used to dissect bone for removal of root tips, foreign bodies, and various types of pathological tissue from the mandible and maxillae. The splattering of the environment by blood and tissue debris is a common occurrence in such operations, as is attested by the spotting of clothing and glasses worn by the operator and his assistant. If hemorrhage and tissue debris from the surgical wounds that contain infectious agents is splattered or atomized, such debris could infect members of the dental health team.

As of 1959, there were 6,688 dental laboratories in the United States<sup>(19)</sup>. Dental laboratories are additional locations where environmental and personal contamination may occur because of the act of manipulating impressions, prosthetic appliances, and other technical components frequently utilized in providing dental care. These components often are sent directly from the mouths of patients in the dental operating room to the dental laboratory. Decontamination procedures are not universally employed to prevent the transfer of infectious agents to the dental laboratory via these fomites. In the

laboratory, the technical component or prosthesis is frequently intermixed with those also being processed from the mouths of other patients. When laboratory processing is completed, components are returned to the various dental facilities from which they originated. Again, decontamination procedures are not universally used to prevent potential infectious material on these objects when they leave the dental laboratory, and presumably present when they arrive in the dental operating room or clinic, from being transferred to the patient's mouth.

There has been much consideration given to microbial air contamination and the airborne mode of disease transmission among hospital patients and staff. Perhaps equal consideration should be given to microbial air contamination and the airborne mode of disease transmission in a dental facility and its effect upon dental patients and the dental health team. There might well be the same, or greater, relationship of air contamination in the environment used to provide dental care to the health and well-being of dental patients and staff as the established relationship of microbial air contamination in hospitals to the health and well-being of hospital patients and staff.

It is not uncommon to find dental care and dental educational facilities physically linked to, or located within, medical facilities. Both advantages and disadvantages of uncontrolled or inadvertent intermixing of the air in such single structures of combined purpose might well be reviewed and evaluated.

A recent survey has shown that 34.5% of the hospitals in the United States have dental facilities<sup>(20)</sup>. There is an increasing demand being placed upon the dental profession to provide more dental health care for the aged<sup>(21)</sup>, the chronically ill or debilitated<sup>(22)</sup> and the institutionalized or home-bound patient<sup>(23)</sup>. The problems of controlling aerosol production or aerosol distribution and exposure in such unique environmental situations may present even greater potential difficulties than those found in the more usual dental office or clinical situation.

Dentistry is more commonly provided in private, self-contained dental offices and clinics on an out-patient basis. At present, there are some 91,000 dentists practicing<sup>(24)</sup> with the aid of some 100,000 auxiliary personnel including dental assistants<sup>(25)</sup>, laboratory technicians<sup>(26)</sup>, and dental hygienists<sup>(25)</sup> in several thousand different

locations, providing for the dental health demands of more than 189 million people in the United States<sup>(27)</sup>. An estimated 238.5 million dental treatments were provided annually in the United States from July 1957-June 1959<sup>(28)</sup>.

The prospects for increasing the number of dentists graduated annually are not great. To compensate for the increased services to be demanded of the dental profession in the future, it has been proposed<sup>(25)</sup> that the number of ancillary personnel utilized be increased and their duties expanded to permit the dentist to provide more dental care per patient<sup>(25)</sup>. Possibly more than 200,000 more extensively trained dental assistants will be required in the foreseeable future to help the dental profession fulfill the needs of its services. Correspondingly, an increase in total number and expansion of individual duties for both dental hygienists and laboratory technicians may be anticipated.

The moral and legal obligation to provide all reasonable protection for his employed auxiliary personnel rests with the dentist. As he employs more paradental personnel, his responsibilities and liabilities will also increase. One may safely predict that in the foreseeable future more than 300,000 people, both professional and auxiliary personnel, will be involved in providing dental health services, directly or indirectly, for our ever-growing population.

Considering the magnitude of present and future demands upon the entire dental profession to provide dental health care, both the dentists and his auxiliary personnel will benefit from a better understanding of the role of aerobiology in dentistry. They may then better protect their health and well-being as well as that of their patients. To assist the dental profession and dental education in achieving this objective, heavy reliance must be placed upon the knowledge and experience of serobiologists as well as other medical and public health specialities. This will provide dentistry with a better definition of the risks of exposure to infectious and toxic aerosols produced in the dental operating room, teaching clinic and laboratory environments.

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#### DISCUSSION

L. Miller: A study was performed by Naval Medical Research Unit No. 4 at Great Lakes concerning the incidence of acute respiratory disease in dentists and their technicians in relation to the types of patients they were taking care of. Dentists and the assistants taking care of recruits in their first 4 weeks of training had a rather high percentage of respiratory infection compared to those taking care of the older recruits. It certainly illustrates the problem involved in protecting dentists and technicians when they are caring for patients who have acute respiratory disease. Further, assistants caring for recruits had fewer instances of respiratory disease than dental officers.

Mazzarella: If assistants have less disease than dental officers, is that because they are less close to the patient?

Burton: Perhaps, also perhaps it is because a dentist is always present in the immediate area of operation, while frequently the assistant will be away from the chair for varying periods of time. Perhaps there is a time factor involved with exposure and resultant infection.

Mazzarella: Perhaps another reason might be that the dental assistant has been through boot camp for some times, whereas dentists are

relatively new to the camp. I know of instances where all the dental officers for a section of the camp were ill with respiratory disease at the same time.

VFTF\*: I wonder if any thought has been given to the wearing of any kind of mask? Has this thought been proposed or considered?

Burton: Yes; however, other approaches might be as effective and more aesthetically satisfactory. We have conducted preliminary tests on only one type of mask, so I can only give you limited information. The mask happened to be of the type issued to the dental students at the U. C. School of Dentistry in San Francisco. It was found to be ineffective in filtering out 1-5  $\mu$  particles when it was placed over the intake orifice of the Andersen Sampler.

Kniazeff: Air screening, and other things, I am sure, would be applicable.

Burton: Yes; perhaps the study of means of preventing the distribution of air within and from environments used for dental diagnosis and treatment would fall in the realm of micrometeorology.

Kethley: I think it would be interesting to note that even with the aerosolization problem, as you presented it, the incidence of disease is not great. Apparently humans are a highly resistant population. It is therefore difficult to understand or to study mechanisms of infection when you have these high levels of exposure.

Burton: This is true. I think one of the problems of understanding what is going on may be related to a scarcity of reports. A great deal of effort and time is required to publish and distribute in the open literature. Then someone must tabulate the several reports. It is interesting to note, however, that in the days when communication was not as efficient as today, more information of this type was published. As communication has improved, and our social and legal responsibilities have changed, we find fewer instances reported.

Harper: I want to ask a very simple question. Has any air sampling been carried out during dental operations to show that there is an increase in microbial bearing particle counts over normal counts during dental operations?

Burton: Yes, there has. In the next paper to be presented, Bob Mil'ner will report what we have done to date.

Morton: The associated fact strikes me as quite odd. I am interested here from the medical viewpoint. Not only dental lesions, but lesions generally in the mouth, seem to cause very little trouble.

Why is this? Is it the mouth environment -- you can't really clean it up, you know -- you can't bandage it -- and you normally get away without an infection; why is this?

Burton: These are good questions. The difficulty associated with recognizing intra-oral signs of pathology may be related to the dilution, abrasion, and removal of debris. This could alter the gross clinical expression of pathology observable in or on other tissues of the body. The bactericidal effect of saliva has been checked, and according to some reports in the literature, has been found to be insignificant. Whether a composite colony of microorganisms resides, is nourished, and multiplies in the oral cavity, but is not recognized as similar in form to pathological situations seen in or on other tissues, is not really known. Whether other events occur that might mask or alter the expression of signs or symptoms -- these are things that one may only speculate upon, for little is known. Why several species of microorganisms that enter the body through the oral cavity or its tissues do not produce symptoms at the site of inoculation is also unknown. Greater research activity is obviously needed to improve our understanding of the several factors involved.

Proc. First Intern. Symp. Aerobiol., Berkeley, Calif., 1963

## AEROSOLS PRODUCED BY DENTAL INSTRUMENTATION

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Occupational hazards form an integral part of the practice of dentistry. Examples of such hazards are the cardiovascular sequelae, postural maladies, radiation and traumatic injuries, allergies, poisonings, and infections suffered by the members of the dental health team<sup>(1)</sup>. Our interest has been in hazards of infection.

Primarily, the dentist's risk of infection manifests itself by two routes. The first is by contact, best illustrated by the cases of non-venereal syphilis<sup>(1)</sup> and viral hepatitis<sup>(2)</sup> contracted by dentists and their ancillary personnel as a result of digital manipulation of the oral tissues of an infected patient. The second is by the airborne route. Because of his proximity to the patient's face while operating, the dentist is exposed to any respiratory pathogens disseminated by the patient. We speculate that he is further jeopardized by exposure to microbial aerosols generated by the use of his instruments in the patient's mouth.

In the practice of dentistry, the dentist often applies rotating instruments, and air and water sprays, to the contents of the patient's mouth. As a result of these applications, microorganisms, tissue fragments, dental restorative materials and oral fluids are driven into an airborne state. Similarly, prosthetic appliances removed from the mouth and laden with oral debris may serve as sources of microbial aerosols when subjected to rotating instrument or air and water sprays.

This paper is a report of two surveys: First, a quantitative study of aerosols produced in the mouths of dental patients during treatment for simple caries; second, a study of microbial aerosols produced by the dental lathe when a denture was polished.

#### SECTION I. THE CLINICAL PROCEDURE SURVEY

Kazantzis<sup>(3)</sup> reported the finding of bacteria in aerosols produced during the use of dental handpieces in the mouths of patients. Stevens<sup>(4)</sup> presented semi-quantitative evidence of the production of microbial aerosols by the air turbine handpiece during use in a patient's mouth and made a plea for further study of the production of these aerosols. This section describes the quantitation of the aerosols generated in the mouths of dental patients during a typical dental procedure.

#### METHODS AND MATERIALS

The six-stage Andersen sampler<sup>(5)</sup> was used to sample aerosols generated in and emanating from the mouths of dental patients during dental operations performed in the clinics of the University of California School of Dentistry, San Francisco. The dental clinics consisted of two large bays, each containing 50 dental chairs and units. When used to maximum capacity, the clinics each accommodated approximately 120 people; patients, dental students, dental assistants, and instructor-dentists.

Andersen samplers were operated at an air flow rate of 1 ft<sup>3</sup>/min, impinging airborne particles on 3% blood agar held in plastic petri dishes. For each operation, air samples were taken about 2 inches from the mouths of 6-10 patients, with the exception of the polishing of restorations. We found that the polishing operations had to be sampled at an 8-10 inch distance for 15 sec to prevent exceeding the maximum capacity of the Andersen samplers. After sampling, agar plates were incubated aerobically for 72 hr at 35 C. The resultant colonies were counted and the numbers of colony forming units per cubic foot (cfu/ft<sup>3</sup>) of air were calculated, using the methods and correction factors of Andersen<sup>(5)</sup>. The median particle diameter (mpd) of the aerosols was estimated by use of probit transformations and the mpd values assigned to various stages of the Andersen sampler<sup>(5)</sup>, viz., stage one, 7.5  $\mu$ ; stage two, 5.5  $\mu$ ; stage three, 3.5  $\mu$ ; stage four, 2.0  $\mu$ ; stage five, 1.0  $\mu$ ; stage six 0.8  $\mu$ .

## RESULTS OF THE CLINIC STUDY

We found that microbial aerosols were generated in and discharged from the mouths of dental patients during performance of dental procedures involving rotating instruments and air and water sprays. Table 1 summarizes the data accumulated during the sampling of aerosols discharged from mouths of dental patients. These aerosols ranged in concentration from 12.5-140,000 cfu/ft<sup>3</sup> with mpd ranging from 1.3-7.0  $\mu$ . All cfu recovered from the aerosols were of a size capable of impinging on or in the human respiratory system, including the conjunctiva. Thirty to 90% of these viable particles were 5  $\mu$  or less in diameter and thereby would have been capable of penetrating the human respiratory system to the level of the terminal bronchioles and alveoli<sup>(6)</sup>.

While no qualitative study of the organisms recovered was conducted, we observed that the environmental air samples taken in the clinic were composed primarily of colonial types commonly associated with air saprophytes. The alpha and gamma hemolytic streptococci that comprised the majority of colonial forms recovered from aerosols discharged from the mouths of dental patients were found in the environmental air samples in low incidence. The environmental air of the clinics had an average of 15.5 cfu/ft<sup>3</sup> (range of 7.3-29.4) with a mpd of 5.9  $\mu$ . We found no correlation between the concentration of bacteria and fungi in the clinic air and the number of people occupying the clinic; however, we did not attempt a complete survey of the environmental air in the clinics. As indicated by the data contained in Table 1, operations performed under conditions of a clinically wet field (saliva, blood, and water present) consistently produced aerosols of higher microbial content than did the same operations performed under dry field conditions. The use of cotton rolls or the rubber dam to maintain a dry operating field was correlated with lower concentrations of microorganisms in aerosols produced during the operations, i.e. 18-226 vs. 11,000-140,000 cfu/ft<sup>3</sup> observed for polishing a restoration with and without the use of the rubber dam, respectively.

With but one exception, the polishing of a restoration, the mpd of the aerosols was smaller for operations performed under wet field conditions than it was for the same operation performed under dry field conditions.

TABLE 1. Summary of concentration and particle size distribution of colony forming units (cfu) in aerosols generated in mouths of patients during selected phases of dental treatment

Operation	Field condition	cfu/ft <sup>3</sup>		Median particle diameter	Percentage of particles less than 5 $\mu$ in diameter
		Low	High		
None (environmental air)	None	7.3	29.4	5.9	40
Polishing teeth using prophylaxis cup and pumice	Wet	35.0	1,488.0	4.9	45
Drying of teeth using air syringe	Wet	98.0	944.0	1.3	90
Cutting of preparation in tooth using:					
Air turbine with air spray	Dry	12.5	42.5	7.0	35
	Wet	16.2	85.3	6.7	33
Air turbine and water spray 557 bur	Wet	40.0	212.0	4.8	52
Contra-angle 557 bur	Variable wet and dry	23.0	194.0	5.5	42
"Finishing" restoration using the contra-angle with 5/8" cuttle disk	Dry	32.0	86.0	5.1	40
	Wet	4,679	6,537.0	2.7	80
Polishing restoration using the straight handpiece with a Robinson bristle brush and pumice	Dry	18.0	226	4.2	55
	Wet	$1.1 \times 10^4$	$1.4 \times 10^5$	6.5	30

Whereas the use of the water spray with the air turbine handpiece has been considered advantageous in reducing the obvious flow of air-borne tooth structure and dental materials from the patient's mouth<sup>(3)</sup>,

we found that when the water spray was used, the microbial content of the aerosols produced was actually increased. While the difference was small, significance lies in the fact that the water spray failed to reduce the content of viable particles in the aerosols. Further, there was a decrease in the mpd of aerosols generated when using the water spray, i.e. 4.8  $\mu$  for the water spray and 6.7-7.0  $\mu$  for air spray, which would not serve to advantage in terms of an inhalatory hazard.

When using rotary instruments, the air turbine did not produce larger quantities of microbial aerosols than the conventional engine-driven handpiece. The higher operating speeds, 200,000-300,000 rpm for the turbine compared to 6,000-8,000 rpm for the engine-driven handpiece, and the exhaust of about 1/2 ft<sup>3</sup>/min of air by the turbine inside the patient's mouth, apparently made little contribution to aerosol production. Data in Table 1 indicate that the use of polishing brushes, disks, and prophylaxis polishing cups consistently produced microbial aerosols of greater concentration than did the #557 carbide dental bur used in all the cutting operations.

The air syringe, used to dry the teeth or clear the operating field of blood and debris, generated aerosols containing up to 944 cfu/ft<sup>3</sup> with a mpd of 1.3  $\mu$ . Ninety per cent of these viable particles were 5  $\mu$  or less in diameter.

The reported values for microbial content of aerosols disseminated by a human being during quiet breathing range from a low of 0.03<sup>(7)</sup> to a high of 0.53<sup>(8)</sup> organisms/ft<sup>3</sup>. For talking, the reported values range from 2.0<sup>(8)</sup>-40.0<sup>(7)</sup> organisms/ft<sup>3</sup>. Bourdillon, Lidwell and Lovelock<sup>(7)</sup> established the microbial content of the sneeze at 77,000-200,000 organisms. Comparisons of these values for natural human activity with our findings in the dental clinic (Table 1) indicated that the "low" concentrations of cfu observed for many of the dental operations were comparable to the act of speaking. The "high" concentrations of cfu observed for the dental operations consistently exceeded the "high" value reported for speaking. In the instance of the polishing of a restoration using a Robinson brush in a wet operating field, the dental operation produced microbial aerosols equivalent to a sneeze.

At the present time it is impossible to assign a risk rate to the inhalation of the aerosols produced by dental instrumentation. We

have found no published reports that definitely link clinical cases of respiratory diseases of dentists with those of their patients. With no standards of air hygiene to act upon, one can be guided only by common sense. Intuitively, one could speculate that a potential health hazard exists for a person exposed to microbial aerosols emanating from a patient's mouth when these aerosols exceed the microbial dissemination normally associated with breathing and speaking, and which, on occasion, reach the proportion of a sneeze directed into his face.

## SECTION II. THE LABORATORY PROCEDURE SURVEY

Dentures taken from the mouths of patients are frequently polished by the dentist as an expedient method of removing oral debris and smoothing areas ground or cut during denture adjustments. The dental lathe is the equivalent of a machinist's bench grinder, fitted with muslin buffing wheels that serve as carriers for a slurry of pumice held in a pumice reservoir pan. The pumice serves to polish the plastic, metal and porcelain components of prosthetic appliances.

In a preliminary study of denture polishing procedures we found that the polishing of a set of dentures taken from a patient's mouth contaminated the polishing pumice in excess of  $1 \times 10^7$  cfu/ml of pumice slurry. Microbial aerosols produced during these polishing operations contained about  $1 \times 10^4$  bacterial cfu/ft<sup>3</sup> as determined by use of Andersen samplers positioned at chest level of the lathe operator. Samples of pumice taken from the pumice reservoir pans of commercial and private dental laboratories had bacterial contents ranging from  $1.4 \times 10^5$  to  $3.0 \times 10^8$  cfu/ml.

The following experiment was designed to determine the extent of airborne contamination occurring in a dental suite when a denture was polished on the dental lathe using tracer organisms in concentrations approximating those found on patient's dentures or in the pumice of dental laboratories.

### METHODS AND MATERIALS

This survey was conducted in a private dental suite consisting of a waiting room, an office area, three dental operating rooms, a laboratory, a dark room and a lavatory. Figure 1 is the floor plan of the suite.

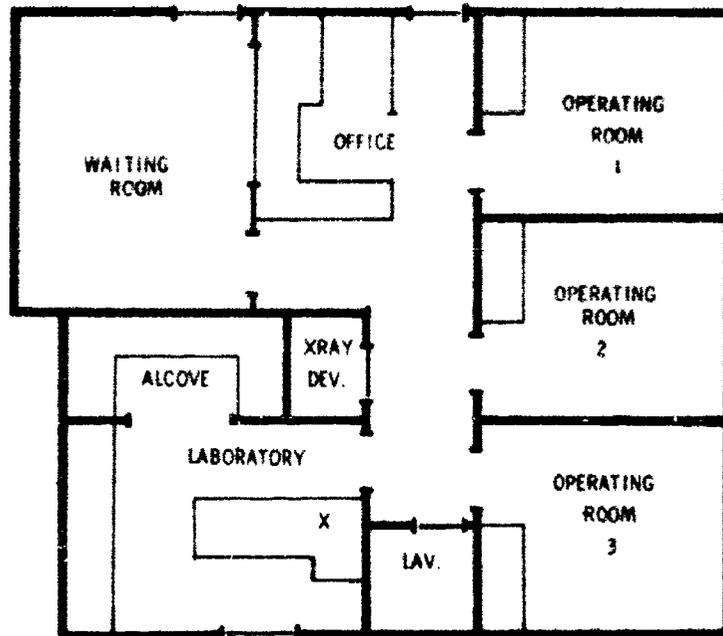


FIG. 1. Floor plan of the private dental suite used in the laboratory study; dental lathe located at position "X".

The suite was served by a 100% air recirculation type "air conditioning" system, which was a simple blower fitted with a heat pump that tempered the air by addition or removal of heat. Fresh air was supplied to the office by peripheral leakage and drafts through the openings of doors and windows. The air flow patterns of the suite were determined by the use of smoke and a hot-wire anemometer. Tempered air was supplied through a grate near the ceiling of each room at velocities ranging from 250-1,100 linear ft/min. The air fanned out and moved diagonally across the upper part of the room, spread down the walls, reversed direction near the floor to pass across the lower aspect of the room and out the door to the hall. In the hall the air was drawn up to a single ceiling-mounted exhaust duct to be tempered and redistributed to the various rooms of the suite. Air moved along the walls at a rate of 10-20 ft/min with drafts occasionally reaching velocities of 50 ft/min. The centers of the rooms were characterized by confused turbulence and low air velocities.

The tracer organisms, Serratia marcescens, strain 8 UK, were grown in Bunting's broth at 31 C for 24 hr on a rotary-type shaker-incubator. Dilutions of freshly harvested cultures were used to "seed" the "test" dentures and polishing pumice used in the experiments. No S. marcescens was recovered from the environmental air of the dental suite during Phase I (see below) of the experiment. It was therefore assumed that S. marcescens recovered during the experiment was the result of our experimental procedure and, unless noted herein-after, concepts of recovery, concentration, numbers, colonies, bacteria, etc. refer to S. marcescens.

Recovery of airborne bacteria was made by mouthwash of human volunteers and by the use of various types of mechanical air samplers. Mouthwash samples<sup>(9)</sup> were taken of all human volunteers immediately before and 15 min after commencing the test polishing operations. Twenty-five ml of Bunting's broth<sup>(10)</sup> was gargled and rinsed about in the mouth of each volunteer for 1 min. Expecterated samples were then treated with 5 ml of saliva liquifying enzyme<sup>(11)</sup> and serial dilutions of the "digested" sample plated out on Bunting's agar. The plates were incubated 48 hr at 35 C and the colonies counted.

Settling plates used in all rooms of the suite were plastic, having a 155 cm<sup>2</sup> surface area of Bunting's agar exposed during each of the polishing operations. Six-stage Andersen samplers, fitted with plastic petri dishes containing Bunting's agar were used in all rooms except the dark room and lavatory. Additionally, Decker Samplers<sup>(12)</sup> were used in the waiting room and operating room II, and a Naval Biological Laboratory slit sampler<sup>(9)</sup> in the laboratory. In each room, sampling stations were set up with air samplers in positions normally associated with the heads of patients or members of the dental health team; i.e. near the headrest of the dental chairs, and at head level for persons seated in the office or waiting room. Mechanical samplers were operated for 1-3 min at a flow rate of 1 ft<sup>3</sup>/min. Sample plates were incubated 48 hr at 35 C. The concentration and the mpd of aerosols were calculated by methods described in Section I of this paper.

To simulate conditions of bacterial contamination observed during polishing of dentures in the private dental laboratory, and to correlate aerosol production with various polishing procedures, we divided our testing procedures into six phases:

Phase 1. Controls.

We monitored the air, using settling plates and Decker and Andersen samplers, to determine the incidence of contamination in the environmental air of the dental suite.

Phase 2. The polishing of dentures after "disinfection" of the dentures was attempted.

A set of steam-sterilized acrylic dentures, the "test" dentures, was dipped into a broth culture giving them a total surface contamination of about  $1 \times 10^{10}$  cfu (which approximated the levels of microbial contamination found for dentures of clinic patients). After "seeding", the "test" dentures were "disinfected" by immersing them into a solution of sodium hypochlorite, 100 ppm available chlorine, for 1 min. Following the "disinfection" step, the "test" dentures were polished on the dental lathe for 5 min, using a steam-sterilized polishing "set-up". The "test" dentures were then set aside. Dentures taken from the mouth of a volunteer, J.B. Sr., were then "disinfected" and polished in a similar manner. After they were polished, J.B. Sr.'s dentures were again "disinfected", rinsed under running tap water and returned to his mouth. A mouthwash sample from J.B. Sr. was taken and assayed immediately after receipt of his dentures. Mouthwash samples were taken of the other volunteers stationed in the various rooms of the suite before and 15 min after the polishing of the dentures was started. Air in the various rooms of the dental suite was sampled for 30 min commencing with the polishing operations.

Phase 3. The polishing of dentures with no disinfection attempted.

The experimental situation was identical to that of Phase 2, except that the "disinfection" step was omitted.

Phase 4. The polishing of dentures simulating the use of contaminated pumice, no disinfection procedures attempted.

The experimental situation was the same as Phase 3, except that *S. marcescens* was added to sterile polishing pumice at a concentration equivalent to the average level of bacterial contamination found in the pumice pans of private and commercial dental laboratories ( $1.7 \times 10^7$  cfu/ml).

Phase 5. The polishing of a denture with "seeded" pumice to characterize the aerosols produced, no disinfection procedures attempted.

The procedures were identical to those used in Phase 4, except

that the pumice was "seeded" to levels about 10-fold higher than the maximum levels of bacterial contamination of pumice we observed in commercial and private dental laboratories ( $2.9 \times 10^9$  cfu/ml). This high concentration was used to generate aerosols containing sufficient viable bacteria to permit estimation of mpd and half-life values.

Phase 6. Polishing of dentures while using disinfection procedures and a ventilated protective hood mounted over the lathe.

A control test was conducted comparable to Phase 5: however, the number of sampling stations was reduced. The volunteer's dentures were not polished to prevent massive contamination of his mouth.

Polishing of dentures using the disinfection steps and protective hood was as follows: "Test" dentures and volunteer J.B. Sr.'s dentures were soaked in a solution of sodium hypochlorite, 1,000 ppm available chlorine, for 1 min before and after being polished. The "seeded" polishing pumice contained  $3 \times 10^9$  cfu/ml. The lathe was isolated by a negative-pressure, ventilated, protective hood that had a face velocity of 300 linear ft/min air flow, with the air wash discharged through a Mine Safety Appliance absolute filter (rated at 99.9% efficiency at 0.3  $\mu$  mpd). After polishing and "disinfection", J.B. Sr.'s dentures were rinsed for 1 min under flowing tap water and returned to his mouth. Mouthwash samples were taken of J.B. Sr., the lathe operator, and two other human volunteers stationed in the laboratory and office area.

The concentration of bacteria in the polishing pumice was determined for all phases of the experiment. During some of the polishing operations, petri dishes were taped to the face, hands, and clothing of the lathe operator to determine the effect of splatter of microorganisms by the lathe.

#### RESULTS OF THE LABORATORY PROCEDURE SURVEY

In all phases of our experiment involving the polishing of dentures using "seeded" dentures of polishing pumice, aerosols were generated by the dental lathe and distributed to the various rooms of the dental suite. Tables 2 and 3 summarize the mechanical and human recoveries of airborne S. marcescens made during the six phases of the experiment.

TABLE 2. Concentration of colony forming units (cfu) found in the air of rooms of a dental suite as determined by Andersen sampler and indicated by settling plates.

Room	Maximum concentration cfu/ft <sup>3</sup> determined by Andersen sampler for Phases:									
	1	2	3	4	5	6: control	6: test 1	6: test 2	6: test 3	
Laboratory	0	13.20	259.20	717.60	2615+	3540	0.4	0.2	0.2	
Office	0	0.40	5.40	29.60	795	745	0	0	0	
Operating Room 3	0	0.20	5.00	24.90	1075	938	0	0.2	0	
Waiting Room	0	0.20	1.80	5.60	132	96	0	0	0.2	
Operating Room 2	0	0.12	0.67	5.00	290	--	--	--	--	
Operating Room 1	0	0.00	0.20	1.00	19	--	--	--	--	

Room	Maximum concentration determined by settling plate cfu/155 cm <sup>2</sup> for Phases:									
	1	2	3	4	5	6: control	6: test 1	6: test 2	6: test 3	
Laboratory	0	16	350	2040	8910	5310	0	0	0	
Office	0	0	17	44	665	1320	0	0	0	
Operating Room 3	0	0	4	58	640	2090	0	0	0	
Waiting Room	0	0	1	--	76	145	0	0	0	
Operating Room 2	0	0	1	12	127	814	0	0	0	
Operating Room 1	0	0	1	3	82	96	0	0	0	
Dark Room	0	0	0	5	3	5	0	0	0	
Lavatory	0	0	0	0	1	0	0	0	0	

TABLE 3. Summary of the recovery of *Serratia marcescens* from the oropharynx of human subjects occupying various rooms of a dental suite following exposure to aerosols generated by the dental lathe

Room	Subject	Experiment Phase									
		1	2	3	4	5	6: control	6: test 1	6: test 2	6: test 3	
Laboratory	R.S.	0	+	0	0	+	+	0	0	0	0
Laboratory	L.T.	0	+	0	+	+	+	+	+	+	+
Laboratory	W.E.B. Lathe Operator	0	0	$9.0 \times 10^2$	$1.8 \times 10^4$	$9.0 \times 10^5$	+	+	+	+	0
Office	J.P.S.	0	0	+	+	+	+	+	+	+	+
Office	R.L.M.	--	--	--	--	--	+	0	0	0	0
Waiting Room	J.T.S.	0	0	+	0	0	--	--	--	--	--
Operating Room 3	J.B. Jr.	0	0	0	0	+	--	--	--	--	--
Operating Room 2	J.B. Sr.*	0	$2.2 \times 10^3$	$3.8 \times 10^5$	$6.9 \times 10^5$	$2.9 \times 10^7$	--	$1 \times 10^4$	$1 \times 10^4$	$1 \times 10^4$	$1 \times 10^4$
Operating Room 1	D.J.	0	0	+	0	0	--	--	--	--	--

\* J.B. Sr. dose was the sum of inhaled dose plus dose transmitted by dentures.

In order of decreasing levels of contamination by the aerosols, the laboratory had the highest content, followed by the office area, operating room 3, waiting room, operating room 2, operating room 1, lavatory and darkroom. The concentration of the aerosols was proportional to the concentration in the polishing pumice, and the distribution of the aerosols was consistent with the air flow patterns maintained by the "air conditioning" system.

In Phase 2 of the experiment, where "disinfection" of the "seeded" denture was attempted prior to polishing, the lathe-produced aerosols reached a maximum concentration in the laboratory of 13.2 cfu/ft<sup>3</sup>. With the exception of operating room 1, airborne S. marcescens was recovered from every room served by the "air conditioner."

Phase 3 (equivalent to Phase 2 with the omission of the "disinfection" procedures) simulated the conditions observed for the polishing of a patient's dentures when using sterile pumice. Here, the aerosols generated by the lathe reached a maximum concentration of 259.2 cfu/ft<sup>3</sup> in the laboratory and were distributed to every room served by the "air conditioner." In a comparison of the results of experimental Phase 2 and 3, we found the use of sodium hypochlorite, 100 ppm available chlorine, to have some value in reducing the numbers of bacteria introduced to the pumice by the "seeded" denture. This reduction in concentration was about 1,000-fold for the pumice and bacteria transferred to the volunteer's mouth by his denture, but only 20-fold for the aerosols produced.

In Phase 4 where the levels of bacterial contamination of polishing pumice of private dental laboratories were simulated, the polishing of the dentures with "seeded" pumice produced aerosols having up to 717 cfu/ft<sup>3</sup> in the laboratory. Again, all rooms served by the "air conditioner" contained airborne S. marcescens. The lavatory, although not served by the "air conditioner", also received airborne S. marcescens.

During Phase 5, where we simulated pumice pan contamination conditions, but used a 10-fold higher bacterial level than "normal", aerosols were generated by the lathe exceeding 2,615 cfu/ft<sup>3</sup> in the laboratory, with all rooms of the suite receiving the airborne tracer.

When the same test procedures were used as the controls for Phase 6, the lathe-produced aerosols reached 3,540 cfu/ft<sup>3</sup> in the laboratory.

When we used the protective hood and sodium hypochlorite "disinfection" procedures during the denture polishing operations (pumice seeded with  $3.0 \times 10^9$  cfu/ml), we recovered only two cfu from the entire suite in each of the three tests (100 ft<sup>3</sup> air sampled per test). Based on the recovery of two cfu in one 5 ft<sup>3</sup> air sample, the maximum concentration observed during use of the hood was 0.4 cfu ft<sup>3</sup>, without the hood, the maximum concentration was 3,540 cfu ft<sup>3</sup>. Therefore, the protective hood and disinfection procedures accounted for better than an 8,000-fold decrease in the dissemination of microorganisms by the dental lathe. Surface contamination of the dental suite without the use of the hood ranged up to 4,310 cfu/settling plate; however, when the hood-and-hypochlorite treatment was used, no S. marcescens was recovered on settling plates.

A comparison of the Andersen sampler data and the settling plate data in Table 2 indicated that a general agreement existed between the two methods of sampling airborne microorganisms. The settling plates proved that surface contamination in the various rooms of the suite resulted from the dissemination of S. marcescens in aerosols generated by the dental lathe in the laboratory.

Twenty-one of the 35 mouthwash samples taken from human volunteers stationed in the various rooms of the dental suite during Phases 2, 3, 4, 5 and the control test for Phase 6 contained the tracer. Obviously, the viable particles in aerosols generated by the lathe were disseminated to the various rooms in concentrations sufficient to be inhaled and deposited in the respiratory system of man. Volunteers J.P.S. and L.T. were our most consistent "samplers"; they were also the only habitual mouth-breathers in the groups of volunteers. The number of S. marcescens recovered from the mouthwashes of the lathe operator and the human volunteer J.B. Sr. who had his dentures polished on the lathe, were directly proportional to the number in the polishing pumice. This relationship is illustrated in Fig. 2. The difference in dose levels observed was due to the lathe operator receiving an inhaled dose, and volunteer J.B. Sr. receiving an inhaled dose plus an oral dose carried to him by his freshly polished denture. In two of the three trials of the hood-and-hypochlorite "disinfection" procedures we recovered S. marcescens by mouthwash of the lathe operator. Later we realized that for these two trials the lathe operator had smoked cigarettes immediately after polishing the dentures. It was therefore

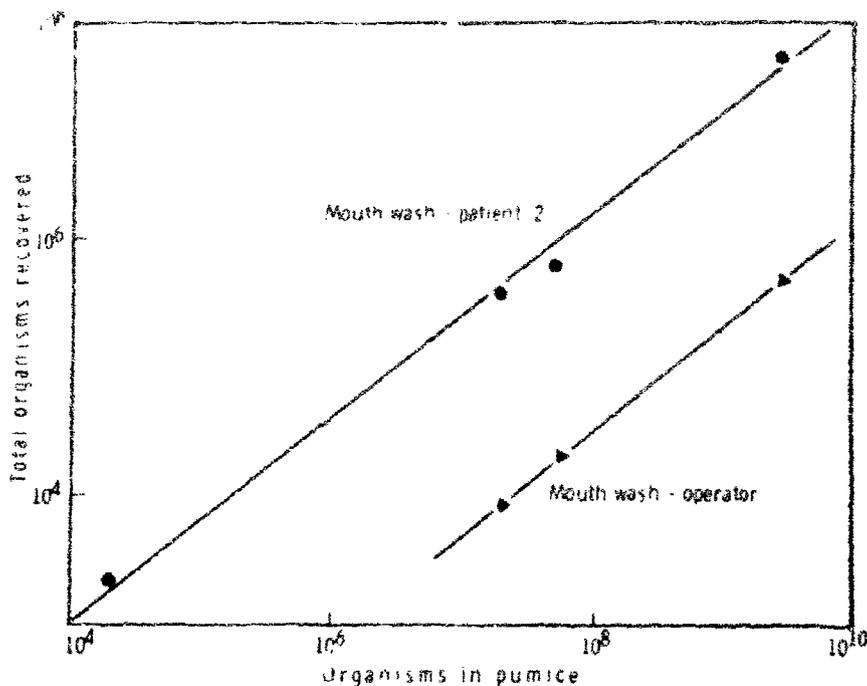


FIG. 2. Recovery of Serratia marcescens from human subjects exposed to aerosols and contaminated dentures. Source of contamination: pumice and dental lathe

possible that he had manually transferred S. marcescens to his mouth from the pumice pan. In each of the three tests of the hood and "disinfection" procedures, more than  $1 \times 10^4$  cfu were transferred to the volunteer J.B. Sr. by means of his freshly polished dentures, despite the dentures having had a 1 min "disinfectant" treatment in sodium hypochlorite solution (1,000 ppm available chlorine) and a thorough rinsing under running tap water before being returned to his mouth. No S. marcescens was recovered from the mouths of the other human subjects occupying the office area and laboratory during the three tests of the hood and hypochlorite treatment.

Thus far, we have reported the magnitude and distribution of aerosols generated by the dental lathe. These aerosols had common characteristics, varying only slightly from one another. Data from Decker and NBL slit samplers indicated that aerosols traveled at velocities equal to those provided by the "air conditioning" system; i.e. aerosols

generated by the father crossed the laboratory in about 15 sec, arrived in the waiting room in 2 min and in operating room 1 in 2-1/2 min.

Figures 1, 4 and 5 are probit transformation plots of cfu recovered on the six stages of the Andersen samplers operated in the laboratory, office area, and waiting room. From these plots, the  $\text{mpd}$  for the aerosols were estimated to be between 2 and 3.5  $\mu$ . Seventy-five to 90% of the viable particles recovered from the aerosols were 5  $\mu$  or less in diameter and were capable of penetration of the human respiratory system to the level of the alveoli<sup>(6)</sup>.

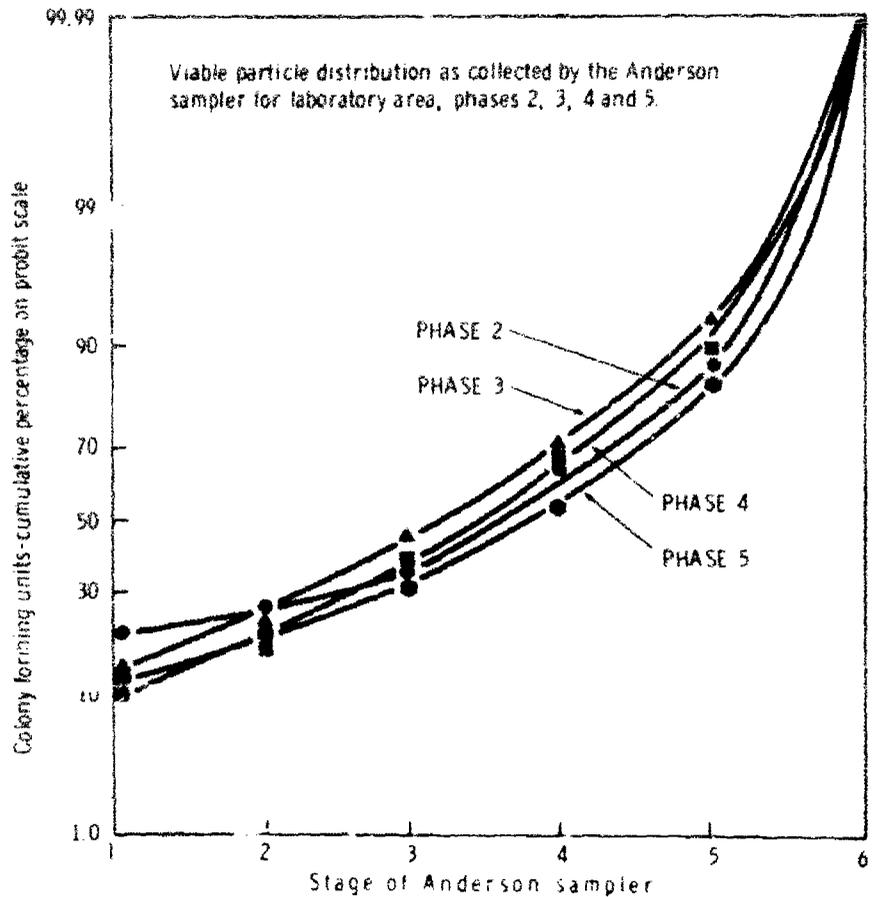


FIG. 3. Viable particle distribution as collected by the Andersen sampler for laboratory area, Phases 2, 3, 4 and 5.

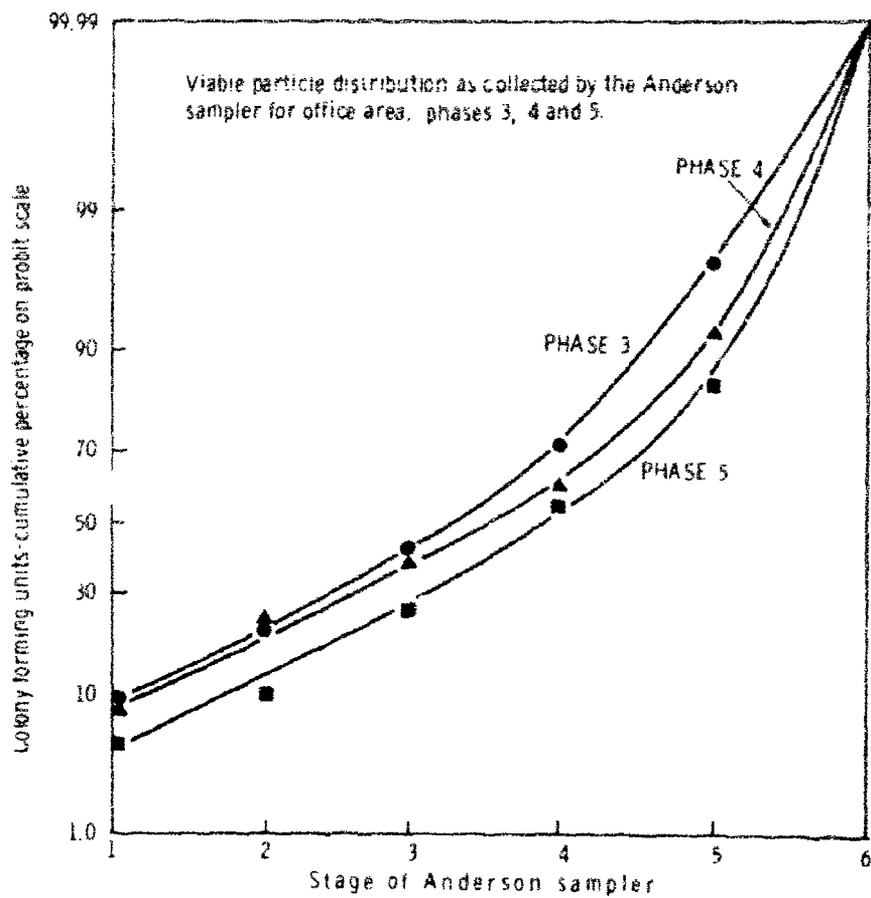


FIG. 4. Viabie particle distribution as collected by the Anderson sampler for office area, Phases 3, 4 and 5.

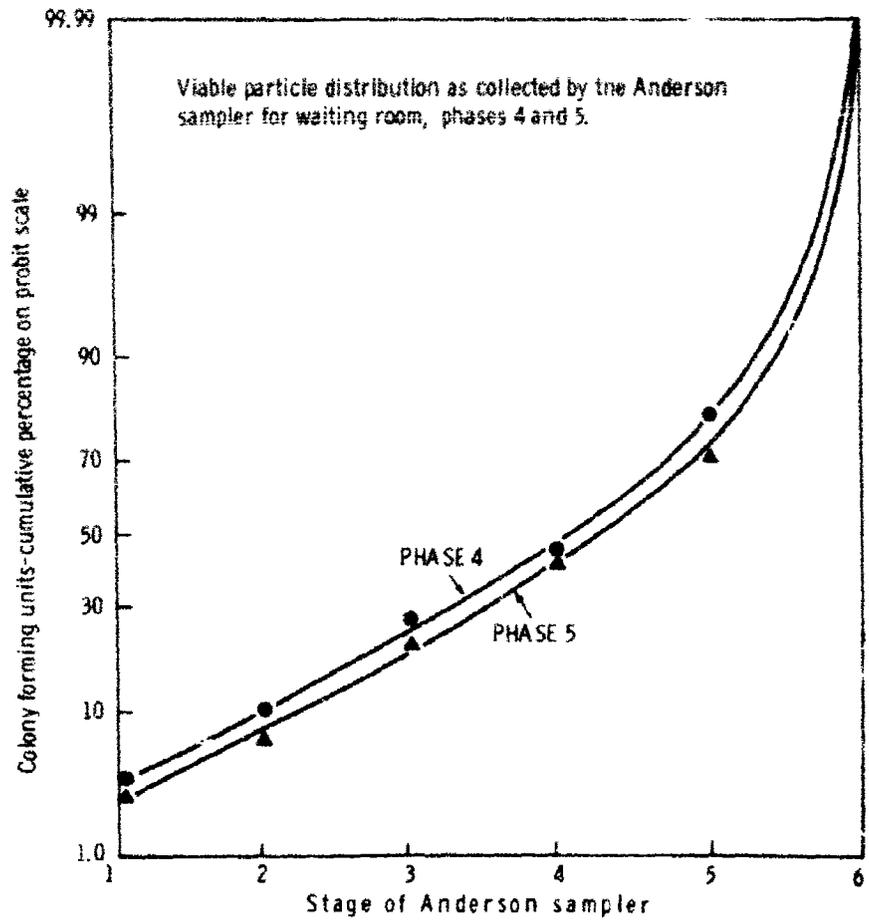


FIG. 5. Viable particle distribution as collected by the Andersen sampler for waiting room, Phases 4 and 5.

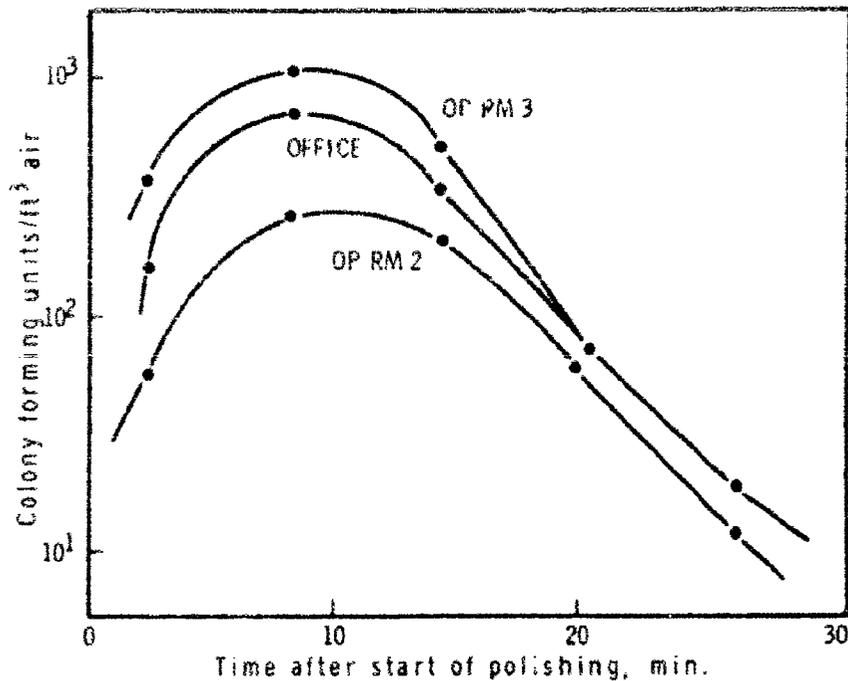


FIG. 6. Numbers of airborne *Serratia* recovered from office, operating room 2 and operating room 3.

The time-course of aerosol build-up and decay in three rooms is illustrated in Fig. 6 where the similarity of the aerosols distributed to the various rooms can be seen. The concentration of the aerosols increased and reached a peak in about 12 min; i.e. 10 min of polishing and 2 min travel time from the laboratory. The viable content of the aerosols then decreased, exhibiting half-life values of about 3-1/2 min. Although particle size did influence the die-away of the aerosols, the rates observed were probably due to loss of viability by the airborne bacteria. In Fig. 7, where the individual slopes of the curves were plotted as a function of the mpd established for the corresponding stage of the Andersen sampler, there was a direct relationship between particle size and die-away rate. However, if one compares physical half-life values taken from the nomograms of Bourdillon<sup>(13)</sup> for aerosols having mpd of 2-3.5  $\mu$ ; viz. 1-1/2 to 3-1/2 hr, to our observed half-life values of 2-5 min, it is reasonable to assume that physical decay played a small role in total die-away rates; organisms less sensitive than *S. marcescens* would have remained airborne for extended periods.

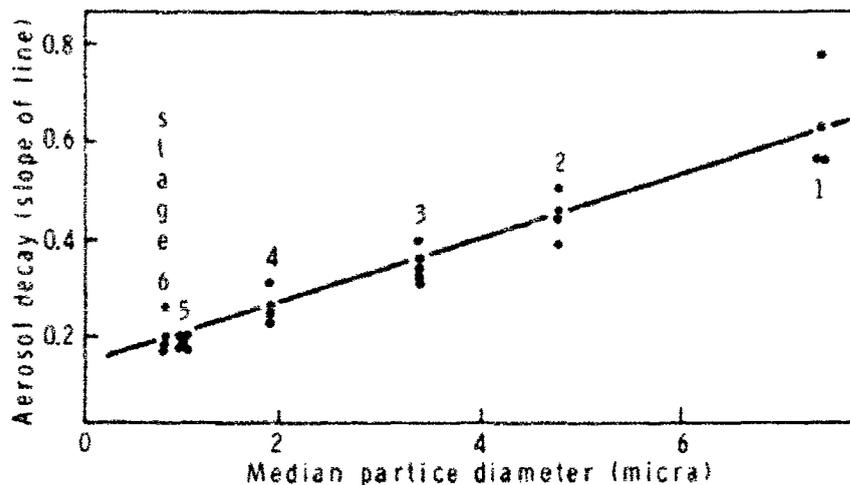


FIG. 7. Relationship of "decay" of aerosols generated in a dental suite by a dental lathe to particle size as collected by the Andersen sampler.

Ancillary to the generation of aerosols, large particles of "seeded" pumice were splattered by the lathe during polishing operations. Plates attached to the face, neck, arms, back, and legs of operators became literally overcrowded with colonies as a result of polishing a "seeded" denture or a denture using "seeded" pumice. When the protective hood-and-hypochlorite-solution treatment was used during polishing operations, no *S. marcescens* was recovered on petri dishes taped to the operator.

#### CONCLUSIONS

We found that microbial aerosols were generated in and discharged from the mouths of dental patients during dental instrumentation in concentrations exceeding those reported for the normal human activities of breathing and speaking. We also demonstrated that bacteria contained in polishing pumice or clinging to the surface of a denture could be atomized by the dental lathe, carried to all rooms of a dental suite by an "air conditioning" system, and deposited in the respiratory

system of human beings occupying the various rooms of the suite.

We hold the opinion that a health hazard could be created by such aerosols if the denture or pumice were to contain pathogenic microorganisms. We know that the normal mechanisms of immunity, innate and acquired, serve to protect man against the vast majority of the microorganisms he inhales. However, we must always keep in mind that the greater proportion of human infectious disease is derived from other human beings<sup>(6)</sup> and that the donors of microorganisms shed to the air may be clinical cases, sub-clinical cases or carriers of infectious disease. It is the pathogen of extraordinary virulence inhaled by the recipient, or the one previously unencountered, which serves as a hazard, e.g., Mycobacterium tuberculosis and the viruses associated with respiratory diseases.

We are confident that the correct and appropriate relationship of these potential health hazards to man can be established with adequate study.

#### ACKNOWLEDGMENTS

The authors gratefully acknowledge the interest and assistance of those who made this work possible: the staff of the Naval Biological Laboratory; the University of California School of Dentistry, San Francisco; the Division of Dental Health, California State Department of Public Health; and those dental students and laymen who, in addition to helping with the technical work, participated as human air samplers.

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#### DISCUSSION

Morton: What does it take to identify the actual bacteria generated in the denture operation? I know you have got *Serratia*, but presumably these entirely alarming figures of yours must relate primarily to non-pathogens or all of our dentists would be sick most of the time.

R. Miller: Our studies have been essentially a quantitation of bacteria in aerosols generated from the patient's mouth by dental instruments. In the few instances where classification of the recovered bacteria was attempted, we noted that the majority were Gram-positive cocci, usually streptococci, *Str. mitis* or *Str. salivarius*. To attempt to classify even a small portion of the organisms recovered would be an astronomical job.

Kethley: I believe about the time I belonged to the Boy Scouts, (my father was a dentist), there was considerable agitation on that

subject in the journals. I think there were also some statistical studies about 30 yr ago. Some basic research work was being done in dental schools -- some critical studies. I imagine they bogged down because there was no supporting aerobiology to lead them. But there were considerable discussions at that time, together with discussions of gauze masks, which were beginning to scare people.

R. Miller: While you are on the subjects of masks: In the different stages of the Andersen sampler, where a mask was used over the sampler, there were no identifiable differences between bacterial counts or number of tooth particles seen during the gross search of petri dishes. In terms of health hazard, these figures are almost wholly non-existent. We know of a case wherein a dentist finally admitted that he had contracted silicosis using the dental lathe and that aerosolized pumice, blowing into his face constantly, reached a point where it affected his lungs. We have a case of a dentist here in this area who has mercury poisoning as a result of breathing aerosols from the air turbine. I can't speak for the dental profession as a whole but only as a student. However, in student health records we find that if you compare the records for respiratory disease (this is a dispensary type of thing), you find that the dental student has a mean figure something like 150-190 visits per 100 students per year to the dispensary for respiratory disease, whereas the pharmacy and medical student, on the same campus, living in the same dorm (an apparently equivalent situation), starts at 40 and goes to 140 visits. Our rates are in a different bracket. Our TB conversion rate has varied from 6-43%. Some pharmacy student groups came up to 25% conversion, but ordinarily we have the highest TB conversion rates on the campus.

VFTF\*: Did you give your results in terms of numbers of organisms or organisms/particle?

R. Miller: The data were presented as colony forming units; actually if one considered the numbers of organisms, the figures undoubtedly would have been higher, for each cfu may have consisted of more than one visible organism.

VFTF: Aren't most of these air turbines operated with a small vacuum cleaner alongside of them? My dentist has a technician standing there with a small vacuum cleaner and whenever he turns the air turbine

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\* VFTF = Voice from the floor.

on, he turns the vacuum on. I am wondering -- in your figures the air turbine does not look so bad -- what happens to the air? Where does it go?

R. Miller: Air evacuated from a patient's mouth by use of an oral evacuator is generally carried through a hose to a trap where liquids and large pieces of debris are removed. After passage through the trap, the air is either discharged into the room or is carried to a sewer line where it is discharged through a roof vent. We do not use an oral evacuator at the University of California Clinics, so I have not had an opportunity to evaluate its efficacy in reducing airborne contamination.

VFTF: Someone at Fort Detrick was interested in personal protection of people in hospital work. I just want to point out (to emphasize that few people are aware of it) that there are currently some masks available which are really quite effective. These devices are now commercially available; they are being tested and are really quite reliable and comfortable. They are plastic devices; you fit them around your nose and they do perform a really acceptable job of filtering. I would recommend them highly to your dental students.

Newton: We can imagine the hazard to the dentist, but would you care to comment about the hazard to the little boy sitting out there in the waiting room, waiting to be treated?

R. Miller: As I say in the paper, the lathe study was strictly a tracer experiment, demonstrating the mechanical dissemination of aerosols produced by a dental lathe. We need a "clean room", or comparable environment, to make a test of spread of organisms from a patient's mouth to other rooms of a dental suite. At the present time, we can not readily distinguish between oral bacteria and the common air saprophytes.

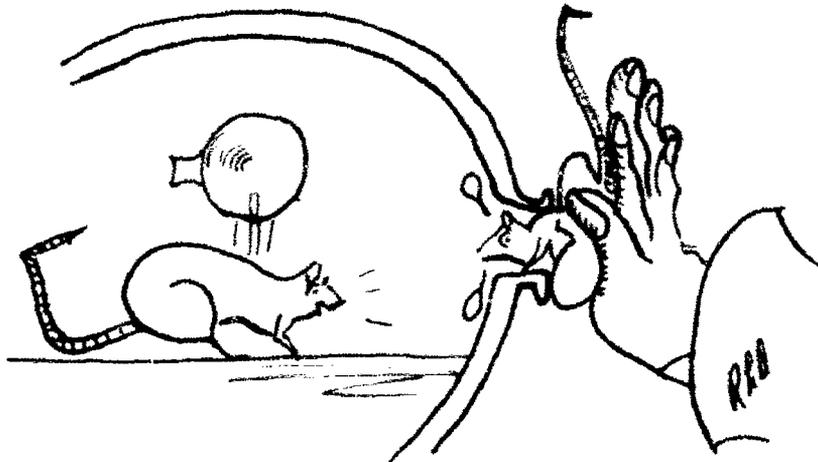
Capt. L. Miller: Your work was all done with bacteria, whereas earlier, in the first paper, viruses were mentioned. I think that oftentimes in aerobiology one forgets that the respiratory system must include the conjunctiva, especially with viruses, so a mask is not going to be the complete answer.

R. Miller: I tend to agree. It has been offered as a strictly clinical observation that dentists stationed at a local Navy base have had an unusually high incidence of eye infections, primarily viral, including a herpes simplex dendritic ulcer of the cornea.

## ECOLOGY SECTION

Elwood K. Wolfe, Moderator

studies of the host-parasite-environment relationship in animals may reveal equivalent relationships in man's environment. This section reports studies of natural and experimentally-induced respiratory infections in animals.



"I told you and told you - you've got to lose ten grams if you expect to get your name on this paper."

## LATENCY OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS\*

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Encephalitis symptoms have been noted in man and animals for many years. Karl Meyer<sup>(1)</sup> in 1932 first described the cases in man and noted that these infections often followed those found in horses in the same area. Howitt<sup>(2)</sup> in 1938 first isolated the Western Equine Encephalomyelitis (WEE) virus from the brain of a child. Since that time it has been ascertained that the WEE virus may periodically be widely distributed among the animal population with wild birds appearing to be the primary hosts for the disease. Man and the horse are believed to be secondary terminal hosts. The disease is arthropod-borne, and although mites and ticks have been shown to be capable of transmitting the virus, they are not believed to be important in transmission. Several species of mosquitoes have been shown to be capable of carrying the virus, but the principal vector at present appears to be Culex tarsalis.

The typical transmission cycle appears to be as follows:

1. A mosquito takes a blood meal from an infected bird.
2. The mosquito matures the virus in its body for several days<sup>(3,4)</sup> depending on the temperature and ingested viral dose.
3. The mosquito bites a susceptible bird and injects some of the virus, initiating a new infection.

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\* Supported in part by a USPHS Grant No. EF 12-828, and completed at the University of Utah during leave from North Dakota State University.

4. The new host develops a viremia after several hours of incubation and this persists for 2-4 days.

5. Following the period of viremia, antibodies that appear in the blood stream are believed responsible for auto-sterilizing the blood.

6. The antibody titer rises in the blood stream and there is some evidence that it remains at a high level for the rest of the bird's life.

The only time the bird may serve as a link in the chain of transmission is during the time that it demonstrates a viremia in its blood stream. Thus, public health surveys that demonstrate a large population of infected or recently infected birds and the presence of large numbers of suitable vectors give occasion for alarm.

The problem in studying the epidemiology of WEE is to determine its reservoir of infection when the disease is not apparent among the bird or animal populations. Where does the virus find sanctuary during the winter? It is obvious that it must have living tissue to survive. Many theories have been advanced to explain the overwintering of the WEE virus, but none have been completely satisfactory. Let us list some of the more prominent theories:

The virus may be harbored over winter in hibernating mosquitoes.

With the advent of cold weather, mosquitoes retreat to such places as rock piles, animal burrows, old cellars, mine shafts, etc. The temperatures in these areas are apparently near freezing and a small percentage of the insects are able to survive. W. C. Reeves and co-workers<sup>(5)</sup> have been able to isolate WEE virus from mosquitoes every month in the year except December. It should be remembered, however, that the mosquitoes apparently had intermittent blood meals through the winter. Each time that WEE virus was isolated from mosquitoes, nucleated red cells were also found in the gut contents of the mosquito, indicating that it had recently fed on either birds or reptiles.

If C. tarsalis is to serve as an overwintering reservoir for encephalitis viruses, it must have an infective blood meal before going into hibernation. There is, however, a strong indication that C. tarsalis does not take a blood meal before hibernation, but feeds on carbohydrates sucked from plant juices<sup>(6)</sup>. Hammon, Reeves, and Golinda<sup>(6)</sup> have even gathered evidence which indicates that a prehibernation blood meal is not compatible with the successful hibernation of

an overwintering of C. tarsalis.

Attempts to isolate WEE virus from hibernating mosquitoes in the more Northern latitudes have, in general, not proven successful. Only one exception to this finding has appeared in the literature: Blackmore and Winn<sup>(7)</sup> found WEE virus in one of 50 pools of female C. tarsalis taken from mine shafts in Colorado. These pools were collected December through February. In any event, most authorities feel that overwintering of the WEE virus in hibernating mosquitoes is not the answer to the problem.

The second theory for overwintering is that the infection chain may be continued through migratory flights of birds. The occasional presence of encephalitis viruses in the bird populations in migratory areas South of the U.S.A.<sup>(8)</sup> indicates that suitable vectors for infection chains may exist there. On the other hand, the failure to isolate virus or detect a high antibody titer in birds enroute from the overwintering areas, or to find it in migratory birds wintering in the Southern U.S.A.<sup>(9)</sup>, argues against this explanation for winter survival and the reintroduction of virus into Northern areas.

Vectors such as ticks and mites transmitting the virus from host to host during the winter. Transovarian passage in ticks is recognized as a mechanism for the perpetuation of the louping ill virus and of Russian spring-summer encephalitis virus. The work of Grundmann et al.<sup>(3)</sup>, however, showed that Dermacentor variabilis and Triatoma sanguisuga would not transfer virus from infected animals to susceptible animals a few days after feeding on infected animals. Thus, apparently these ticks do not mature the virus but only transmit it mechanically. This finding somewhat limits their importance as possible winter-time vectors, although more experimental work should be done on this approach.

WEE virus has been isolated from bird mites<sup>(10)</sup> by Miles et al. in 1951, and for a time it was believed that they might be an important vector. Later investigations by Chamberlain et al.<sup>(11)</sup>, working with Saint Louis Encephalomyelitis (SLE) virus, and Winn and Bennington<sup>(12)</sup> using WEE virus, indicate that detectable virus persisted less than 2 days in mites and no transmissions could be demonstrated after that time. They concluded, therefore, that mites were not an important vector in the transmission of these viral encephalitides.

Reeves et al.<sup>(13)</sup>, in a study of 61 captured wild birds held under

carefully controlled conditions, noted five conversions from a negative to a positive WEE neutralization index during a 4- to 7-month holding period. The only arthropods observed in the aviary were nasal mites and bird mites Ornithonyssus sylviarum. This may indicate that under certain conditions these mites could serve as important vectors and explain the overwintering of the WEE virus.

The fourth hypothesis is that hibernating reptiles might harbor the virus through the winter months and restore it to activity in the spring. Gebhardt and Hill<sup>(14)</sup> and Thomas and Eklund<sup>(15)</sup> in 1960 were able to demonstrate that WEE virus could be carried through the winter by garter snakes under laboratory conditions. In the autumn they injected snakes with virus and allowed them to hibernate under simulated natural conditions. In the spring, when the snakes came out of hibernation, they were shown to develop a viremia as the temperature warmed up. Virus levels in the snakes during the hibernation period were very low or non-detectable but increased with the increase in body temperature that developed during the warm spring months.

Karstad<sup>(16)</sup>, working in Georgia, collected snakes, turtles, tortoises, skunks, anoles, and alligators and tested them for virus and antibody titer. Of the 99 reptiles tested, he found seven (five snakes, one turtle, and one alligator) to possess significant titers of Eastern Equine Encephalomyelitis (EEE) virus-neutralizing antibody at the time of capture. He was, however, unable to isolate any virus.

The possibility that reptiles may serve as overwintering hosts presents a very interesting approach to the problem. Several investigators have studied hundreds of snakes and have failed to find any demonstrable virus. Gebhardt and Hill<sup>(14)</sup>, however, have recently claimed to have found live WEE virus in the blood of a garter snake following hibernation. The importance of this discovery will require further verification but may well be of considerable importance in explaining overwintering.

The fifth hypothesis for overwintering is that bird or animal hosts may harbor the virus as a latent infection which would re-manifest itself at a later date due to some external or internal stimulation. Several instances have been noted in the literature where birds or mammals have been shown to harbor virus several weeks or months after the initial infection. Reeves et al.<sup>(13)</sup> injected 284 birds with WEE

virus and was able to re-isolate the virus from various organs and the blood 1-10 months later. Slavin<sup>(17)</sup> injected 2-week-old mice born to hyperimmune mothers and isolated SLE virus in 3 of 14 pairs 71-162 days later. Using an electrophoresis procedure, Olitsky and Long<sup>(18)</sup> were able to recover vaccinia virus 133 days after its injection into a rabbit. Olitsky et al.<sup>(19)</sup> were also able to recover the poliomyelitis virus 23 days after it had been injected into a monkey, which subsequently exhibited all the symptoms of polio, followed by an apparent full recovery. Here again, electrophoresis was used to separate the virus from the immune serum in which it was suspended. These experiments would tend to indicate that virus can persist in a mammalian host for long periods of time. In many instances detection of virus is apparently masked by the presence of neutralizing antibodies. In other cases the virus apparently reaches some sort of equilibrium with the immune defenses of the body and may appear in a free state after the passage of a period of time. One would think, however, that the latter situation would be the exception rather than the rule.

It has been known for some time that there are several means by which a virus can be dissociated from its combining antibody. The method of electrophoresis has already been cited. Rubin and Franklin<sup>(20)</sup> reported that a small percentage of combined Newcastle Disease Virus can be dissociated from its antibody by dilution. Other workers have noted this same phenomenon with many of the viruses. This mechanism could explain the appearance of virus in an animal system if the animal were passively immunized and produced enough new tissue and body fluids to dilute out the virus-antibody mixture.

Mandel<sup>(21)</sup> has shown that a change in pH will lead to almost a 100% dissociation of poliovirus; perhaps conditions exist for such a phenomenon in the mammalian system.

Hove<sup>(22)</sup>, working with the poliovirus, was able to recover the virus from feces during the period of late convalescence. This recovery was effected by extracting the feces with Freon 113, which is a protein extractant. No virus was recovered without the use of Freon 113. The success of the Freon in aiding the recovery of the virus was believed to be caused by the removal of antibody that was masking recovery by conventional means.

Soave<sup>(23)</sup> performed a very interesting experiment using rabies

virus. He injected a group of 24 guinea pigs with rabies virus and 11 of them subsequently died with the symptoms of rabies. Seven and one-half months later, 12 surviving animals from this group were started on a regimen of ACTH treatments. Three of these 12 guinea pigs subsequently died with the symptoms of rabies. Rabies virus was positively identified from one of these three by sub-passage in mice. This finding indicates that combination of virus-antibody can actually be shifted towards the liberation of free virus by appropriate hormone treatments.

The dissociation of virus from antibody by digestion with proteolytic enzymes offers some very interesting possibilities. Chester<sup>(24)</sup> was able to demonstrate the dissociation of tobacco mosaic virus from antibody combination by pepsin digestion, Kalmanson and Bronfenbrenner<sup>(25)</sup> in 1943 were able to dissociate coliphage from anti-coliphage using either pepsin or papain digestion. The pepsin digestion was much slower and had to be performed at a rather low pH, which is often detrimental to many viruses. The papain digestion, however, proved to be very effective and would liberate phage in a short period of time. They further noted that if the phage were over-neutralized with a large amount of antiphage and allowed to sit for a long period of time, the phage could not be recovered. On the other hand, if the phage was just combined with enough antibody to neutralize it, then it could readily be recovered by papain digestion.

These studies prompted this investigator to wonder what the effect of papain digestion would be on WEE virus combined with antibody. The literature indicated that EEE virus was sensitive to chymotrypsin but not to trypsin. Since Kalmanson and Bronfenbrenner had such good success with papain on the coliphage, an experiment was thus set up to determine the sensitivity of WEE virus to papain. A concentration of papain of 1:10, 1:100, and 1:1000 was set up and WEE virus introduced into these enzyme preparations. The mixture was then digested for 2-1/2 hr at 37 C. The results of this experiment showed that the 1:10 and 1:100 concentration of papain were apparently toxic to the virus but that the 1:1000 dilution yielded as good a recovery of the virus as the untreated control.

Subsequently, an experiment was set up to determine if virus could be recovered from neutralizing antibody. Virus and antibody were combined and allowed to equilibrate for 1 hr at 37 C, then the mixture was subjected to papain digestion for 2-1/2 hr. About a 50% recovery of

virus was obtained using a 1:1000 and 1:3000 dilution when compared with the control. Further experiments have shown that about a 1:600 dilution of papain is optimal for recovery of the virus from the antibody. It has also been noted that the ratio of neutralizing antibody to virus appears to be important. "Overneutralized" virus is more difficult to recover. Further research is required in this area to clarify this problem. In any event, it has been proven that the WEE virus can be dissociated from its neutralizing antibody and recovered in an infective state.

The fact that a proteolytic enzyme can release WEE from its neutralizing antibody and that virus can persist in the presence of antibody for prolonged periods of time, opens up a whole new field of epidemiological possibilities as far as the study of the latency of arthropod-borne viruses are concerned.

Let us reconstruct a hypothetical case of infection from a latent source. Assume that a mosquito takes a blood meal from a bird that has recently returned from a migratory flight (resulting in its having an excess of adrenocorticotrophic hormone). This bird has been infected the previous year with WEE virus and still has combined virus-antibody circulating in its blood stream. The hormonal stimulation effected by the stress of the migratory flight has shifted the virus-antibody equilibrium to the point where the virus is just neutralized. The mosquito ingests this "just neutralized" blood and subjects it to proteolytic enzyme digestion in its gut. This digestion destroys the antibody and liberates the virus. (Scrivani, Reeves, and Brookman<sup>(26)</sup> showed that *C. tarsalis* could destroy the antibody in 24 hr or less.) The virus then undergoes multiplication during the intrinsic incubation period (see the work of Merrill and Ten Broeck<sup>(27)</sup> denoting a 10,000-fold increase). The mosquito then bites another susceptible bird host and the chain of infection is continued through the winter. All the pieces of the puzzle appear to be present. Efforts to reconstruct this entire picture by experimental evidence have not yet met with success but efforts will be continued along this line.

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THE PERSISTENCE OF PASTEURELLA TULARENSIS  
IN SOILS UNDER A VARIETY OF  
CLIMATIC SITUATIONS

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Contaminated water has been implicated in several outbreaks of tularemia in man. While the exact means by which the water became contaminated is not known, several possibilities exist. These include (1) the presence of dead, infected animals in the water, (2) the discharge into the water of contaminated animal excretory materials, and (3) the contamination of water during runoff with soil containing Pasteurella tularensis organisms from dead animals or excrement. That animal discharges can contain large numbers of P. tularensis has been shown by several workers, including some in our laboratory who will present data at these meetings. In considering the third possibility, it became of interest to determine the length of time P. tularensis can persist in soil under a variety of climate and soil conditions. The persistence of P. tularensis in the soil is the subject of the present report.

To investigate this problem, three different soil types were selected. They were all taken from the Dugway, Utah area and were of the general saline-alkaline type. The first, designated as "sandy knoll soil", was a very fine wind-blown sand. It had a soluble salt content of about 0.5% and contained a very small amount of plant debris. The pH of the saturated soil was 7.5. The second type called "clay flat soil" was a silty sand. It contained about 1% salt and a small amount of plant debris. Its pH was 7.9. The third type designated

"salt flat soil" was a silt which contained about 6% salt and was nil in organic matter. Its pH was 7.5.

The variables considered in this study were (1) soil type, (2) surface vs. subsurface contamination, (3) soil moisture, (4) exposure to sunlight vs. shading, (5) depth in the soil, and (6) seasonal changes.

The soil was contained in trays which were sunk into the ground. These trays were covered with hoods to protect the soil from extraneous factors such as wind and rain and access by animals. Metal hoods covered the shaded plots and plastic hoods covered the plots exposed to sunlight. Other plots were covered with screens as a check against any deleterious effects of the metal or plastic hoods. The plastic\* used transmitted about 67% of the germicidally effective energy of summer daylight. This figure was determined by weighing the transmitted intensities of several spectral bands in terms of Luckiesh's (1946) curve for the relative germicidal effectiveness of radiant energy.

The hoods were ventilated by means of louvers in the sides and top of the hoods, and surface and subsurface soil temperatures outside and beneath the hoods were recorded during the experiments. A typical summertime maximum temperature for the surface of the ground was 140 F as compared to 145 F under the plastic hood and 110 F under the metal hood. Subsurface temperatures during the same period were 115, 125, and 100 F for the outside, plastic hood, and metal hood, respectively. The ground temperature got very high during summer days and was only slightly higher under the plastic hood than outside the hood; either temperature, however, would seem to be incompatible with the survival of the organism. Ground temperature under the metal hood remained cooler than the outside, as would be expected. Typical wintertime maximum surface temperatures were 80, 110 and 65 F for the outside, plastic hood, and metal hood, respectively. Here the temperature was considerably increased by the presence of the plastic hood. Subsurface temperatures during this period were 70, 85, and 60 F for the outside, plastic hood, and metal hood, respectively.

Surface inoculation was effected by aerosolizing a suspension of the virulent Schu strain of P. tularensis with a Chicago atomizer

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\* "Flex-O-Glass" plastic film. Thickness 4 mils. Warp Brothers, Chicago, Illinois.

over the filled trays contained in a special inoculating hood. The mixed inoculation was accomplished by aerosolizing the material with a Chicago atomizer into a modified concrete mixer containing the soil and then placing the inoculated soil into the trays. Plots which were watered were sprayed with distilled water periodically to maintain a moist condition of the soil.

In order to study all combinations of the factors: soil type, type of inoculation, soil moisture, sunlight exposure and shading, a split plot type of experimental design was chosen. Soil plots were set up in two replicates. Each replicate contained five whole-plots or "environments". Each "environment" consisted of a particular hood and moisture combination. Whole-plots were divided into two split-plots, one being the surface inoculation and the other the mixed inoculation. Each split-plot in turn was divided into three split-split-plots corresponding to the three soil types. Each soil was divided into eight compartments. One of the compartments of each soil was sampled each time. All units were randomly arranged. In this setup each soil was represented in each type of inoculation for each environment and each environment had two replications.

Sampling was done by taking a 3/4 inch diameter core of the soil. The surface layer down to a depth of 1/4 inch and the layer between 1 and 1-1/4 inch from the core were assayed. A 20% suspension of the soil was made in gelatin phosphate diluting fluid containing 0.2% gelatin and 0.4%  $\text{Na}_2\text{HPO}_4$ . Appropriate dilutions were made and plated on Peptic Digest Starch medium (Levin, Trupin, and Cabelli, 1962). The plating medium contained 500 units of penicillin/ml, 500  $\mu\text{g}$  of streptomycin/ml and 300  $\mu\text{g}$  of actidione/ml to inhibit the normal soil microflora.

In the first experiment, which was conducted in June 1962, the maximum temperatures ranged from 94-99 F and the relative humidity (RH) ranged between 5 and 45%. In this experiment it became immediately evident that *P. tularensis* would not survive longer than 1 day in soils exposed to sunlight under these hot, dry conditions. This was true down to a depth of at least 1-1/4 inch which was the deepest that the soil was sampled and it did not matter if the soil was watered. However, when the soil plot was shaded by the metal hood, no recovery was obtained from the surface sample after 24 hr, but viable *P. tularensis* was recovered up to 72 hr in the subsurface samples.

Since it was apparent that the only recovery of the organism after 24 hr in soils exposed to these harsh conditions would be confined to the subsurface layer of soils shaded from sunlight, all other summer-time experiments were limited to soils inoculated by mixing and shaded from sunlight by metal hoods. Five hoods were employed, each hood being a replicate, and the split-plots were the watered and non-watered treatments. Experiments conducted in the wintertime included surface inoculation and sunlight exposure as previously described.

The second experiment was initiated on August 27, 1962. It was concluded on August 31. The weather was again hot and dry. Temperature at the time of setup was 60 F and RH was 27%. Maximum temperatures during the experiment ranged between 74 and 88 F with lows down to 48 F. RH during the experiment ranged between 7 and 48%. The soils were initially dry. All plots were shaded with metal hoods and the soil was inoculated by mixing only. Forty-five ml of a suspension containing  $27 \times 10^{10}$  organisms/ml was inoculated into 38.4 kg of each soil, giving an initial concentration of  $30 \times 10^7$  organisms/g of soil.

The average persistence time of the organism in salt flat soil of 39 hr was significantly longer than in the other two soils. The persistence time of 26 hr for clay flat soil was also significantly longer than that for sandy knoll soil which was only 12 hr. Watering had a significant effect, the persistence time being longer for soils which were watered than for soils which were not watered. There was a watering-by-level interaction, in that watering increased the persistence time in the surface layer more than it did in the subsurface layer. The persistence time for the individual soils is the average between watered and non-watered plots, since there was no interaction of watering with soil type; in other words, all soils responded to watering in a similar manner. Likewise, the persistence times for watered and non-watered soils is the average for all three soil types.

The third experiment was initiated on November 13, 1962, and lasted until February 18, 1963. During this prolonged period much freezing weather was encountered. The temperature at the time of setup was 46 F and the RH was 44%. Temperature and humidity varied considerably over this long period. Temperature in November ranged from 15-63 F, in December from -5 to 57F, and in January and February from -16 to 50 F. Soils were initially moist. All plots were shaded by metal hoods and the soil was inoculated by mixing only. Forty-five ml of a

suspension containing  $28 \times 10^{10}$  organisms/ml was inoculated into 38.4 kg of soil, giving an initial concentration of  $33 \times 10^7$  organisms/g of soil. The persistence time for sandy knoll soil was 210 hr or about 9 days. That for salt flat soil was 280 hr or about 12 days. These soils were not significantly different. However, for clay flat soil the persistence time extended to 2200 hr or about 92 days.

Water caused a significant difference but, strangely, in this experiment the persistence time was decreased in soils which were watered. This phenomenon might be explained by the fact that continued watering over an extended period might leach the soluble salts from the soil, decreasing the osmotic pressure of the soil solution. Leaching of these saline-alkaline soils also causes a rise in the soil pH which might be harmful to the organism. Levels were significantly different; the organism persisted longer at the lower level. Again, there was a watering by level interaction. When the soil was watered, the persistence time was about the same in both layers, but when the soil was not watered, the persistence time was less in the top layer.

It is my opinion that the initially moist condition of the soil in this experiment is the predominant factor which led to the long persistence time of the organism. Another experiment which was conducted during September and October 1962, when conditions were considerably more hostile, supports this impression. In that experiment the organism persisted in moist salt flat soil for 35 days as compared to less than 1 day in dry clay flat soil.

In an experiment conducted in December 1962, when plastic hoods and surface inoculation were again employed, there was no significant effect of sunlight exposure at the deeper soil level, but there was some evidence that sunlight exposure decreased the persistence time of the organism on the surface. In the wintertime the temperature of the subsurface soil does not attain the lethal degree that it does in the summertime. Sunlight exposure in the summer definitely has a deleterious effect on the microorganism in the soil. On the surface, the proportion of this effect due to heat and that due to germicidal radiation would be difficult to ascertain. Certainly the soil in the summertime reaches temperatures that are incompatible to the organism. Any lethal effect of sunlight at the subsurface level is most certainly due to heat alone, since any germicidal radiation cannot penetrate soil to this depth.

Differences between soil types are inconclusive in these experiments. It is quite likely that each soil has its own optimum range of moisture for the survival of the organism. Since the initial moisture content of the soils varied between experiments, the reason that one soil gave a longer persistence time than another may simply be because the moisture level of the soils in that particular experiment was nearer the optimum for the favored soil.

Watering, in general, had the effect of producing higher recoveries at least in the earlier sampling times, but in one experiment in which the persistence time was considerably extended, continued watering caused a significant decrease in the recovery. This phenomenon might be explained by the fact that prolonged watering leaches the soil of its soluble salt and decreases its osmotic pressure and also that leaching of these saline-alkaline soils causes a rise in the soil pH.

There was a consistent watering-by-level interaction in these experiments, in that recovery in the surface layer of dry soils was much less than in the subsurface layer, whereas in soils which were watered the recovery was more nearly equal in the two levels. This effect was perhaps more pronounced in the coarser sandy knoll soil which tends to dry out faster than the other two soil types. This effect is probably due to the rapid drying of the surface of the soil. Applying water keeps the surface in a moist condition more nearly equal to that at the subsurface level.

In summary: P. tularensis, when inoculated by the aerosolization of a concentrated suspension onto the surface of dry soil, whether or not the soil was subsequently watered, would not survive longer than 1 day under hot, summertime conditions, and when it was mixed into the soil it would not survive longer than 1 day down to a depth of 1-1/4 inches if the soil was exposed to sunlight; however, if the soil was shaded from sunlight, the organism could persist up to 72 hr at this lower depth. When the soil was initially moist and protected from sunlight and the organisms were mixed into the soil, P. tularensis could persist in the subsurface soil under hot, summertime conditions for at least 35 days and under wintertime conditions for at least 92 days.

Subsequent watering of the soil tended to prolong the persistence of the organism in the surface layer of the soil more than it did at

the deeper level. The initial moisture condition of the soil appeared to be an important factor influencing the persistence of the organism.

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#### DISCUSSION

Derr: As most of you probably know, a number of years ago it was found, I think, that streptococci lying in the dust or on the floor of the barracks, though they retained their viability for prolonged periods of time, rapidly lost their ability to infect men. A number of people have since been involved in studies wherein the association of viability and infectivity of P. tularensis is rarely demonstrable within relatively brief periods of time in the airborne state for both experimental animal and man. The question that I think is crucial about your studies on viability is, what do we know about the relationship of viable P. tularensis under these storage conditions, if you will, to the soil and their infectivity?

Thorne: The recovered organisms in these experiments were spot-checked for virulence by guinea pig inoculation as well as being identified by slide agglutination. Virulence was demonstrated in every case; however, no titration of virulence was attempted.

Hatch: Are you aware of the fact that P. tularensis has been isolated from the Bitter Root Valley, Montana, in soil samples containing cystine? Did you attempt to add cystine to your soil samples? Did you use other media to determine viability?

Thorne: No, we have done neither of these. The only medium we used was Peptic-Digest-Starch.

Proc. First Intern. Symp. Aerobiol., Berkeley, Calif., 1963

## EXPERIMENTAL INFLUENZA VIRUS INFECTION OF MICE

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For a long time it has been recognized that mice are susceptible to infection with influenza viruses. Strains of virus that have never been previously passaged in mice readily multiply to high titer in the mouse respiratory tract, and strains adapted to mice by serial passage produce typical areas of pulmonary consolidation that frequently lead to death of infected animals. Nevertheless, it has proven experimentally difficult to induce mice to transmit influenza virus infection from one to the other with reproducible results. A number of years ago, Eaton observed transmission of influenza virus infection when he housed infected and uninfected mice together in small jars. The spread of infection was determined by the presence of pulmonary lesions in the contact animals. Since that time, however, other investigators have encountered difficulty in obtaining similar results.

For the past 3 yr we have been working with an experimental model to study transmission of influenza virus infection in mice. In the course of these investigations we have defined a number of factors which significantly influence the rate at which infection is transmitted - factors that may in part explain the difficulty that other investigators have found in the past.

Mice were infected with influenza virus by exposure to an aerosol mist of virus in a closed chamber. The chamber was simply an old autoclave that had been modified so that a nebulizer could be introduced

at right angles to a room air inlet, and an exhaust opening could be attached to a vacuum source. Twenty-five liters/min of air were evacuated from the chamber; 20 liters/min entered the chamber as room air and 5 liters/min were introduced through the nebulizer. During a 40-min period mice were exposed to approximately 100 mouse infective doses of influenza A2 (Asian) virus.

Contact between infector mice and previously uninfected animals was established according to one of two experimental designs. In the first of these, infector mice were placed, two each, in small stainless steel cages shortly after infection. Twenty-four hr later, contact was initiated by adding two previously uninfected mice to each cage. A 24-hr period of contact was permitted, after which contact mice were removed and isolated in individual cages for 48 hr. Ground lung suspensions from contact animals were then prepared and inoculated into chick embryos to demonstrate the presence or absence of virus. This particular time period for contact was chosen for specific reasons. To determine the period of maximum infectiousness, four successive groups of contact mice were exposed to the same group of infector mice for 24-hr periods beginning immediately after initiation of infection. Virtually all transmitted infections occurred among mice exposed to infector mice during the period from 24-48 hr after the infection of the infector mice.

This selective period cannot be related directly to virus titers in the tissues of infector mice at various periods during the course of infections. Peak titers of virus were not attained in the lungs until 48-72 hr after initiation of infection, and titers were not appreciably lower at 96 hr. Titers of virus in the trachea were likewise still at peak levels at 96 hr, and appreciable titers of virus in the nose did not appear until long after transmission ceased. In addition, we obtained throat swabs from infector mice at 24-hr intervals following infection. The percentage of swabs from which influenza virus could be isolated was as high (60%) 96 hr after infection as after either 24 or 48 hr.

If I may digress for a moment, I think that these observations pinpoint a frequent conceptual error. We tend to equate infectiousness with the ability to isolate organisms from an appropriate site in a potential infector. Obviously, the presence of such organisms in sites from which the infection can be effectively transmitted is essential

to such transmission. However, the mere presence of the microorganism does not prove that the subject is capable of effectively introducing that microorganism into the environment and infecting other subjects. We have observed that, in fact, some animals in our experimental model transmit influenza virus infection much more effectively than others. I have already explained that in the first of our experimental models, two contact animals are housed with two infector mice during the contact period. In 28 individual experiments, we have examined 511 pairs of contact mice to determine the frequency with which both, neither, or one of a pair acquired infection. We found that there were more pairs in which both mice, or neither, became infected than was predicted. Conversely, fewer pairs than predicted were found in which one animal acquired infection and the other did not. Our interpretation of these results is that the infector mice vary in their ability to transmit infection. In cages where there was a good transmitter, both contact mice tended to acquire infection, whereas in those cages where neither infector mouse was a good transmitter, neither exposed contact animal acquired infection. In some of these experiments we examined the tissues of infector mice for influenza virus at the end of the contact period. Infector mice from cages in which one or both contact animals acquired infection did not have higher titers of virus in the nose, trachea or lungs than infector mice that did not transmit infection. The varying ability to transmit infection is not, therefore, simply a consequence of varying titers of microorganisms in the tissues of potential infectors.

I mentioned earlier that we employed a mouse-adapted strain of influenza A2 (Asian) virus. We chose this virus after comparing it to the CAM strain of influenza A1, a strain of virus which has been extensively passaged in mice but even on initial isolation was found to be quite virulent for mice. We found that the CAM strain multiplied to greater peak pulmonary virus titers (72 hr after infection), that it produced more extensive pulmonary lesions and caused a higher mortality rate than the A2 virus. Nevertheless, the A2 strain proved to be more readily transmitted from one animal to another and was therefore chosen on that basis. I believe that these results point to the need to dissociate transmissibility from other parameters of virulence such as lesion production and mortality.

The second type of experimental design was employed to study the

mechanism of spread of infection, whether by large droplets introduced into the immediate environment of the infectors or by tiny droplet nuclei which are more freely dispersed. In this model, contact was initiated in a large cage inside a closed chamber through which the air flow could be controlled. In each experiment we maintained a constant ventilation rate (rate of air flow through the chamber) during the entire 24-hr period of contact. As we increased the air flow from one experiment to another, from 213-230 liters/min, the rate of transmitted infection declined. We feel that this inverse relationship between ventilation and transmission strongly suggests that infection is spread by droplet nuclei. One would not expect spread by larger droplets to be influenced appreciably by ventilation, since the particles are airborne for only very brief periods. In addition, the cage was divided by a double mesh wire screen. On one side we placed all the infectors and half of the contacts and on the other side the other contacts. In this arrangement, some of the contact mice were allowed to mingle freely with infectors, whereas the other group of contacts were physically separated. The actual amount of physical separation was not sufficient to absolutely preclude spread of infection by large droplets, but it did insure a significant difference in the mean distance between infectors and contacts on one side of the cage as compared to the other. If infection were spread by droplets, this should have produced an appreciable difference in infection rates in the two groups of contacts. In fact, no such difference was found - an observation that we believe supports the hypothesis that infection is spread by droplet nuclei freely dispersed in the environment.

In the course of these latter experiments, we were able to measure the relative humidity (RH) inside the chamber during the contact period, and found that the mean RH varied from 40-70% in different experiments. We found that as the RH increased the transmission rate tended to decrease. These observations are in accord with those of Harper and Hemmes et al. who showed that airborne influenza virus survived less well at higher RH.

In the course of experiments in which contact was initiated in small cages, we found wide seasonal variations in the rates of transmitted infection. During the first summer we found that only one of 120 animals exposed to transmitted infection acquired the infection in experiments carried out between July and September. This was in

contrast to the preceding December and January, when 22% of the exposed contacts became infected. These differences were not associated with seasonal differences in peak virus titers or pulmonary lesions. Part of this striking difference seemed attributable to the influence of RH. These early experiments were conducted in animal rooms equipped with ordinary air conditioning and with steam and electrical heating during the winter. In the next year, however, we equipped two of the animal rooms so that a year-round temperature of 72 F and 50% RH was maintained. Under these conditions, seasonal variations in transmission were far less striking. Nevertheless, even with year-round controls of temperature and RH, transmission still occurred appreciably more frequently during winter as compared to summer months.

In this experimental model we have excluded the effects of variation in crowding and stress as a result of exposure to cold, factors which are so often incriminated in the seasonal variations in human respiratory infection. Only part of the seasonal fluctuations in our experimental model seem to be related to the effect of humidity, and we are left with variations for which we have no ready explanation. One theoretical possibility, however, is that the release of virus from the infector mice is influenced by their respiratory tract secretion, and that these secretions are, in turn, influenced (during the course of influenza virus infection) by the bacterial flora.

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## DISCUSSION

VFTF\*: In the older literature, with regard to plague, reference is made to the individual who had pneumonic plague with a very frothy-type sputum. I wonder if there is any change in the character of the mucus secretion in the transmitters vs. the non-transmitters. Perhaps it is the bacterial flora that might be involved in these mice in the winter-time -- it may be a lot different than in the summer-time.

Schulman: We have not made any observations relative to the changes in mucus secretion; that is a pretty difficult thing to study in the mouse other than by crude methods. We also think that mucus secretions might be very important. We have observed, however, that pulmonary lesions in the good transmitters are no greater than in the poor transmitters. What we did was to kill all of the infector mice at the end of the contact period, assay the virus in their lungs, and make estimations as to the extent of pulmonary lesions. There was no difference in the two groups. As to bacterial flora, we also feel that this may play a significant role, at least with regard to the seasonal variations. The mice were not born in our laboratory; they were purchased on the outside at 4 weeks of age, so that all sorts of seasonal variations were possible before they were actually delivered to us. We have not, thus far, been able to demonstrate any difference in bacterial flora, although we haven't studied it very extensively. One observer has shown in the past that the bacterial flora in the respiratory tract of mice does vary seasonally. In some experiments we have infected the infector mice with a gram-negative rod, Bordetella bronchiseptica, an organism which multiplied quite well in the mouse's respiratory tract without killing the animal. It produces small pulmonary lesions which subside. Three days later, we infected those animals with influenza and found that the doubly infected animals were much poorer transmitters of influenza than animals that had not previously been infected with the bacterial organism. This is in agreement with observations made years ago by Glover, that animals infected doubly with streptococci and influenza, transmitted the streptococcus much better, but were poorer transmitters of the influenza infection.

Shinefield: Did you have an opportunity to study factors relative to age and susceptibility of transmission; the capacity to transmit vs. susceptibility of the mice?

Schulman: Yes; I haven't presented those data. We found that older mice were more susceptible to acquiring the transmitted infection than younger mice. There was no difference with regard to age and the ability to transmit the infection, but there was in the susceptibility to acquiring the infection.

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\* VFTF = Voice from the floor.

## STUDIES ON EXPERIMENTAL RESPIRATORY INFECTIONS IN MONKEYS

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One principle I learned as a graduate student from Dr. N. Paul Hudson was that if you ever talk to a group, remember there is somebody in the audience who knows more than you do about the subject. I have always respected this, even when talking to a small county medical society or to students. It is obvious that I am now addressing a group highly skilled and accomplished in the field of aerobiology.

Our work with monkeys for some 25 yr has included studies of influenza and streptococcal infections, singly and in sequence<sup>(1-3)</sup>, nutritional studies with Vitamin M<sup>(4)</sup> and folic acid deficiencies<sup>(5,6)</sup>, effects of total body irradiation<sup>(7,8)</sup>, tolerance of chloramphenicol<sup>(9)</sup> and studies of histoplasma infections<sup>(10)</sup>. More recently, we have been impressed with the correlation between what we found in tularemia infections in humans as compared to what we observed in monkeys<sup>(11,12)</sup>. We like to work with monkeys because the clinical observations simulate pretty closely what one sees in a patient. Monkeys also lend themselves well to hematologic and serologic studies.

Studies we did some 25 yr ago on influenza in monkeys were done by intranasal instillation under so-called "light ether" anesthesia, and we lost a few monkeys to that "light anesthesia". The threshold was a bit precarious. They coughed and gagged if not adequately anesthetized and one wasn't certain of dose absorbed. Under these conditions we found that the PR8 strain of influenza virus did not produce

obvious illness. We did get a leukopenia and serologic evidence that we had introduced the agents. When we gave them a type C streptococcus, they likewise exhibited no obvious illness, but had a leukocytosis and serologic evidence of infection. When we gave both agents together, nothing unusual happened. Influenza virus followed by the streptococcus 2-3 weeks later, however, occasionally resulted in streptococcal septicemia. Since these studies were done by rather crude methods, we felt that now, with newer methods, we had the chance to re-evaluate some of these data as well as working with other organisms in anticipation of doing some work in monkeys on mixed infections. The studies we are going to describe today are of a preliminary nature wherein aerosol challenge was conducted in a Model 3 Henderson apparatus.

First of all, after giving them a hemolytic streptococcus aerosol containing about  $4.3 \times 10^6$  organisms, we found really nothing clinically happening in the animals. No illness, no fever, no positive blood culture, although there was a significant rise in antistreptolysin titer and a modest leukocytosis.

Going over to the pneumococcus with the aerosol, again we drew a blank giving  $1.0 \times 10^4$  with a Type 3 pneumococcus. No illness was observed and everything else was negative; blood cultures and even nasopharyngeal cultures were negative.

Then, using an 80/81 staphylococcus aerosol,  $1.5 \times 10^7$  organisms, again, clinically the animals looked well. No blood cultures were positive, but nasopharyngeal cultures were positive from 1-3 days. Thus, the gram-positive cocci studied did not produce any appreciable disease following aerosol challenge.

In preliminary studies with influenza, a mouse lung aerosol containing  $1.7 \times 10^5$  mouse  $LD_{50}$ 's of the PR8 strain was used. A very mild illness was observed in two of seven monkeys. All developed antibodies and showed the leukopenia observed when intranasal instillation was used.

Next, preliminary studies with Rickettsia rickettsii were performed, employing yolk sac material and containing 6-67 yolk sac  $LD_{50}$ 's per liter. These monkeys were exposed for 3 min, so potentially

they had an exposure of three times this amount. With the smallest dose, two of eight became ill and died. As the dose was increased, invariably most of them developed clinical illness, comparable to what you see in a human as far as Rocky Mountain Spotted Fever is concerned, with about a 75% mortality.

To date vaccination of monkeys prior to challenge afforded solid protection. Therapy with broad spectrum antibiotics usually resulted in complete recovery.

In summary, aerogenic challenge of monkeys with staphylococci, pneumococci and streptococci produced no obvious clinical symptoms. The influenza virus challenge resulted in minor symptoms of anorexia and listlessness for 2-4 days in two of seven monkeys. The aerosol challenge with *R. rickettsii* produced severe, often fatal disease similar to that observed following naturally-acquired disease. Vaccine afforded complete protection. Tetracycline therapy, started 48 hr after symptoms, was usually followed by complete recovery.

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## THE SUSCEPTIBILITY OF BIRDS TO TULAREMIA

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Naturally-occurring infections with Pasteurella tularensis have been observed in several species of gallinaceous birds. These include the bob-white quail<sup>(1)</sup>, the ringneck pheasant<sup>(2)</sup>, several species of grouse<sup>(3,4)</sup> and probably the sage hen<sup>(5)</sup>. Several of these same species have been reported as being relatively susceptible to infections with P. tularensis from experiments in which the organisms either were injected into the birds or were fed to them in contaminated grain or infected tissue<sup>(6,7,8)</sup>. However, the ringneck pheasant and the sage hen, which were shown to be naturally infected and were implicated in the transmission of tularemia to man<sup>(2)</sup>, have been reported as being relatively resistant to infection with P. tularensis<sup>(5,7)</sup>. Most of these early reports do not contain information on the dosage used to infect the animals or the number of organisms which were recovered from the blood and excreta of the infected birds. In the studies to be described with the mourning dove, Zenaidura macroura, the above information was obtained in order to define the susceptibility of this species to infection with P. tularensis and to obtain preliminary information concerning the potential of this species to transmit the disease.

The doves used in this study were trapped in the areas in and around Dugway, Utah. The ten doves available for experimentation were divided into two groups, one of which was to receive a respiratory dose of approximately  $10^5$  viable P. tularensis, strain Schu, cells and

the second group of  $10^1$  organisms. The sera from an additional twelve unexposed birds were also tested for the presence of antibodies against this organism. Prior to aerosol exposure, the ten birds were bled, and antibody titers against P. tularensis were determined using a tube agglutination test. Spray suspensions of the organism were prepared from 14-16 hr cultures grown in modified casein partial hydrolysate (MCPH) medium. Respiratory exposure was accomplished with aerosols continuously generated from these suspensions into a modified Reynier chamber by a University of Chicago Technical Laboratory (Chicago) atomizer. The numbers of organisms in the aerosols, and thereby the numbers of organisms inspired, were determined from air samples collected in the all-glass impinger and plated on peptic-digest starch (PDS) medium<sup>(9)</sup>. The day following aerosol exposure, and periodically thereafter, blood and cloacal swab samples were taken and processed to determine the numbers of P. tularensis organisms present. Samples of the cloacal contents were removed using cotton-tipped applicator sticks. The swabs containing the cloacal material then were placed in tubes of collecting fluid<sup>(10)</sup> which were shaken to suspend the organisms, serial 10-fold dilutions were prepared, and aliquots of these dilutions were plated on PDS medium to which streptomycin had been added. By using a streptomycin-resistant strain of P. tularensis and adding penicillin, streptomycin and actidione to the medium, the quantitative recovery of the organism was obtained in the presence of the indigenous microbial flora of the cloaca. Blood samples, removed by intravenous or intracardial puncture, were diluted and plated in a similar manner. Upon autopsy of the birds, the numbers of organisms in their tissues were quantitatively determined. The sensitivity of the assay system for the recovery of the P. tularensis cells from the blood was 50 orgs/ml, from the cloacal swab 20/swab, and from the tissues between 25-2500/g of tissue.

The sequence of events, in terms of bacteremia, excretion of organisms into the cloaca, mortality and serological response, during the first 14 days subsequent to a respiratory challenge dose of  $10^{5.18}$  organisms is shown graphically in Fig. 1. Data are not presented for birds exposed to the low dose ( $10^1$  organisms) since no signs of infection were obtained. Of the three birds tested prior to aerosol exposure, none showed a serum titer of 1:20 or greater; furthermore, titers of 1:20 or greater were not obtained from the other 18 dove serum

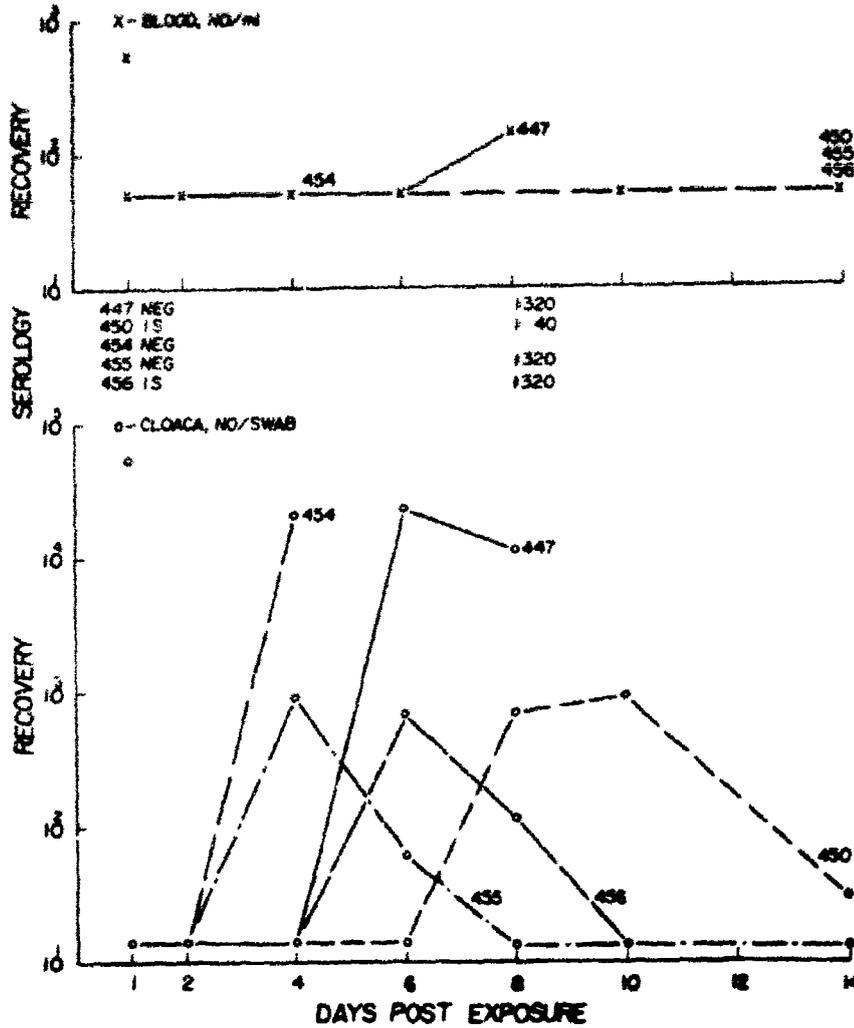


Fig. 1. Recovery of *Pasteurella tularensis* from the blood and cloacal contents and the serological response of mourning doves exposed to  $10^{5.18}$  organisms via the respiratory route. Baselines of 50 organisms/ml of blood and 20 organisms/cloacal swab were used since these represent the sensitivities of the assay systems used. IS = insufficient serum.

samples examined. However, P. tularensis agglutination titers of 1:40 or greater were seen in all the infected birds that survived to the 8th day post-exposure. Two of the doves excreted relatively large numbers of organisms into their cloacae and died between the 4th and 8th days. A detectable bacteremia was demonstrated in one of these two birds. The three remaining birds showed transient low levels of P. tularensis in the cloacae and survived the 14-day observation period. The recoveries of P. tularensis from the tissues of the birds which died of the disease are shown in Table 1.

TABLE 1. Recovery of Pasteurella tularensis from tissues of aerosol-exposed mourning doves

Tissue	Log recovery/mg tissue*
Lung	2.06
Spleen	3.65
Liver	2.86
Kidney	1.32
Proventriculus	2.53
Intestine	3.22
Cloaca	3.17

\* Geometric means of recoveries from two birds; dose  $10^{5.18}$ .

Thus, it appears to be quite certain that the mourning dove is relatively insusceptible to disease but not infection with P. tularensis, even when large numbers of organisms are introduced via the respiratory route. This relative insusceptibility does not appear to be acquired during the lifetime of the bird but rather is innate to the species. Thus, the mourning dove is similar to the pigeon, to which it is closely related taxonomically, in being relatively resistant to tularemia<sup>(7)</sup>.

The existence of the mourning dove for many generations in areas endemic for tularemia raised the possibility that the relative resistance of this species to infection with P. tularensis may have arisen through a process of selection in populations initially susceptible to

this disease. In this regard, information concerning the susceptibility of species having little, if any, contact with P. tularensis was considered of interest. Furthermore, the more frequent recovery of P. tularensis from the cloacal contents than from the blood of doves exposed to aerosol suggested that fecal transmission could be significant in the dissemination of tularemia by birds. The presence of P. tularensis has been reported in the feces and urine of naturally and experimentally infected mammals, frogs and turtles (11-16). However, there is little information concerning the levels of this organism in the feces of infected birds and no data on the numbers of P. tularensis discharged by highly susceptible avian species. The widespread distribution of tularemia made the investigation of land birds for these purposes somewhat less than desirable. However, the species of terns and noddies used in the following experiments were considered appropriate test subjects, on the assumption that these sea birds have had little, if any, contact with tularemia for numerous generations, and they could be gathered in large numbers at their nesting sites.

Preliminary investigations with the white-cap noddy, Anous tenuirostris, revealed this bird to be exquisitely susceptible to the lethal effects of tularemia when the birds were infected via the respiratory route. Of even greater significance was the observation that, in contrast to the situation with the mourning dove, extremely large numbers of viable P. tularensis organisms could be isolated from the blood, tissues and, especially, the cloacal contents of the infected birds. The possibility that in highly susceptible birds such as these fecal transmission, as well as arthropod vectors, could be significant in the dissemination of tularemia was studied further using two species of noddies, the common noddy (Anous stolidus) and the white-cap noddy (A. tenuirostris), and two species of terns, the white tern (Gygis alba) and the sooty tern (Sterna fuscata). These birds were examined for their susceptibility to respiratory exposure with varying doses of P. tularensis and to rechallenge with a relatively high respiratory dose. Information also was obtained concerning the sequence of events following challenge and rechallenge in terms of the levels of organisms in the blood, cloaca and various tissues, the immune response following challenge, the susceptibility of the birds to subcutaneous and oral infection and the transmission of the disease from respiratory-infected birds to susceptibles housed with them.

Following their capture and transport to these laboratories, the birds were maintained in wire cages, the floors of which were covered with sand. They were fed a diet of ground squid. With the exception of the white tern, which did very poorly in captivity and whose death following exposure to aerosols of P. tularensis could not be attributed definitely to the disease, the remaining species of birds did reasonably well during all but the terminal stages of their captivity (up to 6 months). During this latter period the birds were in poor condition, and some of the resulting deaths could not be attributed directly to tularemia. During this period, the experiments on the susceptibility of the birds to subcutaneous and oral infection and the investigation of transmission efficiencies were performed.

Respiratory infection was accomplished as described previously. Rechallenge of the surviving birds was performed between the 13th-15th days after the initial exposure. Subcutaneous inoculation of appropriate dilutions of the suspensions referred to previously was accomplished by the injection of the material into the breasts of the birds. A tube was used to introduce suspensions of the organism into the esophagi of the birds when oral infection was being studied.

To investigate secondary transmission of tularemia from infected to susceptible birds, two birds were exposed to appropriate numbers of P. tularensis via the respiratory route. These birds previously had been brushed to remove ectoparasites. Following respiratory exposure, the birds either were air washed in clean air or were placed in the sunshine for 1-2 hr to reduce the number of viable organisms which might have contaminated their feathers. On the 2nd day post-exposure, the susceptible birds were introduced into the room housing the two birds exposed to aerosol. The room was approximately 10 ft long by 8 ft wide by 8 ft high and was ventilated along its walls. In the first trial, the floor was left bare; in the second trial, the floor was covered with sand to a thickness of about 1/4 inch.

Blood samples for serological analysis were taken prior to challenge of the birds and again about the 17th and/or 14th day post-exposure. In those instances when rechallenge experiments were performed, surviving birds again were bled on the 7th and/or 14th day post-exposure. In general, the day following aerosol exposure and periodically thereafter blood and cloacal swab samples were taken and assayed as described previously.

In the first experiment on the respiratory exposure of white terns to varying doses of P. tularensis, no definitive information on their susceptibility could be obtained due to the inability to maintain the birds in a healthy condition while in captivity. However, viable organisms were recovered from the blood and/or the pooled cloacal swabs taken from birds which had received a challenge dose of as few as 200-250 organisms via the respiratory route. Furthermore, P. tularensis was recovered in the large majority of instances from the brains, hearts, kidneys, livers, lungs and spleens of the birds which died subsequent to the 3rd day post-challenge. Further experimentation with this species was discontinued because of the questionable nature of the results obtained.

Since significant numbers of viable P. tularensis cells were recovered from the cloacal contents of the white-cap noddy terns in the preliminary experiment referred to previously, assays were performed on the individual cloacal swabs. Also, there were indications, confirmed in later experiments, that the levels achieved on a given day were dependent on the number of organisms to which the bird had been exposed. The LD<sub>50</sub> estimate of 246 organisms with 95% confidence limits of 25-2130 is somewhat questionable since, even with the white-cap noddy which survived in captivity much better than the white tern, constant handling required to obtain the necessary samples may have contributed to their deaths. However, Table 2 shows a strong indication that the day of death of the birds was dose-dependent.

TABLE 2. Death of white-cap noddies following respiratory infection with Pasteurella tularensis as a function of dose and time

Resp dose	Number dead on day post-exposure										Dead/Total	
	5	6	7	8	9	10	11	12	13	>13		
10 <sup>5.41</sup>		1	3	1								5/5
10 <sup>3.70</sup>	1			2	1						1	4/5
10 <sup>2.70</sup>						1	1		1		2	3/5
10 <sup>2.00</sup>					1	1					3	2/5
10 <sup>1.04</sup>									2	1		3/3

LD<sub>50</sub> - 246 viable cells.

95% -CL -25 - 2130

To clarify the extent to which the white-cap noddy excretes P. tularensis into the cloaca, an additional experiment was performed in which the cloacal swabs were assayed individually. In view of the high degree of susceptibility of this species to respiratory exposure shown in the previous experiment, only two groups of birds were used and they both received relatively small respiratory doses of P. tularensis. The results of this experiment in terms of lethality and the recovery of organisms from the blood and the cloaca are shown in Table 3. Beginning about the 3rd day post-exposure and extending through the 13th day, extremely large numbers of organisms could be recovered from the cloacal contents of the individual birds. In some of the birds, the recovery of P. tularensis from the cloacal contents preceded, or was greater than, that from the blood. The estimated LD<sub>50</sub> was 9.92 organisms with confidence limits of 0.393-387. The organisms were recovered from the brains, kidneys, livers, lungs and spleens of almost all the birds which died from the 3rd through the 13th day. As described previously, because of the wide variation in response from one bird to another, the geometric rather than the arithmetic means of the recoveries from the blood and cloacal contents were used to define the response of the species to a given respiratory dose. Five birds survived the 13th day post-exposure observation period; data on the rechallenge of these birds will be presented later in this paper. The susceptibility of a common noddy to respiratory exposure with P. tularensis was investigated in the next experiment. Six groups of six birds each were exposed to graded doses, ranging from 10<sup>0.52</sup> - 10<sup>5.0</sup> organisms. The estimated LD<sub>50</sub> was 10.8 viable cells with 95% confidence limits of 6.8-17.3. The time of appearance of the organisms in the blood and the cloacal contents and the levels achieved (Table 4) as well as the time of death (Table 5) appeared to be dose dependent. The geometric means of the numbers of organisms recovered from the cloacal contents and the blood of the birds as a function of time and respiratory dose are shown in Fig. 2 and 3. Pasteurella tularensis was isolated from almost all the tissues of those birds which died between the 5th and 13th day post-exposures (Table 6).

TABLE 3. Lethal effects and blood and cloacal levels of Pasteurella tularensis following respiratory exposure of white-cap noddies

Resp dose	Day Post Exposure	No. of Surv.	BLOOD			CLOACAL CONTENTS		
			Log, Org./ml			Log, Org./swab		
			+ / total	Mean	range	+ / total	Mean	range
10 <sup>2.30</sup>	1	10	0/10			0/10		
	3	10	0/10			1/7*	0.63	4.38
	5	9	7/9	3.75	4.23-5.42	7/9	3.26	2.38-6.34
	7	6	6/6	4.18	1.88-7.40	6/6	4.28	2.20-6.78
	9	5	3/3*	4.17	3.59-4.78	5/5	4.88	4.08-6.04
	11	2	2/2	4.67	4.30-5.04	2/2	3.34	2.53-4.15
	13	1	1/1	3.58	3.58	1/1	4.52	4.52
10 <sup>1.53</sup>	1	12	0/12			0/12		
	3	12	0/12			1/7*	0.19	1.30
	5	11	3/11	1.17	1.70-6.46	3/11	0.43	1.30-1.85
	7	9	3/8*	1.58	2.76-5.41	4/9	1.93	3.36-5.60
	9	7	5/7	2.86	2.00-6.81	2/7	1.74	5.72-6.45
	11	5	2/5	1.13	2.81-2.83	2/5	1.23	2.53-6.19
	13	4	2/4	1.93	2.47-5.25	2/4	2.18	1.96-4.18

\* Unusable samples due to assay plates overgrown by normal flora or contaminants accounts for difference between the number of surviving birds and the "total" samples taken.

LD<sub>50</sub> = 9.92.

95% CL = .393-387.

TABLE 4. Lethal effects and blood and cloacal levels of Pasteurella tularensis following respiratory exposure of common noddies

Resp dose	Day Post Exposure	No. of Surv.	BLOOD Log, Org/ml			CLOACAL CONTENTS Log, Org/swab		
			+/total	mean	range	+/Total	mean	range
10 <sup>5.00</sup>	1	6	0/6			0/6		
	3	6	0/6			3/6	2.45	3.53-5.60
	5	5	4/5	3.44	2.45-5.93	5/5	4.66	3.15-6.81
	7	1	1/1	4.96	4.96	1/1	5.67	5.67
	9	0	0/0			0/0		
10 <sup>3.70</sup>	1	6	0/6			0/6		
	3	6	0/6			2/6	0.97	1.60-4.23
	5	6	5/6	3.23	1.76-7.26	6/6	4.68	2.90-7.00
	7	3	3/3	4.46	3.08-6.65	2/2	4.29	3.64-4.94
	9	1	0/0			1/1	6.30	6.30
10 <sup>2.60</sup>	1	6	0/6			0/6		
	3	6	0/6			3/6	1.62	1.95-4.61
	5	5	2/5	1.72	3.40-5.20	4/4	2.32	1.30-3.53
	7	3	3/3	4.54	3.15-6.53	3/3	3.98	1.85-6.45
	9	2	2/2	5.58	4.75-6.42	2/2	4.66	4.43-4.88
10 <sup>2.30</sup>	1	6	0/6			0/6		
	3	6	0/6			0/6		
	5	6	0/6			1/5	0.84	4.20
	7	6	5/6	3.39	2.48-6.42	1/5	0.79	3.95
	9	4	3/4	3.56	4.23-5.56	3/4	2.93	2.41-4.98
10 <sup>1.64</sup>	1	6	0/6			0/6		
	3	6	0/6			0/6		
	5	6	0/6			0/6		
	7	6	5/6	3.46	3.08-4.80	2/5	1.66	4.08-4.23
	9	4	3/4	4.52	5.20-6.46	3/4	3.70	3.48-6.23
10 <sup>0.52</sup>	1	5	0/5			0/5		
	3	5	0/5			0/5		
	5	4	0/4			0/3		
	7	4	3/4	2.19	2.30-4.18	3/4	2.50	2.18-4.04
	9	4	1/4	1.62	6.46	1/4	0.87	3.47
	11	3	0/3			0/3		
	13	3	0/3			0/3		

LD<sub>50</sub> = 10.8

95% CL = 6.78-17.3

TABLE 5. Death of common noddies following respiratory infection with Pasteurella tularensis as a function of dose and time

Resp Dose	Number dead on day post-exposure							Dead/Total
	2	4	6	8	10	12	>12	
10 <sup>5.00</sup>		1	4	1				6/5
10 <sup>3.70</sup>			3	2	1			6/6
10 <sup>2.60</sup>			2	1	1	1		5/5
10 <sup>2.30</sup>				2	2	1	1	5/6
10 <sup>1.64</sup>				2	3		1	5/6
10 <sup>0.52</sup>					1		3	1/4

TABLE 6. The isolation of Pasteurella tularensis from the tissues of common noddies exposed to aerosols of the organisms

Days to death	Sacrificed* or Died	<u>P. tularensis</u> recovered from				
		Brain	Kidney	Liver	Lung	Spleen
1	Sacrificed	0/2	0/2	0/2	1/2	1/2
2	Sacrificed	0/2	1/2	0/2	2/2	2/2
3	Sacrificed	0/2	1/2	1/2	2/2	1/2
4	Sacrificed <sup>§, ¶</sup>	0/2	2/2	1/2	2/2	2/2
5-13	Died	27/29	28/29	29/29	29/29	29/29

\* Data from a separate experiment in which the birds were exposed to 10<sup>5.0</sup> organisms.

§ Cloacal swabs and blood samples assayed on sacrifice day were negative.

¶ Log recovery from one cloacal swab was 2.08.

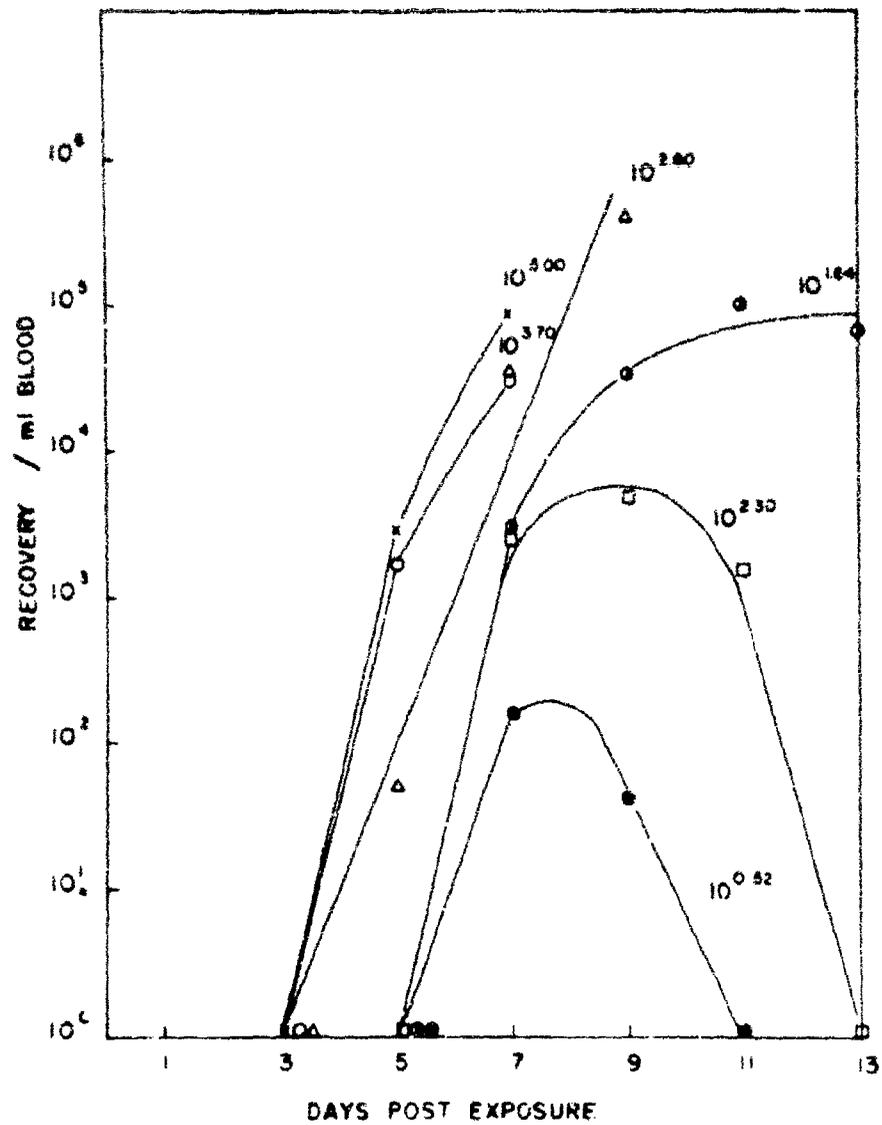


Fig. 2. Levels of Pasteurella tularensis recovered from the blood of common noddies following respiratory challenge with  $10^{0.52} - 10^{5.00}$  organisms.

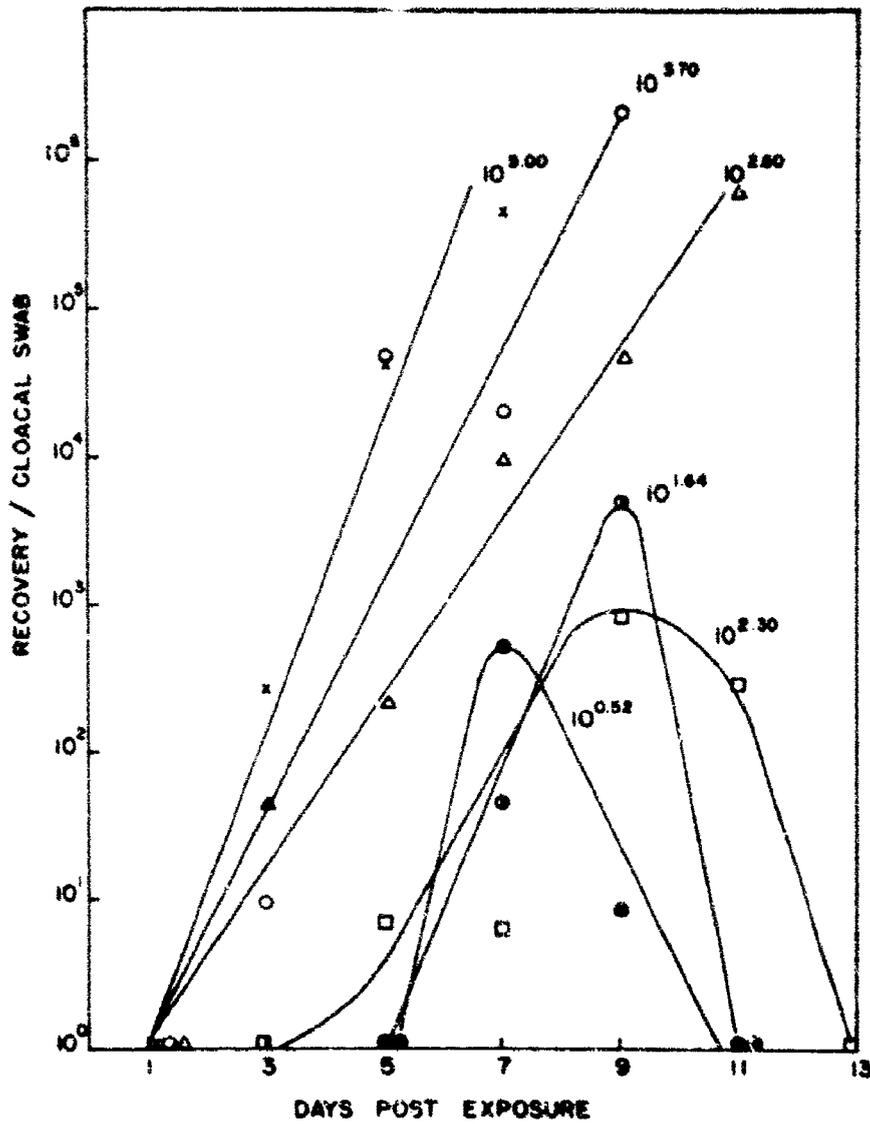


Fig. 3. Levels of Pasteurella tularensis recovered from the cloacal contents of common noddies following respiratory challenge with  $10^{0.52} - 10^{5.00}$  organisms.

Two sooty terns were available for experimentation. These were exposed to approximately  $10^5$  organisms via the respiratory route. One of the two birds did not have an antibody titer against P. tularensis at the time of exposure. It developed extremely high levels of the organism in both the blood and the cloacal contents and died within 5 days after respiratory challenge. The second bird, which had a significant anti-tularensis titer, did not develop a bacteremia, discharged only a few organisms into the cloaca and survived the post-exposure observation period (Fig. 4).

In order to obtain an insight into the sequence of events in the various tissues following respiratory exposure of the birds to P. tularensis, to obtain information as to the means by which the organisms are excreted into the cloaca and to explore the hazard to other birds or mammals upon the ingestion of the tissues of birds which have died of tularemia, an additional experiment was performed with six white-cap noddies exposed to approximately  $10^1$  organisms via the respiratory route. Upon the death of the birds, or at sacrifice, tissue homogenates were quantitatively examined for the number of P. tularensis organisms present. The quantitative recovery of the organisms from the various tissues as a function of time is shown in Fig. 5. It can be seen that massive involvement of the lung, liver and spleen occurred early in the disease process. This was followed by the isolation of large numbers of P. tularensis from the gall bladder and then the intestine. Organisms were recovered from the pancreas, kidney and finally the cloacal contents in large numbers subsequent to their isolation from the liver. Thus, it appeared that with small doses of P. tularensis introduced via the respiratory route, massive involvement of the liver and kidney occurred during the intermediate stages of the disease process and was probably the source for organisms which were discharged into the intestine and cloaca. Furthermore, from the results with white-cap noddy #176 (Table 6) chronic, persistent infections of the liver and kidney with concurrent discharge into and excretion from the cloaca is a distinct possibility.

As the first step towards determining the potential for vector transmission by the single species of soft-shelled ticks (Ornithodoros capensis) removed from terns and noddies, a trial was performed in which approximately  $10^{4.6}$  organisms were injected into common noddies via the subcutaneous route. The numbers of P. tularensis organisms

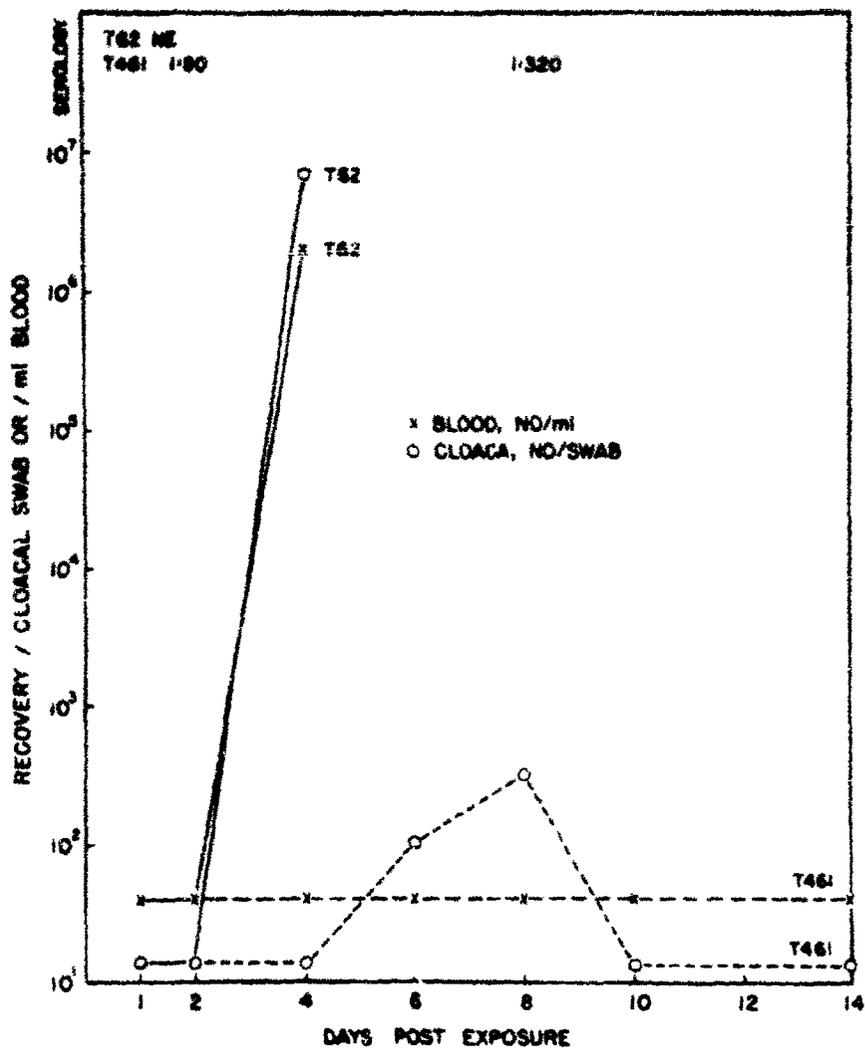


Fig. 4. Recovery of *Pasteurella tularensis* from the blood and cloacal contents and the serological response of sooty terns exposed to  $10^{5.18}$  organisms via the respiratory route.

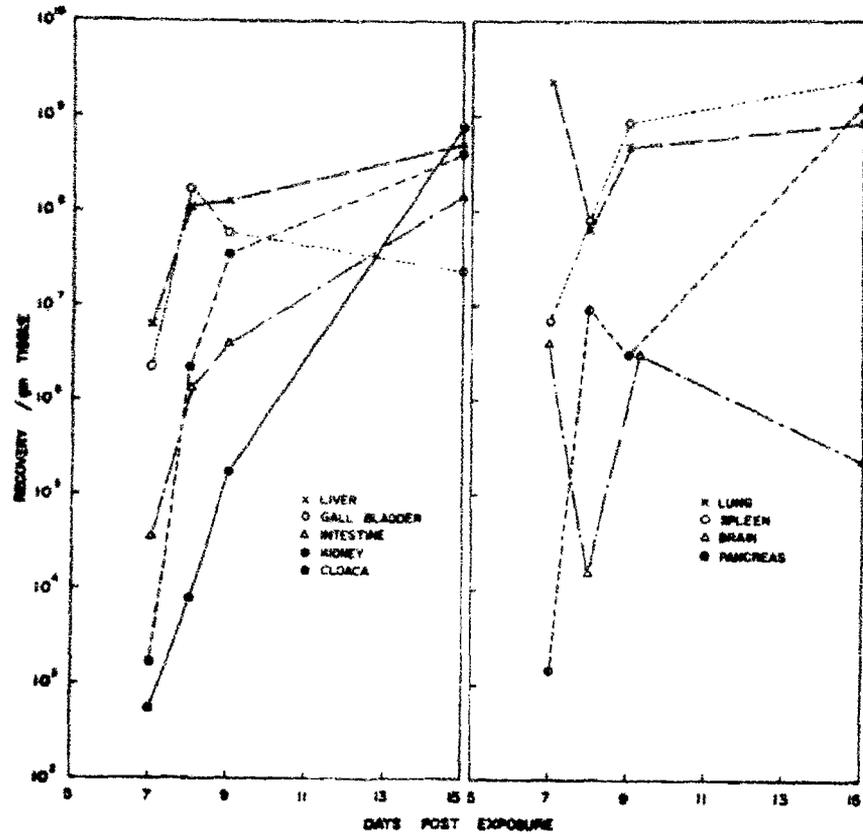


Fig. 5. Levels of *Pasteurella tularensis* recovered from the tissues of white-cap noddies exposed to  $10^1$  organisms via the respiratory route.

recovered from both the blood and the cloacal contents between the 4th and 7th days were comparable to those obtained from the birds infected via the respiratory route (Fig. 6). On autopsy, large numbers of *P. tularensis* organisms were recovered from the lungs, livers, kidneys, gall bladders, intestines and cloacae of all the injected birds.

The possibility of transmission from bird to bird by the ingestion of contaminated feces was examined initially by an experiment in which approximately  $10^{3.3}$  organisms were fed by tube to five common noddies. In only one instance were viable cells recovered from any of the birds; on the 10th day post-exposure a cloacal swab from one of the birds

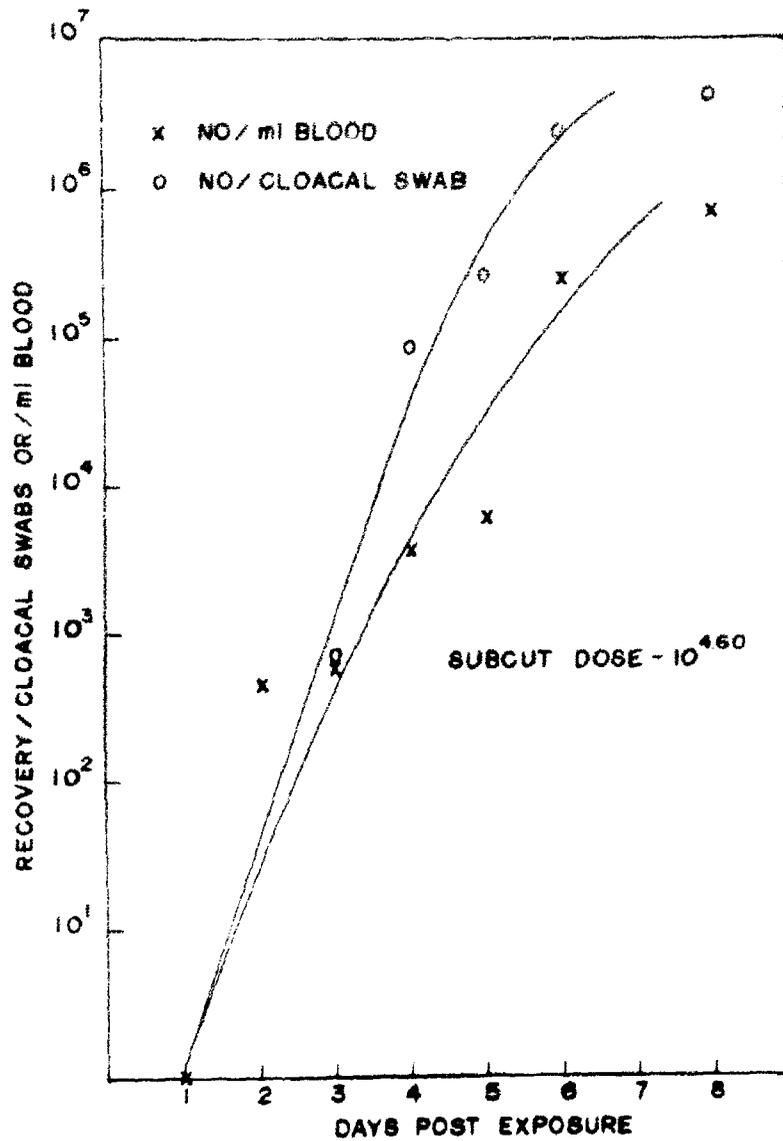


Fig. 6. Levels of Pasteurella tularensis recovered from the blood and cloacal contents of common noddies following a subcutaneous inoculation of  $10^{4.60}$  organisms.

yielded P. tularensis. No increase in antibody titer was observed in any of the birds which survived to the 8th day, nor could the organisms be recovered from the tissues of the birds when they died -- presumably due to causes other than tularemia. This subject was pursued further in two secondary transmission experiments in which "susceptible" birds were placed with aerosol-infected birds under the conditions described previously. In the first experiment the floor was bare; in the second, it was covered with sand. Although P. tularensis could not be recovered from the blood, cloacal contents or tissues of the two birds exposed to a respiratory dose of approximately ten organisms, evidence for secondary transmission to the susceptible birds housed with them was obtained on the 10th day post exposure when both the blood and cloacal contents of one bird and the blood sample from a second bird yielded significant numbers of P. tularensis organisms. The sporadic appearance of the organisms in the blood and cloacal content of the two susceptibles did not manifest itself further, either by a rise in antibody titer or in the persistence of the organisms in the tissues of the birds as seen from the autopsy data. Conversely, in the second experiment P. tularensis was recovered from the blood, cloacal contents and tissues of two birds exposed to approximately  $10^5$  organisms via the aerosol route. However, the organisms were not isolated from the blood or cloacal contents of the susceptible birds during the 15th day post-exposure observation period, nor was there any rise in antibody titer in either the susceptibles or the birds exposed via the respiratory route.

As noted previously, serological analysis and rechallenge procedures were performed to determine the immune status of the surviving birds. In the preliminary experiment with the white-cap noddies, those birds which had survived through the 14th day again were bled for sera and then were re-exposed to a challenge dose of  $10^{5.70}$  organisms. Data on individual birds treated in this manner (Table 7) indicate that some of the birds had demonstrable titers by the 8th day post-exposure, and that all the surviving birds had antibody titers against P. tularensis by the 15th day. With the exception of bird #174, these birds were relatively insusceptible to rechallenge in the sense that the organisms could not be recovered from the blood and, with one exception, from the cloacal contents. Two birds which developed transient bacteremias to the initial challenge, which developed antibody titers by the 15th day, and from which the organisms could not be

TABLE 7. Antibody titers against, and blood, cloacal and tissue levels of, *Pasteurella tularensis* prior to and following respiratory challenge of white-cap noddies

Days post-exposure	Log recovery per ml blood or per pool of cloacal swabs or serum agglutination titer			
	Bird #165		Bird #170	
	Blood	Sera	Blood	Sera
0				
2	C*	< 1:20	--	< 1:20
6	C	3.52	--	--
8	4.20	1:40	--	2.00
10	C			> 3.30
15	5.30			1:160
Respiratory dose 10 <sup>3.70</sup>				
15				
17	5.11			
20	3.30			
23				
28				
35				
Respiratory challenge 10 <sup>5.70</sup>				
Isolation of <i>P. tularensis</i> from tissues of dead birds				
Liver		+		
Lung		C		C
Spleen		C		C
Kidney		C		C
Brain		C		+

\* C = Plates overgrown by contaminants or indigenous flora.

... continued ...

TABLE 7. (CONTINUED)

Days post-exposure	Log recovery per ml blood or per pool of cloacal swabs or serum agglutination titer											
	Pool of cloacal swabs		Bird #113		Bird #159 <sup>†</sup>		Bird #161		Bird #162		Bird #163 <sup>††</sup>	
	Blood	Sera	Blood	Sera	Blood	Sera	Blood	Sera	Blood	Sera	Blood	Sera
0		< 1:20		< 1:20		< 1:20		< 1:20		< 1:20		< 1:20
2	C	-		-		-		-		-		-
6	C	1.70		-		-		-		-		-
8	C	4.49	< 1:20	2.60	1:20	3.20	< 1:20	3.68	< 1:20	< 1:20	< 1:20	< 1:20
10	> 4.20	4.20		-		4.00						> 3.30
15	-			-	1:160							1:20
Respiratory dose 10 <sup>2.70</sup>												
15												
17												
20												
23												
28												
35												1:160
Respiratory rechallenge 10 <sup>5.70</sup>												
15												
17												
20												
23												
28												
35												
Isolation of <i>P. tularensis</i> from tissues of dead birds												
Liver		+										
Lung		+										
Spleen		+										
Kidney		+										
Brain		-										
† Died 102nd day post-exposure. †† Died 83rd day post-exposure.												

continued

TABLE 7 (CONTINUED)

Days post-exposure	Log recovery per ml blood or per pool of cloacal swabs or serum agglutination titer											
	Pool of cloacal swabs		Bird #173		Bird #174		Bird #176*		Bird #180		Bird #193	
	Blood	Sera	Blood	Sera	Blood	Sera	Blood	Sera	Blood	Sera	Blood	Sera
0			< 1:20		< 1:20			< 1:20		< 1:20		
2	C	-		2.00	-							
6	C	2.40			C					2.30		
8	C	5.00	1:20			2.95		< 1:20		3.88		
10	> 4.60	> 6.60				4.95				4.23		
15	5.30							1:160		C		
Respiratory dose 10 <sup>2.00</sup>												
15												
Respiratory rechallenge 10 <sup>3.70</sup>												
15												
17	6.30											
20	5.15											
23	5.30			3.98				1:160				
28	2.72			4.60								
35	4.77											
Isolation of <i>P. tularensis</i> from tissues of dead birds												
Liver		+										+
Lung		+										+
Spleen		+										+
Kidney		+										+
Brain		+										+

\* At sacrifice, log recovery from about 100 mg tissue as follows: Liver, 3.51; gall bladder, 2.00; blood, 0.0; kidney, 3.04; duodenum, 0.0; intestine above ureters, 3.18; cloaca, 4.46.

† Organisms recovered from tissue smear.

recovered from the blood or cloacal contents, survived the entire re-challenge observation period. When they died on the 83rd and 102nd days post-challenge (presumably due to prolonged captivity), their tissues were negative for the presence of the organism. Bird #176 also showed a transient bacteremia between the 8th and 10th days post-exposure and developed significant antibody titers by the 15th day. It was insusceptible to rechallenge both in terms of surviving the 18th day post-rechallenge observation period and had no detectable bacteremia during that period. However, both this bird and bird #165 excreted P. tularensis into the cloaca. On the 35th day post-exposure, when bird #176 was sacrificed, significant numbers of the organisms were recovered from the liver, gall bladder, kidneys, intestine and cloaca. The brain was negative and very few organisms could be recovered from the lungs, heart and spleen. From these results it would appear that this bird was suffering from chronic infections of the liver and the kidneys because P. tularensis was being discharged into the GI tract from these organs.

Large numbers of P. tularensis were recovered from the blood and cloacal contents of bird #384 which survived the second experiment with the white-cap noddies and which had a significant antibody titer by the 13th day (Table 8). The organisms persisted in the blood and the cloacal content following rechallenge at the 14th day post-exposure, and the bird expired the following week, at which time P. tularensis was isolated from all the tissues tested. P. tularensis was recovered from the blood and cloacal contents of birds #394 and #395 late in the post-exposure observation period. They died before any data could be obtained concerning the presence of the organisms in both these areas following respiratory rechallenge with  $10^{3.40}$  organism. Neither could the organisms be recovered from the blood or the cloacal contents of the remaining two birds during the 13th day post-exposure observation period, nor did these birds develop significant antibody titers by the 13th day. One of these birds (#405) did develop a significant bacteremia following rechallenge. The organisms could be isolated from the tissues of both these birds when they died following respiratory rechallenge.

Data from five common noddies which survived the 13th day post-exposure observation period are shown in Table 9. Three of the birds, #344, #340 and #357, exhibited only transient or no bacteremias, or



TABLE 9. Antibody titer against, and blood, cloacal and tissue levels of, *Pasteurella tularensis* prior to and following respiratory challenge of common noddies

Days Post-exposure	Log recovery or agglutination titer in																				
	Respiratory dose 10 <sup>2.30</sup>				Respiratory dose 10 <sup>1.64</sup>				Respiratory dose 10 <sup>0.52</sup>												
	Bird #344	BI*	CI†	Sera	Bird #340	BI	CI	Sera	Bird #352	BI	CI	Sera	Bird #357	BI	CI	Sera	Bird #359	BI	CI	Sera	
1	-	-	-	< 1:20	-	-	-	< 1:20	-	-	-	< 1:20	-	-	-	< 1:20	-	-	-	1:160	
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
7	-	-	-	-	-	-	-	-	2.30	4.04	-	-	4.18	3.48	-	-	-	-	-	-	
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
11	-	-	-	< 1:20	5.10	-	-	< 1:20	-	-	-	-	-	-	-	-	-	-	-	-	
13	-	-	-	< 1:20	4.80	-	-	< 1:20	-	-	-	1:160	-	-	-	1:20	-	-	-	1:160	
Respiratory challenge 10 <sup>3.40</sup>																					
14																					
17	2.26	C			4.43	3.46			-	-											
19	3.76	C			5.28	4.78			-	-											
22	1.0	3.15			ND	5.11															
24	2.94	3.48			5.11	5.28															
26	2.95	3.91																			
Isolation of <i>P. tularensis</i> from tissues of dead birds																					
Liver	+																				+
Lung	+																				+
Spleen	+																				+
Kidney	+																				+
Brain	+																				+
Pancreas	+																				+

\* BI = per ml blood.

† CI = per cloacal swab.

discharge of the organisms into the cloaca during the period and did not have significant antibody titers by the 13th day following exposure. When they were rechallenged with  $10^{3.40}$  organisms, P. tularensis was recovered from the blood and cloacal contents of all three birds and from all their tissues on autopsy. Two birds, #352 and #359, had significant anti-tularensis antibody titers prior to initial exposure. The organism was absent or appeared sporadically in the blood and/or cloaca during the 13th day post-exposure observation period; it was not recovered from either bird following rechallenge. In contrast to those of the remaining three birds, the tissues from birds #352 and #359 were not uniformly positive for the presence of P. tularensis. In fact, it would appear that organisms were being cleared from the tissues when the birds died -- presumably due to natural causes.

The sooty tern (#461), which had a prechallenge antibody titer and survived a high challenge dose (Fig. 4), subsequently was rechallenged with approximately three organisms. P. tularensis was not recovered from the blood of this bird but was isolated from the cloacal contents on one occasion. A white-cap noddy (#505), which had a questionable pre-exposure antibody titer against P. tularensis, also was exposed at this time. It too survived the observation period; on only one occasion were the organisms recovered from the blood and cloaca. No rise in antibody titer was detected in either bird. These two birds again were challenged with a respiratory dose of  $10^{6.58}$  organisms. Bird #505 was infected and died of the disease. The sooty tern (#461) survived the challenge dose, did not excrete organisms into the blood or cloacal contents, and showed a rise in antibody titer when tested on the 7th post-exposure day. It was again challenged with a subcutaneous dose of  $10^5$  P. tularensis cells. The results of this and previous challenge experiments with birds #461 and #505 are shown in Table 10.

The relative insusceptibility of the mourning dove to tularemia referred to previously can be seen more clearly in the light of the later observations with the highly susceptible terns and noddies. While these findings do lend some weight to the hypothesis that prolonged habitation in areas endemic for tularemia selects for avian species and/or populations more innately resistant to this disease, other factors such as diet may be more determining. Many more species of land birds, especially gallinaceous game birds and sea birds, will

TABLE 10. Respiratory and subcutaneous rechallenge of immune birds

Days Post-exposure	Dose	Route	Log recovery or agglutination titer in							
			Bird #505			Bird #461				
			Bl $\diamond$	Cl $\ast$	Sera	Bl	Cl	Sera		
2	$10^{0.41}$	Resp			1:10 $\ast$			1:80		
3			-	-		-	-			
4			-	-		-	-			
5			-	-		-	-			
6			NS	-		NS	-			
7			-	-		-	-			
8			-	-		-	-			
10			4.05	4.58		2.84	-			
12			-	-	1:20	-	-	1:80		
1			$10^{6.58}$	Resp			1:20			1:80
2					-	-		-	-	
3					-	-		-	-	
4	2.88	2.70				-	-			
5	-	-				-	-			
6	3.30	3.32				-	-			
7	dead $\ddagger$					-	-			
14	-	-				-	-	> 1:320		
1	$10^{5.0}$	Subcut				All samples	> 1:320			
14						negative 1-14 days	> 1:320			

$\ast$  Weak reaction

$\ddagger$  On autopsy large numbers of *P. tularensis* isolated from lung, liver, kidney, spleen, cloaca and gall bladder.

$\diamond$  Bl = blood/ml

$\ast$  Cl = cloacal swab.

have to be studied similarly before any judgement can be made on this subject. Investigations with the bob-white quail are now in progress; and, from preliminary data, it appears that they resemble the doves by being relatively resistant to tularemia. It is not inconceivable that the accumulation of quantitative data on the comparative susceptibilities of avian species to tularemia when correlated to their phylogenetic histories would give an insight into the history of this disease

The excretion of the organisms into the cloacal contents is not surprising because organisms have been isolated from the feces and/or urine of hares, rabbits, guinea pigs, buffaloes, turtles, and frogs

experimentally or naturally infected with P. tularensis. But the large numbers of organisms regularly recovered from terns and noddies was unusual. Even in the relatively resistant mourning dove exposed to large numbers of organisms via the respiratory route, P. tularensis was recovered from the blood in only one instance, but was recovered from the cloacal contents of all five of the birds tested.

Although the potential for fecal transmission of tularemia was realized in one of the three transmission experiments performed, further experimentation will be necessary to establish the probabilities for these events. In these studies, the ratios of infective to susceptible birds will be varied. The successful transmission of tularemia in only one of three attempts is understandable, since the ratio of infective to susceptible birds was low in these experiments and since the two methods by which fecal transmission could have been accomplished from bird to bird, inhalation and ingestion probably are marginally effective. Even though large numbers of organisms were present in the fecal material, as demonstrated in these studies, and despite the fact that terns and noddies were susceptible to artificially induced infection when P. tularensis was introduced via the aerosol route, before respiratory infection could have taken place in nature, the dry fecal material must have been dispersed in some way as particulates small enough to permit penetration to the lower reaches of the respiratory tree. In addition, the efficiency of fecal transmission would depend upon the length of time P. tularensis survived in bird feces. Preliminary information indicates that, in contrast to the long (26 days) survival times of this organism in tick feces<sup>(11)</sup>, P. tularensis cannot be recovered from bird feces after 3 days. Infection of susceptible birds by the ingestion of fecal material containing viable P. tularensis organisms is limited by the number of organisms required. Saval'eva<sup>(17)</sup>, working with mammals, demonstrated that rather large numbers of cells ( $10^4$ - $10^6$ ) are required to produce tularemia when the organisms are introduced into the gastro-intestinal tract by the ingestion of tissue from animals dead of this disease or by tube into the stomach. The results described herein with the highly susceptible noddies are in agreement with these findings.

Although arthropod vectors must be considered the major means by which tularemia is maintained within a given geographic area and spread peripherally to adjacent locales, it is interesting to speculate on the

role fecal transmission by birds may have played in the world-wide distribution of this disease. Compared to the direct transmission of disease by arthropod vectors because of the drive of the arthropod to obtain a blood meal, fecal transmission must be considered inefficient. However, with "virgin" populations of highly susceptible migratory birds from which large numbers of P. tularensis organisms could be intermittently discharged over a wide geographic area, the probability of transmission and dissemination should increase appreciably. Furthermore, with fecal transmission, problems of host-vector-parasite specificity and the requirement for "lighting" of the birds are eliminated.

While a correlation of antibody titer to immune status is beyond the scope of the present investigation, it does appear that, in general, recovery from an infection, as demonstrated by the presence of an antibody titer against P. tularensis, renders the bird at least partially resistant to subsequent challenge either by the respiratory or the subcutaneous route. In this case, there may or may not be a temporary carrier state, not unlike that in typhoid fever, in which the organisms are discharged into the feces.

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#### DISCUSSION

VFIF\*: Did you take any second swabs from birds after they were exposed?

Cabelli: Following exposure, the birds were "air-washed". In the first series of experiment, concurrent with the taking of the blood and cloacal samples, the birds were placed in a chamber and "air-washed" for some 30 min. We could not recover P. tularensis from any of the impinger and Andersen samples collected from the effluent air during this period. Maybe our technique was not good enough. Maybe we didn't sample long enough.

VFIF: Did you use feather swabs?

Cabelli: No.

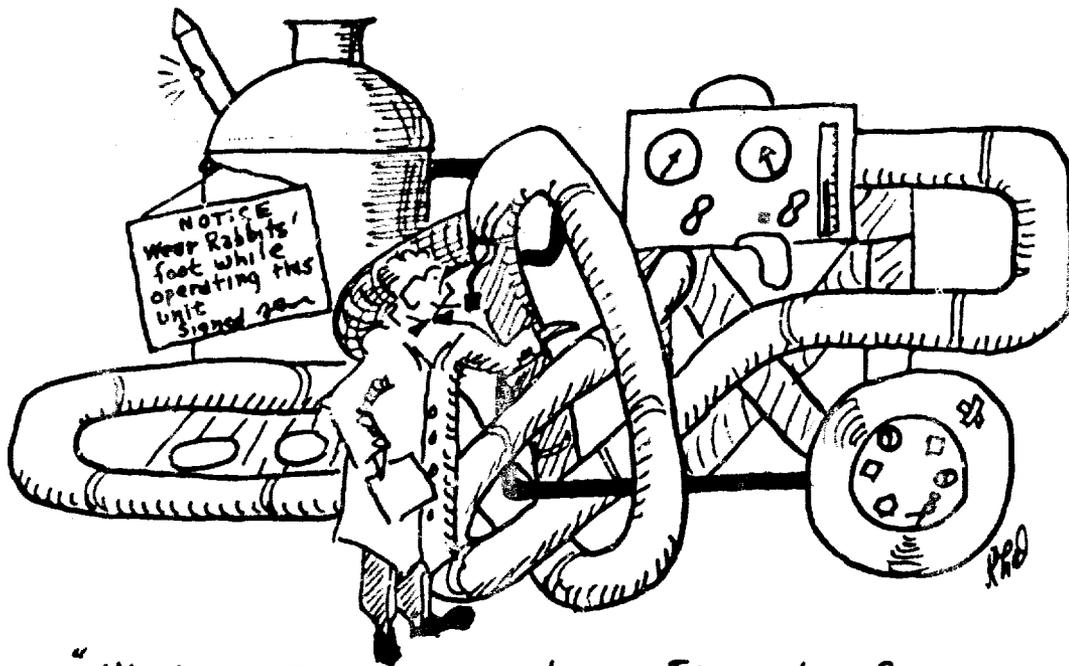
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\* VFIF = Voice from the floor.

## EXPERIMENTAL AND TECHNICAL SECTION

John D. Morton, Moderator

Without ideas leading to the creation, proper understanding and application of new tools, progress in research diminishes to only speculative endeavor. In this section new apparatus, new techniques and new approaches are discussed and reviewed.



"We built this one ourselves. It works fine, but there's a technician lost in it - somewhere."

## REMARKS FROM THE CHAIR, A CRITIQUE

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I should like to start by saying how very pleased I am to be here. When I started thinking about the invitation, I decided this was the perfect opportunity to give you the benefit of my critical comments on all sorts of things! After what Mr. Wolfe said this morning, however, I shall have to cut it short. I dare not go into more than 10 minutes or I shan't hear the last of it for years.

It is time, I believe, to stand back from the aerobiology scene, to take a critical look at our efforts and to consolidate our gains. This is particularly apropos because at Melpar we are discussing extending our aerobiology facilities; in particular we are considering the use of apparatus and techniques developed by many of you learned gentlemen now in the audience. You may be able to tell us whether we are right. Perhaps we should re-evaluate the techniques and methods now being used. I would like to review briefly the sort of thoughts going through my mind regarding such an evaluation.

In a typical experiment as you commonly do it, you take a stock culture, maintained perhaps for years on artificial medium, grow it in an artificial medium, and harvest the bacterial suspension. You take this resting suspension, and spray it from something like a Collison atomizer, which gives you a substantially single-celled aerosol. You then proceed to hold the cloud -- in something like Goldberg's brilliant invention of the rotating drum -- in the dark, and probably in

very closely controlled constant temperature and relative humidity conditions; quite likely it stays there for many hours. Then you take a sample from this by sonic impingement into an artificial fluid, which is probably at room temperature or below, and is unlikely to be of "physiological" tonicity. Finally, you take this impinger sample and place it on an artificial medium; you count the colonies and you call this a viability or viable count.

Well, now; you know very well what I am about to say here -- this is not a "natural" state. But we might as well remind ourselves of it in relationship both to normal transmission of airborne disease and also to what one might call "defense" aspects.

Consider first artificial maintenance and culture medium: these are relevant to the laboratory picture but not to natural transmission, and they are important because both can affect the response to environment and the calculated infectivity of virulence of a bacterium.

Consider the resting suspension: this is interesting from two points of view. Firstly, it has been shown (several people in this room have done it) that actively dividing log-phase cells are far more susceptible to environmental stress than resting cells; and, secondly, there is some evidence -- I think this is perhaps more doubtful -- that cells in an actively dividing state may be more capable of resting, cells of initiating growth in the host animal; i.e. they have a shorter lag-phase, and are likely therefore to display higher infectivity. Here again, the "standardized" experiment is relevant only to the "defense" picture and not to natural transmission from an infected host.

The single-cell cloud is typical of neither side. As for constant temperature and humidity -- I am looking forward very much indeed to hearing Drs. Hatch and Dimmick on the subject later because, of course, there is conflicting evidence about what happens when you play about with environmental conditions of an aerosol. But, whatever we find in experiments, the condition of constant temperature and humidity is typical neither of natural transmission nor of the field.

Now we come to the most critical phase -- the sampling. In normal impinger sampling you give your aerosol a considerable badgering. You accelerate it to sonic velocity -- even that process is violent enough to make, as some people know, an audible noise with a particle a few microns in size -- so there must be some stress on it. You

instantly stop it again with a most horrid bash, and you drench it with fluid which is of the "wrong" temperature and probably the wrong tonicity; and this is all pretty tough on it. And this, I think, makes the results remarkably irrelevant to either of the above circumstances we were considering, especially when you are really interested in what will happen to a bacterium that is deposited gently onto the body-warm surface of the lung and into a "physiological" environment.

Finally, viability -- well, we know that can catch us either way. We have had reference already today to experiments with aged tularensis clouds -- being that the count can give you an exaggerated idea of their infective capacities: in other words, you require more viable cells to produce the same effect than you do with the unaged aerosol. Now, you can also observe the other effect, the viable count giving you an apparently increased infectivity. Possibly George Harper is the only one here to remember work we did about 10 years ago when we were disseminating a culture, and we gave it a very violent shock. Animals and impingers were exposed to the same cloud, and the count in the impingers indicated an effective dose about 1/10 of that ever observed anywhere else. But obviously there was something wrong with this; it seemed perfectly plain that the brutal treatment in the impinger was killing off about 9 out of 10 of the cells (which could not have been more infective than they ever were before). So we see that plating of impinger samples may give viable counts that are irrelevant to either "defense" or natural transmission.

In summary, a typical aerosol experiment of the kind I outlined gets about half marks for relevance to defense problems and fails miserably with respect to natural transmission of disease (Table 1).

Now I'll leave it at that and touch on just one other thing that I particularly wanted to say. Here I am a little more diffident because I am not in any sense claiming the competence of a medical man. In studies on the airborne transmission of disease, I would like to see a little more direct evidence that airborne organisms are involved. We keep hearing indirect evidence of airborne transmission, for example, that bacteria survive better in the cold when airborne -- that there is more transmission of disease in the cold -- therefore they are airborne. All kinds of inferential and indirect evidence is there. I would like to see more direct evidence, and I think that we need somebody -- in fact, a body like this -- to lay down something rather

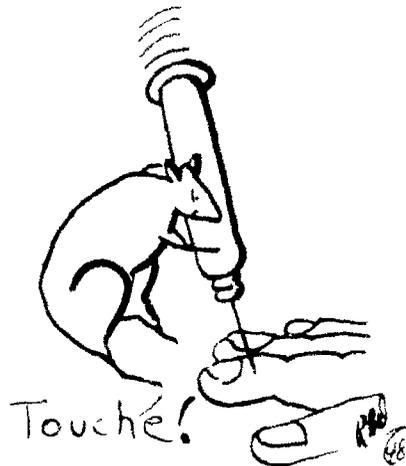
analogous to Koch's postulates for demonstration of airborne transmission of disease. For example\*: (a) you must demonstrate the presence of airborne viable infective organisms; (b) you must measure concentrations and particle sizes; (c) you must demonstrate experimentally that concentrations and particles of this sort can cause infection; and (d) I think we finally ought to show directly, and not by inference, where they have come from, whether from the respiratory tract, or skin, or feces, or what have you.

In designing future experiments I believe we should give these problems our utmost attention.

TABLE 1. Relationship of microbiological techniques to useful situations.

Condition	Relevance to		
	Medicine	Defense	Theory
Transmitted on artificial medium in vitro	No	Yes	Yes
Grown on artificial medium	No	Yes	Yes
Stored in resting suspension	No	Yes	Yes
Sprayed from artificial menstruum	No	Yes	Yes
Held in the dark	1/2	1/2	Yes
Held at controlled temperature and relative humidity	No	No	Yes
Held in monodispersed cloud	No	No	Yes
Samples impinged violently	No	No	No
In fluid of "unnatural" osmotic strength	No	No	Yes
At "unnatural" temperature	No	No	Yes
Samples plated on artificial medium for "viability" (not infective power)	No	No	Yes

\* Editor's Note: These proofs later became known as "Morton's Postulates" during numerous spirited, off-the-floor discussions.



## PHYSICOCHEMICAL PROPERTIES OF BACTERIAL CELL WALLS

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The ability of a bacterium to survive drastic environmental changes, such as drying in the aerosolized state and rehydrating again in a collection liquid, is likely to be determined in part by the peripheral permeability barriers which it possesses. The crucial structure in this regard is the thin cytoplasmic membrane enclosing, and in intimate contact with, the cytoplasm of the cell. By exercising a highly selective control on the passage of materials into and out of the cytoplasm, this membrane maintains the internal environment necessary for the life of the cell; damage to it by mechanical or chemical means allows internal diffusible materials to escape and the cell dies. Distinct from the cytoplasmic membrane, but necessary under normal circumstances to its integrity, is the overlying, thicker, more robust cell wall. This relatively rigid structure maintains the characteristic shape of the cell and prevents lysis which would ordinarily occur as the result of osmotic influx of water (Mitchell and Moyle, 1956). In addition to its mechanical role the cell wall may also have selective permeability properties which come into play under conditions of interest to aerobiologists (Record, Taylor, and Miller, 1962). It is the purpose of this paper to summarize some of the physical and chemical information available on bacterial cell walls which may be useful in discussions of the survival of airborne microorganisms.

## PHYSICAL DESCRIPTION

The cell wall can be differentiated from the underlying cytoplasmic membrane and from externally adhering slime and capsular components by biological stains, plasmolysis (Knaysi, 1951), differences in chemical composition, by electron microscope examination, and by its isolation as a coherent structure (Salton, 1961). The wall constitutes about 20% of the dry weight of the cell but the percentage depends on the growth phase and the nutritional state of the cell (Shockman, Kolb, and Trenchies, 1958). The thickness of the bacterial cell wall is about 20  $\mu$ m for Gram-positive organisms and somewhat less, 10-15  $\mu$ m for those which are Gram-negative (Salton, 1961). These estimates are made from electron micrographs of air-dried and shadowed, isolated cell walls or from thin sections of fixed, dehydrated, embedded intact bacteria. In view of the shrinking and deformation which are likely during the preparation of either of these kinds of specimens for electron microscopy, it would not be surprising if the functional thickness of normal hydrated cell walls exceeded these estimates by a significant amount (Murray, 1960).

Convincing evidence for macromolecular heterogeneity in bacterial cell walls comes from electron microscope investigations. In general the walls of Gram-positive bacteria have appeared less complex than those of Gram-negative bacteria. Air-dried, shadowed preparations frequently appear homogeneous in structure, but it is unjustifiable to conclude that this is the case without employing other techniques of specimen preparation. Thus isolated walls of the Gram-positive organisms, Staphylococcus aureus and Bacillus megaterium, may appear nearly structureless in shadowed preparations (Dawson, 1949; Salton, Kolb, and Trenchies, 1958), but in thick sections the cell walls of both organisms show obvious layers (Piekariski and Giesbrecht, 1956; Saganuma, 1961). The "negative staining" technique, developed by Brenner and Horne (1959), appears to have great value in revealing fine structure, judging from the complex hexagonal array of interconnected aggregates shown in a recent electron micrograph of the cell wall of a micrococcus organism (Horne, 1961).

In Gram-negative bacteria a variety of fine structure has been seen. In shadowed specimens a regular array of spherical particles, roughly 100  $\text{\AA}$  in diameter, has been reported by Houwink (1953), and by Salton and his co-workers (1961, 1954). Layered structures are

evident in some cases and pictures of thin sections confirm this. Kellenberger and Ryter (1958) concluded from their work that the cell wall of Escherichia coli and possibly other Gram-negative organisms is composed of three layers, an electron-transparent layer sandwiched between two electron-dense layers, each being 20-30 Å thick. Ogura (1963), using high-resolution techniques, observed that the outer layer of the cell wall of E. coli is really a network of interwoven fibers embedded in the same matrix material that constitutes the middle layer.

#### CHEMICAL COMPOSITION

The investigation of the chemical composition and the biosynthesis of bacterial cell walls has been very actively pursued in a number of laboratories in recent years. Several excellent reviews of this aspect of cell wall properties have appeared and no more than a cursory treatment is attempted here (Perkins, 1963; Salton, 1961).

The chemistry of bacterial cell walls was placed on a solid footing by the development of mechanical methods of preparing isolated walls and by the use of the electron microscope to demonstrate homogeneous preparations free of cytoplasmic or other contaminants (Dawson, 1949; Salton and Horne, 1951). Cell walls are now usually prepared by disintegrating cells in suspension by vigorous agitation with small glass beads, followed by filtration to remove the beads, and repeated differential or density-gradient centrifugation to remove unbroken cells and cytoplasmic constituents. After additional washings, the cell wall preparation is checked for homogeneity by examining preparations with the electron microscope. Although such preparations are usually considered representative of the structure and composition of the wall of the intact cell, it is apparent from a recent paper of Weidel, Frank, and Leutgeb (1963) that autolytic enzymes may remove certain components of the wall during the preparative procedure unless precautions are taken to inactivate them.

Hydrolysis products of the walls of Gram-positive bacteria invariably include three or four principal amino acids and two amino sugars, glucosamine and muramic acid. These building blocks are combined in an insoluble peptide-substituted amino polysaccharide and the resulting "mucopeptide" or "mucopolymer" appears to constitute the rigid component common to all Gram-positive bacteria. The mucopolymer makes up almost the entire wall of some organisms such as Micrococcus lysodeikticus

but most Gram-positive species also contain polysaccharides and teichoic acids in their walls. The work of Baddiley and his colleagues has established the teichoic acids to be ribitol or glycerol phosphodiester polymers with alanine and hexoses or amino sugars as substituents (Baddiley, 1962). These compounds are extractable from cell walls by mild procedures, which suggested to Baddiley that they are held by ionic and hydrogen bonds to the mucopeptide framework; but recent work by Ghuyssen and Strominger (1963) shows that in *S. aureus*, at least, the bonds are covalent. The polysaccharide constituent is also extractable from cell walls but its mode of attachment to the mucopolymer or other component is not known with certainty.

In accord with their more intricate physical structure the cell walls of Gram-negative bacteria are markedly more complex in chemical composition than those of Gram-positive organisms. Hydrolysates of the cell walls yield a variety of sugars and amino sugars and the full range of amino acids usually encountered in typical proteins. In addition, substantial amounts of lipid (as much as 22%) are also present. Ample evidence is now available to show that a mucopolymer similar in composition to that found in Gram-positive bacteria is present also in all Gram-negative bacteria but in reduced amounts (Mandelstam, 1962; Work, 1961). It is remarkable that the same type of macromolecule, unique to bacteria, should be present in a wide variety of bacterial species and that it should serve the common function of providing the cell wall with a rigid structural component (Work, 1957).

Selton (1960) has found several non-nitrogenous sugars in the cell walls of various species of Gram-negative bacteria and concludes that they are present as polysaccharides constituting an integral part of the wall. Although they are at least partially extractable from the walls by gentle procedures, the extent and manner of the association is not defined. Nor is the composition and anatomical status of the lipid in bacterial cell walls well understood.

In view of the cell wall structure visible in electron micrographs, it is appropriate to inquire whether particular chemical compositions can be assigned to these structures. Apart from the elegant work of Weidel and his co-workers (1958, 1960) on *E. coli* (strain B) very little definitive information seems to be available. These investigators used the specific adsorption properties of the T-series of bacteriophage in combination with solvent extraction and electron

microscope observation to arrive at their conception of the macromolecular structure of the cell wall. They interpret their results to mean that underneath this is a layer of lipopolysaccharide. Nearest the cytoplasmic membrane is still another layer composed of mucopeptide (R-layer). The latter constitutes the main rigid structural component of the cell wall. In order to account for the adsorption of bacteriophages which have sites in the lipopolysaccharide layer, they postulate the existence of discontinuities in the lipoprotein layer. This triple-layer structure thus has a counterpart in the electron micrographs of thin sections of E. coli obtained by Kellenberger and Ryter (1958). However, considerations of the electron scattering power of macromolecular constituents likely to be in the cell walls of their bacterium led these workers to suggest that polysaccharide formed the middle layer and protein or lipoprotein coated this inside and out. Ogura (1963) suggests that his high-resolution electron micrographs of thin sections of E. coli show only two cell wall layers, an inner, electron-dense, lipoprotein layer and a less dense outer layer probably composed of mucopeptide and mucopolysaccharide containing embedded fine fibers which might be polysaccharide or scleroprotein. Additional work with a greater variety of tools is obviously necessary to resolve the discrepancies in these different concepts.

The problem of locating the macromolecular components of the cell walls of Gram-positive bacteria has not received much attention. Hay, Wicken, and Baddiley (1963) have noted in the case of a B. megaterium organism that, although the isolated cell walls contained no teichoic acid, degradation of the walls of the intact organism by lysozyme released substantial amounts of teichoic acid which could not have come from the cytoplasm. This suggested to these workers that the "intracellular fraction" of teichoic acid must be located between the wall and the cytoplasmic membrane.

#### ELECTROPHORETIC RESULTS

The electrophoretic mobility of a particle in an electric field is a function of the charge carried on the surface of the particle. The charge may arise from dissociable chemical groups which are part of the surface structure of the particle or from the adsorption of ionized solutes present in the suspending medium. The microscope method of electrophoresis has been used extensively over the last 40 yr to

explore the nature of the surface of many kinds of single cells, sometimes with conspicuous success. Reviews of the results of electrophoretic studies on bacteria have been given by James (1957) and by Brinton and Lauffer (1954).

In spite of the complex and variable nature of the surfaces of bacteria, experience has shown that the mobility of a particular bacterium is characteristic of that strain and is reproducible through many subcultures if no genetic changes affecting mobility occur and if growth conditions and suspending media remain the same (Lerche, 1953; Lowick and James, 1952). The surface charge of nearly all microorganisms studied has proved to be negative under physiological conditions. The isoelectric points are generally lower than those of most ordinary proteins but they do not give useful distinguishing criteria because the mobilities are frequently altered irreversibly by exposure to acidic media. There appears to be no clear-cut difference in the mobility behavior of Gram-negative and Gram-positive organisms, a rather surprising finding in view of the marked differences in chemical composition of their cell walls.

It is of interest to compare the concepts of the composition of bacterial surfaces derived from electrophoretic investigations with those obtained by other methods. This is possible in the case of E. coli. The results of a thorough electrophoretic study by Davies, Haydon, and Rideal (1956) led to the view that the external surface is dominated by polysaccharide which carries carboxyl groups. On the basis of an entirely different experimental approach, Weidel and his collaborators (1958, 1960) concluded that the exterior surface must be lipoprotein. This marked discrepancy may be due to the use of different strains of E. coli in the two investigations. However, electrophoretic observations on other strains of this bacterium by Alexander and McMullen (1949), Dyar and Ordal (1946), and by the author (unpublished) support the picture of the surface given by Davies et al. (1956) and are inconsistent with either a lipidic or protein surface. A more likely explanation for the disagreement is that during the preparation of the cell walls of Weidel et al., a thin layer of perhaps loosely held polysaccharide was removed. Some electron micrographs of thick sections of E. coli, in particular the high-resolution illustrations of Ogura (1963), show a diffuse material of irregular thickness on the surface with only slightly more electron density than the embedding

plastic. Unfortunately, no companion pictures of isolated cell walls seem to exist so one cannot determine whether the cell wall preparative procedures remove this material. Another approach is to study the electrophoretic mobility of isolated cell walls. Such work has been started by the author and will be reported in the near future.

Electrophoresis has an advantage over other methods of investigating the surface properties of bacteria in that it permits study under conditions closely approaching the natural environment of the cell. However, it yields information only about charged groups and this is often not specific enough to enable one easily to decide what species of ions are present or whether they are part of the structure of the particle or adsorbed from the suspending medium. Uncharged groups or moieties are revealed only through the adsorption of ionized additives in the suspending medium which have known affinities for them; e.g. the adsorption of surface active agents on lipidic constituents. Such information is useful but it is neither very specific nor quantitative. A difficulty of another sort enters in when attempts are made to calculate the surface charge density on a bacterium from electrophoretic data and conventional electrokinetic theory. As Haydon (1961a, 1961b) points out in a recent treatment of this matter, a major part of the difficulty arises because bacterial surfaces do not have their charges distributed on a smooth, impenetrable surface as the theoretical model requires.

The value of electrophoresis as a tool for investigating the surface properties of bacteria would be clearly enhanced if it were used in combination with other chemical and physical methods. A development which shows some promise is the use of specific chemical (including enzymatic) reactions to produce a change in the charge of a particular constituent (Douglas, 1959; Cittens and James, 1962; and James, 1957). Brinton and co-workers (1954, 1959) have demonstrated the value of the electron microscope as a supporting tool by showing that electrophoretic mobility differences between rough and smooth variants of an *E. coli* bacterium were due to the absence or presence, respectively, of filaments or fimbriae on the surface. Electrophoretic investigations would become more meaningful if it were always established whether flagella, fimbriae, capsules, or cell walls should be considered mobility-determining features.

## POROSITY AND PERMEABILITY

The cell walls of bacteria, though relatively rigid structures, must be porous since high molecular weight extracellular products such as proteins and polysaccharides synthesized in the cell or on the cytoplasmic membrane, presumably pass through it. The wall must also be permeable to transforming deoxyribonucleic acid and to deoxyribonucleic acid from bacteriophage in instances where no wall-degrading enzymes have been detected (Fujimura and Kaesberg, 1962). It is difficult to reconcile the degree of cell wall porosity seemingly necessitated by these phenomena with the deplasmolysis experiments of Record, Taylor, and Miller (1962), which indicate that even a comparatively small molecule such as raffinose passes through the cell wall of *E. coli* very slowly and that polyethylene glycol of mol wt 10,000 does not penetrate at all.

Work has been started in this laboratory (NRL) to determine explicitly the porosity of isolated cell walls. To do this, the extent of penetration of dextran, or other solute of known molecular weight, into the water space of the cell wall is determined by mixing a known amount of the solute with a thick, aqueous, wall suspension, centrifuging, and then analyzing the supernatant solution. Since the total volume of water in the system is known, the extent of equilibration of solute with the water of the cell wall can be calculated. Results with a series of dextrans and *B. megaterium* cell walls are shown in Table 1. The third column shows the water volume per gram of dry cell walls which is not available to solute. The last column expresses this as a percentage assuming the dextran with an average mol wt of two million is completely excluded.

Two observations can be made on the basis of these preliminary data. The cell wall is highly porous, being over 90% water, and the porosity is heterogeneous since the availability of included water to solutes of increasing molecular weight decreases over a wide range of molecular weight. Further work is in progress using carefully fractionated dextrans and selected proteins as porosity determinants to learn whether there are discontinuities in the distribution of pore sizes and to assess whether the concepts of the physical structure of cell walls derived from studies on isolated cell walls can be applied to intact cells.

TABLE 1. Water space of cell walls of  
*Bacillus megaterium* (NM)

Solute	Molecular weight	Water space unavailable	
		ml/g cell walls	%
Glucose	180	1.0	7
Dextran	80,000	6.9	47
Dextran	150,000	9.6	66
Dextran	$2 \times 10^5$	14.6	100 (assumed)

It should be pointed out that porosity determinations, particularly on isolated cell walls, may not give information on permeability since thin layers may exist in the wall which limit permeability but occupy only a small volume of the cell wall. It is also possible that the electrical charge carried by the walls might influence their permeability. Acidic groups in teichoic acids (Baddiley, 1962) and phospholipids particularly, would be expected to impart a strongly negative charge which might exert significant effects on the permeability of ionized constituents; osmotic effects might also be anticipated since it is known that charged artificial membranes, even though highly porous, can produce pronounced anomalous osmotic effects in solutions or ordinary electrolytes (Sollner et al., 1955).

The older view of the bacterial cell wall as an inert boundary of living protoplasm, a boundary that had to be postulated in order to account for non-spherical cell shapes, has been greatly enlarged and embellished in recent years by the advent of the electron microscope and modern biochemistry. The wall is a structure of surprising chemical and physical complexity. It must be considered an integral part of the cell, necessary for the protection of the cytoplasm, but dependent upon it for its maintenance and growth.

Remarkable as this change in the concept of the nature of the cell wall has been, there is still little understanding of its macromolecular structure, of the biochemical mechanisms responsible for its synthesis, or of its biophysical properties. While there is ample evidence that active biochemical work will be continued in this field, there is less indication that biophysical approaches, other than those

employing the electron microscope, will be widely used. One cannot now predict with any certainty what value a characterization of bacterial cell walls in terms of porosity permeability, charged groups, swelling properties, etc., will have on future conceptions of the biofunctions of these structures either under normal growth conditions or under conditions of stress such as drying and rehydrating. Two examples, in addition to those already mentioned, are offered to indicate that such studies may play a significant role. Gerhardt and Black (1961), in a thorough study of the porosity of spore walls by the water-space technique already described in this paper, have shown that the conventional conception of an impervious spore coat must be replaced since permeation of molecules up to 10,000 mol wt was demonstrated. In another recent paper Salton (1963) surveyed the evidence supporting the many theories advanced to explain the Gram stain reaction and concluded that cell wall permeability is the critical factor. Provocative findings such as these strongly suggest that biophysical approaches to the study of bacterial cell walls may be essential to a satisfactory understanding of their function.

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#### DISCUSSION

Shon: Since in this space determination technique you are necessarily dealing with large concentrations of cells, and you have relatively low concentrations of solutes, to what extent does metabolic utilization of glucose influence your determination of porosity?

Neihof: All of the studies reported here were done on isolated cell walls so that problems associated with utilization of solutes, such as glucose, or of leakage of internal constituents from the cell, which would interfere with the analyses, are obviated. Eventually, parallel experiments will have to be done with intact cells, and then these difficulties will have to be dealt with.

Silver: Would you be prepared to say what sort of molecular size would just squeeze through the cell wall?

Neihof: You may have access to more information on this than I. The paper of Record, Taylor, and Miller (1962) indicates that even sucrose has a little difficulty getting through the wall of E. coli. The cell wall porosity which we measure should be considered as something different from permeability. It is quite conceivable that the cell wall is composed of a voluminous, loose, gel-like network and a relatively thin layer of denser material. The thin layer could be the limiting permeability barrier, but our space studies would not reveal it. For this reason I am not willing to say what the maximum molecular size might be which is just able to cross the cell wall.

Silver: You have to consider at what stage extracellular material can take part in metabolic or other activity of the cell. In other words, when is the substance outside the cell and when does it truly become available to the cell? One would expect this availability to be related to the molecular size of the substance. We like to think that anything above, let's say, 1,000 or 1,500 in molecular weight constitutes the maximum size of a substance that can cross the cell wall. After that we think in terms of absorption.

Neihof: Again, I cannot shed much light on this from the results of our experiments. If I had to state on the basis of our data the molecular weight of a substance which cannot penetrate the cell wall, I would say about two million. I think I should mention that in the preparation of cell walls one makes a sizeable hole in the wall. Therefore, in our space experiments the solute can permeate not only from the outside, it can get inside and permeate the wall from that side also. The experiment yields a value for space available to the solute being used and reveals little or nothing about what can cross the wall of the intact cell.

THE DEVELOPMENT OF  
THE MULTIPLE ORIFICE IMPINGER (MOI)  
AS AN ANSWER TO THE PROBLEM  
OF PROLONGED OPERATION (ASPIRATION)  
IN THE ALL-GLASS IMPINGER

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In the course of aerosol investigations, it becomes necessary sometimes to operate sampling devices for prolonged periods of time. It has been observed in several laboratories that with the critical orifice impinger this procedure reduces the viable recovery of some microorganisms. In this context, a distinction should be made between the lethal effects occurring during the course of collection (biological efficiency) and the decrease in the recovery of viable microorganisms during the operation of the impinger subsequent to collection (aspiration). The former process has been studied quite extensively in several laboratories with the universal conclusion as stated by May and Harper<sup>(1)</sup> that sonic velocity impingement is shown to have a lethal effect on the more sensitive type of bacterial cell. Lethal effects can be attributed to impaction of the organisms on the bottom of the glass bottle, to their passage through the capillary critical orifice and/or to their violent impingement into the liquid collecting solution. It has been observed by the aforementioned workers that the biological efficiency of the impinger can be increased by decreasing the jet velocity (sub-sonic flow) or by raising the impinger tip. However, after a given point, further decreases in jet velocity or increases in the tip-to-bowl distance (stem height) reduce the physical collection efficiency, especially in the small particle range. In our laboratories, we chose to increase

the stem height of the 6 liter/min all-glass impinger (AGI) to 15 mm (AGI 6-15)<sup>(2,3)</sup> in an effort to reduce the lethal effects. It was observed, however, that there was a significant decrease in the recovery of Pasteurella tularensis in this raised-stem impinger during the course of prolonged aspiration.

The present study was directed towards modifying the AGI 6-15 to minimize the aspiration effect both in regard to the loss of collection fluid and the decrease in viable recovery. The approach taken in this work has been to decrease the jet velocity while increasing the surface-to-volume relationship of the air streams by using multiple numbers of smaller jets. This was accomplished, while maintaining the essential configurations of the impinger, by replacing the capillary orifice in the impinger stem with a metal cap containing an appropriate number of holes (Fig. 1) and by placing a critical orifice downstream from the exhaust arm. The size and number of holes (jets), the fluid volume and the stem height were investigated for the purpose of determining those characteristics that would minimize the aspiration loss of sensitive organisms -- in this case P. tularensis. Bacillus globigii spores or sodium fluorescein were used to indicate the physical loss of organisms under the various test conditions.

The influence of variations in jet diameter from 0.023-0.014 inches was investigated first. The size and number of jets in a given sampler were selected to complement each other such that jet velocity and flow rate were kept nearly constant at 0.1 sonic velocity (78.8 mph) and 6 liters/min, respectively. Over two trials and two replicate samplers per trial, no appreciable differences in recovery attributable to differences in jet diameter were observed. The modified samplers operating at 0.1 sonic velocity were about 55% as efficient as the AGI 6-15 in the collection of sodium fluorescein and about 75% as efficient in the collection of P. tularensis. After a 45-min aspiration period, the survival of P. tularensis cells in the modified samplers was between 93 and 111%; whereas in the AGI 6-15 it was 75%. The jet diameter was maintained at 0.023 inches in subsequent experiments, since the larger size jet could be drilled and cleaned with greater ease, and since no appreciable differences in recovery were observed over the range of jet diameters studied.

In a subsequent experiment, jet velocity was varied between the 78 mph tested previously and 200 mph, which May and Harper<sup>(1)</sup> indicated

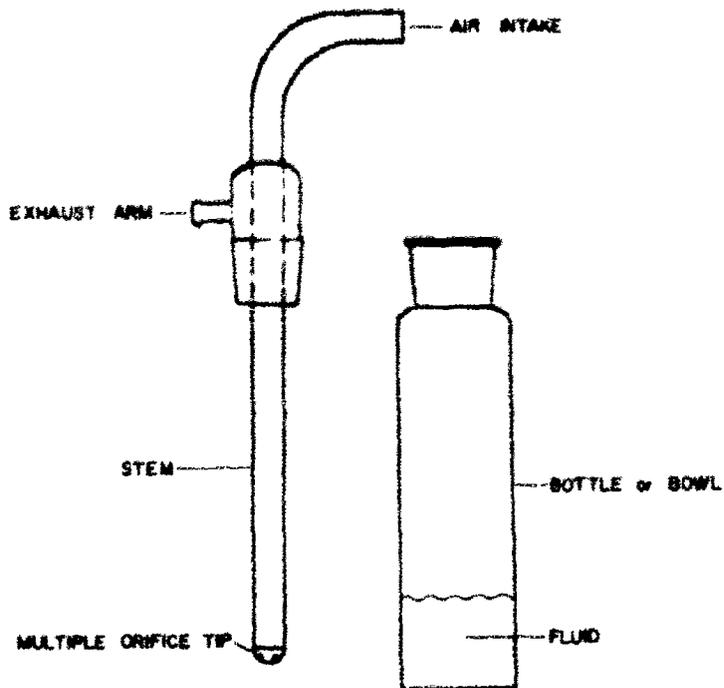


Fig. 1. The multiple orifice impinger (MOI)

as the optimum impingement velocity. To accomplish this, the number of jets per sampler was varied between 12 and 4 with the resulting jet velocities indicated in Table 1. The data shown represent the results of several experiments in which multiple orifice impingers (MOI) and the AGI 6-15 were used to sample dynamic aerosols generated from a B. globigii spore-P. tularensis mixture into a modified Reynier aerosol chamber with a Chicago atomizer. SYN<sup>(4)</sup> was used as the collecting fluid in all the samplers. The four-jet impinger was comparable to the AGI 6-15 for the collection of B. globigii spores and superior to it by 12% when collecting P. tularensis. Consequently, it was compared with the AGI 6-15 in a similar experiment in which both dynamic and 20 min aged aerosols of P. tularensis were sampled. The ratios of the initial recoveries in the four-hole impinger to those in the AGI 6-15 were as follows: Dynamic cloud, 1.03 -- Aged cloud, 1.02.

The optimum stem-height was determined in an experiment with P. tularensis aerosols in which the distance was set at 7, 10 and 15 mm.

All the samplers contained 18.5 ml of SYN fluid. Assays were performed following a 2-min sampling period and again after 60 min of aspiration in "clean" air. The ratios of the recoveries in the modified samplers relative to those in the AGI 6-15 are shown in Table 2.

TABLE 1. The initial recovery of aerosolized Pasteurella tularensis and Bacillus globigii spores as a function of jet number and velocity in the multiple orifice impinger

Test organisms	Jet characteristics *							
	Velocity †	No.	Velocity	No.	Velocity	No.	Velocity	No.
	218	4	159	6	109	8	70	12
<u>P. tularensis</u>	1.13 <sup>⊗</sup>		.99		.92		.88	
<u>B. globigii</u>	1.03		1.06		1.12		.61	

\* Jet diameter - 0.023 inches; sampler flow rate - 6 liter min.

† Velocity - Jet velocity given in miles per hour.

⊗ Obtained as  $\frac{\text{Recovery in test sampler}}{\text{Recovery in AGI 6-15}}$ .

TABLE 2. The recovery of aerosolized Pasteurella tularensis in the multiple orifice impinger as a function of stem height

Stem height	Recovery ratio*	
	Initial	After 60 min aspiration
7 mm	1.52	1.59
10 mm	1.18	1.25
15 mm	1.25	1.49

\* Obtained as  $\frac{\text{Recovery in test sampler}}{\text{Recovery in AGI 6-15}}$ .

Several experiments then were conducted to determine the optimum amount of fluid to be used with the four-hole, 7 mm stem-height sampler. Collection efficiency decreased with volumes of 15 ml or less.

Two trials were performed to compare the aspiration losses in the four-hole, 7 mm stem-height sampler containing 18.5 ml of fluid (MOI) and the AGI 6-15. Dynamic and 30 min aged aerosols were generated from *B. globigii* spore and *P. tularensis* mixed suspensions and sampled as described previously. The samplers were aspirated for 30, 60 and 120 min. An analysis of the data revealed a highly significant difference in residual fluid volumes of the MOI and AGI samplers following aspiration. The rate of fluid loss in the MOI was 0.0124 ml/min, which is approximately 20% the rate for the AGI 6-15. The MOI sampler obtained at 5.6% higher initial or non-aspirated recovery of *P. tularensis* than did the AGI sampler for the dynamic cloud and a 16.2% greater recovery with the aged aerosol. The exponential aspiration loss of viable *P. tularensis* in the AGI sampler was 0.56%/min of operation time; it was .08%/min in the MOI sampler. These data are shown graphically in Fig. 2.

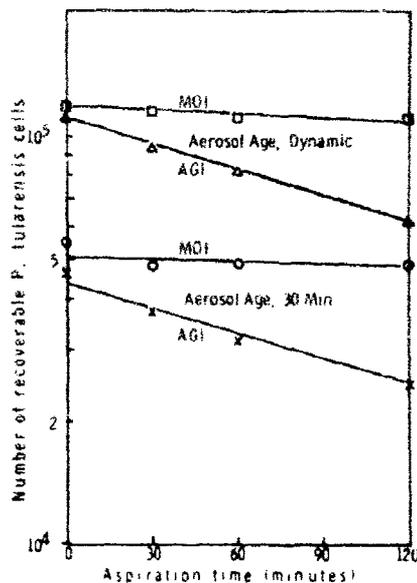


Fig. 2. The effect of prolonged sampler operation (aspiration) on the collection of aerosolized *Pasteurella tularensis* in the MOI as compared to that in the AGI 6-15.

In this experiment the initial recovery of *B. globigii* spores in the ACI 6-15 was about 12% higher than that in the MOI. Aspiration losses in both samplers were comparable, at a highly significant and appreciable rate of 1.87%/min. This has been shown in our laboratory by Levin and Cabelli<sup>(5)</sup> to be due to germination of spores under the influence of hyperoxygenation in SYN fluid and the loss of viability in the highly sensitive, germinated spore during aspiration. Figure 3 and Table 3, taken from their report, document the germination of the spores during aspiration as seen by the release of dipicolinic acid and the dependency of the germination on hyperoxygenation. Germination but not aspiration losses was observed when GMP fluid was used instead of SYN.

To conclude, the MOI recovery data show a significant increase over those of the ACI 6-15 after prolonged aspiration times due to the initial viable cell recovery and the decreases of the loss of collection fluid.

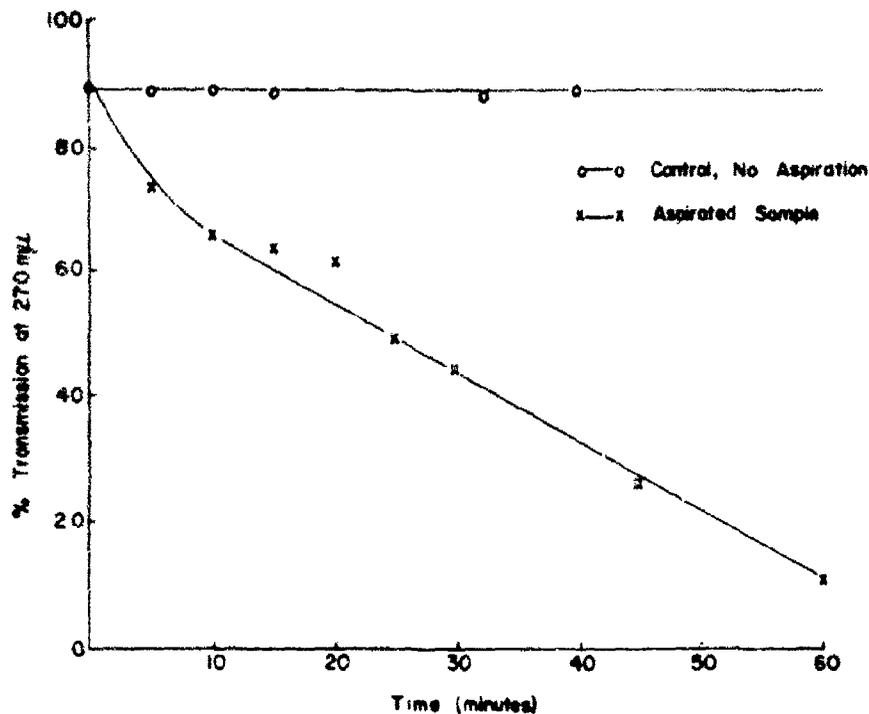


Fig. 3. The germination of *Bacillus globigii* spores during aspiration as seen by the release of dipicolinic acid.

TABLE 3. Aerosolization and aspiration\* of Bacillus subtilis spores in an atmosphere of nitrogen

Trial No.**	% Survival <sup>†</sup>	% Change in DPA
1	92 <sup>‡</sup>	0
2	91	0
3	92	0
4	92	0

\* Aspiration interval - 60 min

\*\* Each trial represents a separate aerosol at conditions of 50% RH, 50 F and dynamic conditions.

† % survival - recovery after aspiration/recovery before aspiration X 100.

‡ Each value represents the mean survival taken from four samplers.

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#### DISCUSSION

VFIF\*: We found a similar experience; there seemed to be some increase in aspiration losses with the age of the aerosolized cell. Did you take measures to check this at all? Do you think selection played any part in this?

Cabelli (for George): We did not try it in this context. We tried it in terms of the growth of P. tularensis on a deficient medium. Thus, the progeny of those aerosolized cells able to multiply on the deficient medium (peptic digest without starch) were no different in this respect than the parent population. This lack of stability suggested that the heterogeneity in regard to this characteristic was environmental rather than genetic. Other explanations are possible, but certainly we did not achieve greater stability by the process of selection.

Morton: A number of groups have aerosolized S. marcescens in the field and collected organisms which managed to survive for considerable distances down-wind, and there was no change whatsoever in the decay of subcultures -- it was precisely the same. I don't know if any one of you has had any luck in this regard. You tried it, Goldie, didn't you, from organisms surviving for several days in a drum? Did you have any luck in improving the breed?

Goldberg: I would like to sketch this out so you can see the dramatic nature of it. If you consider a scale of about 5 logs, and you have a curve that tails at about 1 log, we always make the obvious inference that such a sub-population is different. Now, if it is a genotype, there is no question that it would occur there, but the other postulate reasonably assumed is that you have one genotype and several phenotypes. So far as we know, if the lone survivor is grown up, it generates the same curve back, and the most amazing thing is that you are dealing with 1 part in a 100,000 population. The reason I point out this phenomenon is that people sometimes don't realize that it can occur in one part in a 100,000; even one part in a million; yet in what you might term a phenotypic way. This same type of behavior occurs in all sorts of stress conditions; for example, in the preparation of a polio vaccine you get that same sort of thing.

VFIF: We conducted some studies in which aerosolized cells were exposed to multiple stresses in sequence and the survival percentages

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\* VFIF - Voice from the floor.

were examined. It appeared that, following the initial stress, the population was altered so that the effect of the subsequent stresses seemed to always be less.

Sliver: We visualize the possibility that organisms that survive aerosolization for, let's say, 30 or 60 min, might either represent individuals which have an altered or a specialized genotype, or they might be phenotypic; but there was a possibility that throwing them back on medium and growing up a new culture from them might result in the dissipation of their specialized capacity to survive aerosolization. Consequently, Dr. Harper has prepared aerosols, and obtained the survivors from these aerosols after 60 min of duration, and immediately re-aerosolized them in the thought that, as you pointed out, he might come up with a nice flat line; but he came out with that same boomerang-shaped curve, which seemed to indicate that there was something specialized about the properties of the cell in the aerosol -- the organisms which can survive aerosolization. But that specialization is a property of the organism while it is in the air; when you get it back in the sampling fluid, its characteristics change back to that of a normal individual.

VFTF: In this area perhaps we should use a synchronous culture and then aerosolize them.

Shon: Derr, in my laboratory, had a paper out several years ago on that with Pasteurella pestis, and there seemed to be some physical damage to individual cells that required some hours to repair. There seemed to be no final genetic damage to the progeny. It indicated that you sometimes need an enriched medium -- there have been several papers on that -- to increase viability. Years ago Karl Persichetti passed an organism for 23 cycles of aerosolization and regrowth, and we were right back where we started.

PRELIMINARY STUDIES WITH A CONTINUOUS IMPINGER  
FOR COLLECTION OF  
BACTERIAL AND VIRAL AEROSOL SAMPLES

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This report presents data on the efficiency of a continuous impinger device. It was possible to sample the viable particulate content of 50 cu ft of air (at 1 ft<sup>3</sup>/min) into a terminal recycled fluid vol of less than 2 cc.

An axial section of the current model of the NBL Continuous Impinger (NBL-CI) is illustrated in Fig. 1. If one considers air at sonic velocities as an incompressible fluid, the flow pattern is conceptually as follows:

The air flow through the entrance jet nozzle is restricted to an orifice of 0.078 inch diameter or an area of

$$A_1 = \pi \frac{(.078)^2}{4} \quad \text{Equation 1}$$

The annular effluent air stream is restricted to an area of

$$A_2 = \pi (.156)h \quad \text{Equation 2}$$

where h is the pedestal-nozzle clearance. Area A<sub>2</sub> may be re-expressed as follows:

$$\begin{aligned} A_1 &= \pi \frac{(.078)^2}{4} = \pi \frac{(.156)^2}{16} \\ &= \pi (.156) (.010) \quad \text{Equation 3} \end{aligned}$$

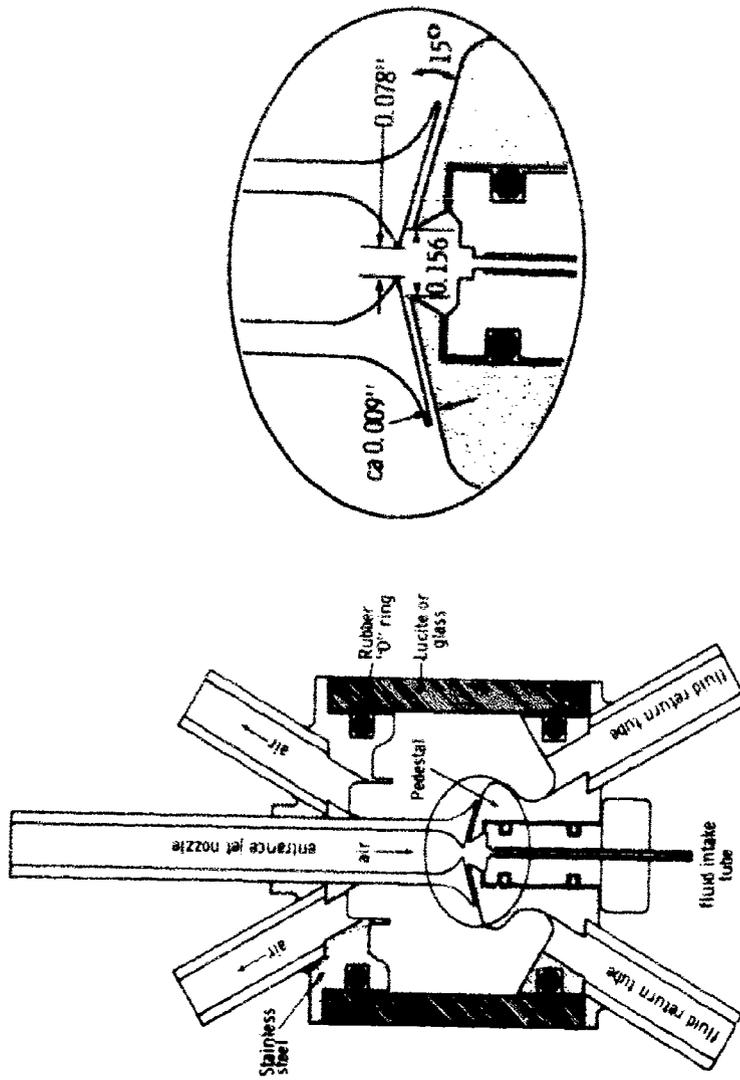


FIG. 1. NBL Continuous Impinger.

Now set  $A_1 = A_2$ . This suggests an annular opening or a pedestal clearance of .010, since the liquid feed hold is .156 inches in diameter, in order to approximate nonturbulent flow over the pedestal.

One notes that the base pedestal and top nozzle have a downward taper of  $15^\circ$ . This taper achieves, at the air entry nozzle, a clearance of 0.020 inches, permitting nonrestrictive air flow through a nominal  $90^\circ$  change in direction.

Another requirement is that the liquid flow over the base pedestal be smooth and unbroken immediately under the air jet, so as to provide an intact fluid front for particulate impaction. This condition is sensitive to pedestal clearance. It was experimentally observed that the desired even liquid flow was obtained for a pedestal clearance corresponding to a nominal 10% reduction of the unrestricted air flow.

As a result of the above approach, a unit was constructed and tested. The impinger operates at an air sampling rate of  $1 \text{ ft}^3/\text{min}$  with a continuous liquid flow of nominally  $1 \text{ cc}/\text{min}$ . For 24 hr continuous operation, a liquid flow of approximately  $1.5 \text{ cc}/\text{min}$  is required to prevent a particulate accumulation from slowly building up on the sampling pedestal. A peristaltic pump was used to transport the impinger fluid.

Preliminary tests for collection efficiency were made with dye (malachite green) solutions aerosolized by a Wells'-type atomizer. Solutions with nominally 5% solids will result in an aerosol with a mass mean diameter of 1-2  $\mu$ . Tests for biological collection efficiency were performed with Serratia marcescens and with Vesicular Stomatitis virus (VSV).

S. marcescens was inoculated from a seed slant into heart infusion broth (Difco) and incubated on a shaker at 37 C for 24 hr. The aerosolized organisms recovered in a nutrient broth (Difco) were assayed on nutrient agar petri plates. After incubation for 18-24 hr at 37 C, colonies were counted.

VSV was harvested from and assayed in chick fibroblast tissue culture monolayers (CFTC). Monolayers employed for virus production, or for assay, were grown in 16- or 3-oz prescription bottles, respectively, for 24 hr at 37 C in 0.5% lactalbumin enzymatic hydrolysate in Earle's balanced salt solution (LE) at pH 7.2-7.4 to which was

added 6% heat-inactivated calf serum and antibiotics. For production of plaques, suitable dilutions of virus samples were permitted to adsorb to drained unwashed CTPC monolayers for 1 hr at room temperature. Subsequently the cells were overlaid with LE fortified with 0.1% bovine albumin Fraction V at pH 7.8, plus 1% nutrient agar, plus 2% fetal calf serum. After 24 hr incubation at 37 C, 1:6000 neutral red was added to monolayers to aid in counting plaques. Endpoints were expressed in terms of plaque-forming units (PFU) per standard volume of aerosol samples.

It was observed that particulate recovery was sensitive to the depth of the fluid feed cavity. It was further noted that the condition of maximum turbulence in the fluid feed cavity, as evidenced by minimum air flow, resulted in maximal recovery.

Tables 1 and 2 summarize the results of two experiments using VSV aerosols. The AGI-30 capillary impinger was used as a parallel, simultaneous, comparative sampler. It is evident that the NBL Continuous Impinger was as efficient as the AGI-30.

Tables 3 and 4 present the results of a similar set of experiments using S. marcescens. Again, no significant differences between the two sampling techniques were noted.

The NBL Continuous Impinger has been demonstrated to sample airborne particulates of 1-2  $\mu$  diameter as efficiently as the standard AGI-30 (12.5 liters/min). The feature of continuous operation has applicability in many sampling problems.

One of the interesting applications of the NBL-CI is in the selection of mutant organisms for study in experimental aerobiology. The entire residual viable content of an aerosol in a holding chamber of approximately 50 ft<sup>3</sup> may be sampled into less than 3 cc of sampling fluid. This should allow one to assay for mutants resistant to the imposed environment with a capability of detecting a mutation ratio of 1/10<sup>8</sup> to 1/10<sup>10</sup>.

One additional comment should be made. Due to the liquid flow characteristics of the NBL impinger, there is no requirement for an antifoam. As a result, the fluid used for collection of virus aerosol may be directly utilized on the tissue culture monolayer used for plaque assay.

TABLE 1 Comparative aerosol recoveries of Vesicular Stomatitis virus employing the NBL-CI and AGI-30

NBL-Continuous Impinger* (26.5 liters/min)			AGI-30* (12.5 liters/min)		
Sample No.	Fluid recovered (corrected to assay vol. of 20 cc)	Plaque count <sup>‡</sup> -0.5 dil.	Sample No.	Fluid recovered (corrected to assay vol. of 10 cc)	Plaque count <sup>‡</sup> -0.5 dil.
2	3.8 cc	1,0	1	9.6 cc	1,9
4	3.6 cc	9,25	3	9.8 cc	4,3
6	3.6 cc	13,22	5	9.2 cc	20,16
8	4.0 cc	14,17	7	9.2 cc	20,12
10	4.0 cc	20,20	9	9.2 cc	16,19
12	3.8 cc	14,3	11	9.2 cc	16,7
14	4.0 cc	23,19	13	9.2 cc	5,18
16	4.0 cc	9,18	15	9.0 cc	19,19
18	3.8 cc	8,10	17	9.0 cc	6,14
20	3.8 cc	16,13	19	9.2 cc	7,18
22	3.8 cc	11,10	21	9.0 cc	13,9
24	3.8 cc		23		
		<u>297</u>			<u>271</u>

\* Operated at 20 inch Hg vacuum.

‡ Assayed plaque count/250 cc of aerosol.

TABLE 2. Comparative aerosol recoveries of Vesicular Stomatitis virus employing the NBL-CI and AGI-30

NBL-Continuous Impinger* (26.5 liters/min)			AGI-30* (12.5 liters/min)		
Sample No.	Fluid recovered (corrected to assay vol. of 20 cc)	Plaque count <sup>‡</sup> -1.0 dil.	Sample No.	Fluid recovered (corrected to assay vol. of 10 cc)	Plaque count <sup>‡</sup> -1.0 dil.
2	3.6 cc	31,21	1	7.0 cc	20,15
4	3.4 cc	15,16	3	9.0 cc	22,17
6	4.0 cc	35,26	5	9.0 cc	23,24
8	4.0 cc	33,25	7	9.0 cc	21,21
10	3.8 cc	26,23	9	9.0 cc	20,21
12	4.0 cc	34,22	11	9.0 cc	16,15
14	4.0 cc	26,22	13	9.0 cc	14,19
16		<u>20,26</u>	15	9.0 cc	<u>20,19</u>
		401			307

\* Operated at 18 inch Hg vacuum.

‡ Assayed plaque count/750 cc of aerosol.

TABLE 3. Comparative aerosol recoveries of *Serratia marcescens* employing the NCL-CI and AGI-30

NBL-Continuous Impinger* (26.3 liters/min)			AGI-30* (12.5 liters/min)		
Sample No.	Fluid recovered (corrected to assay vol. of 42 cc)†	Viable count 0 dil.	Sample No.	Fluid recovered (corrected to assay vol. of 20 cc)	Viable count 0 dil.
1	7.8 cc	88,88,98	1	17 cc	73,82,84
2	7.8 cc	72,84,90	2	18 cc	85,96,89
3	7.8 cc	72,81,89	3	17.5 cc	88,79,86
4	8.0 cc	78,54,88	4	18 cc	70,83,81
5	8.2 cc	55,58,52	5	18 cc	73,82,82
		1147			1233

\* Operated at 20 inch Hg vacuum

† Pump set to deliver 1-1/2 cc/min

TABLE 4. Comparative aerosol recoveries of *Serratia marcescens* employing the NBL-CI and AGI-30

NBL-Continuous Impinger* (26.7 liters/min)			AGI-30 (12.5 liters/min)		
Sample No.	Fluid recovered (corrected to assay vol. of 42 cc)†	Viable count 0 dil.	Sample No.	Fluid recovered (corrected to assay vol. of 20 cc)	Viable count 0 dil.
1	7.6 cc	115,126,114	1	18.5 cc	167,129,161
2	7.4 cc	122,112,125	2	18.0 cc	116,122,135
3	7.6 cc	131,111,128	3	18.5 cc	137,149,160
4	7.4 cc	139,123,142	4	18.0 cc	105,132,141
		1488			1659

\* Operated at 20 inch Hg vacuum.

† Pump set to deliver 1-1/3 cc/min.

## DISCUSSION

VFTF\*: Could you give us some of your thoughts on critical parameters of impingers in regard to viability?

Goldberg: Dr. May did some early work on the cascade impactor which indicated that the critical parameter involved is the impaction parameter characterized as that size collected with 50% efficiency. If you will carefully note this impaction parameter -- in particular, if you hold it near  $0.6 \mu$  -- you will find that rupture of the cell due to impaction will be reduced. Now this means that you can operate at, let's say, sonic velocity with a larger hole, if you will, i.e., you can go to smaller holes but less than sonic velocity. I think its best to consider your sampling in terms of this impaction parameter rather than simply in terms of either velocity or jet diameter or width, depending on whether you have a hole or slit. It turns out with the 12.5 liter/min AGI, that the impaction parameter is almost ideal. When you use the AGI with a 6 liter/min sampling rate, the impaction becomes a little too severe. As a result it appears that the 12.5 liter/min all-glass impinger is superior as a general air sampler. When one restrains the impaction front, one can achieve sampling rates of 25 liters/min. In the AGI, the fluid front is not restricted and as a result, the impaction parameter in effect drops off. So the net result of all of this is that you end up with about the optimal impinger type of device at 12.5 liters/min.

Silver: How do you know that everything is working properly? In the case of the ordinary glass impinger, of course, you get to look at it. How do you know yours is always working?

Goldberg: The wall of the present device is a clear plastic so you can see in and note if it is working properly.

VFTF: In that particular study between the 12.5 liter raised impinger and the 6 liter/min raised stem impinger, I believe there was no difference whatsoever with two organisms, tularensis and BG spores. Actually there were slightly higher, and significantly so, recoveries in the 6 liter than the 12.5 liter impinger.

VFTF: What about the sampling fluid -- isn't this part of the critical parameter?

Goldberg: Yes, this is one of the things that comes up. The

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\* VFTF = Voice from the floor.

sampling fluid is also very important. And I think you may find higher recoveries with sampling fluids other than those I mentioned. We kept one constant during the comparison but, nonetheless, this is a situation where, in a sense, we are trying to compare different results and it is very difficult to relate the two unless you do the experiment under identical conditions again.

VITF: I seriously disagree with a previous statement that the 6-15 and the 12.5 are doing the same job. Those of us who are doing some research in the field of aerosol standardization techniques know that we sometimes get some most odd results. When we collected P. tularensis aerosols, we would end up with about 50% RH when sampling immediately after spraying. I think that under these conditions the 6-15 impinger kills tularensis cells at a greater rate than the 12.5.

Goldberg: We duplicated some of your findings and are not sure whether it is the organism or the sampler causing the trouble. Unfortunately, there is no immediate answer to this type of a dilemma.

## INACTIVATION OF VIRUS AEROSOLS BY ULTRAVIOLET LIGHT IN A HELICAL BAFFLE CHAMBER

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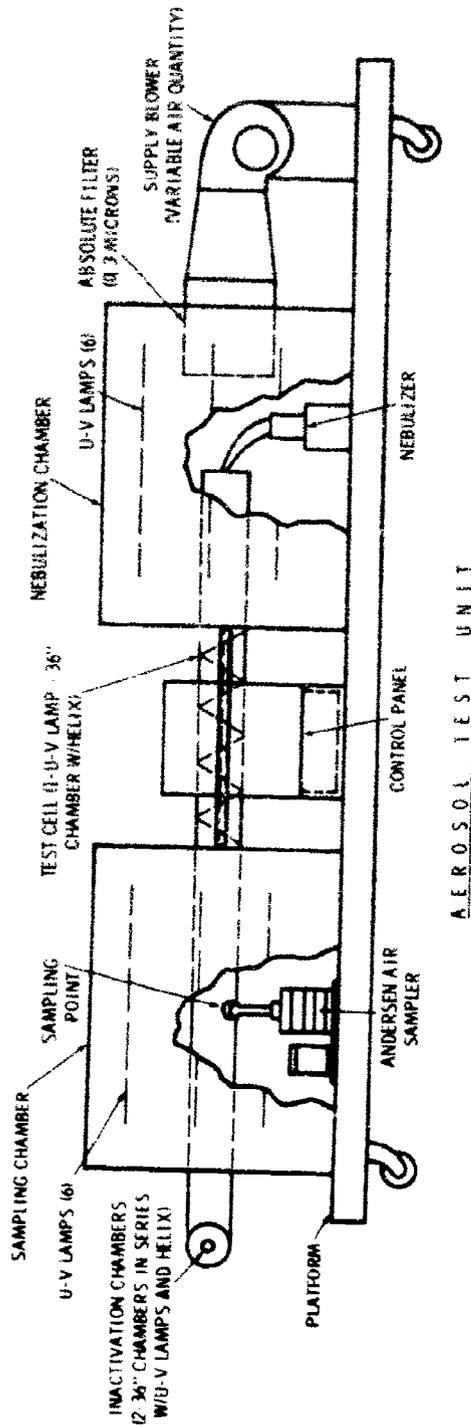
The following report describes preliminary studies which were undertaken to determine the effectiveness of a high-intensity ultraviolet (UV) chamber to inactivate viruses in aerosol suspensions.

### MATERIALS AND METHODS

Aerosol Test Unit. The aerosol test unit designed for this study is shown in Fig. 1. The unit consists of the following components:

1. Aerosol generation chamber.
2. UV test chamber.
3. Sampling chamber.
4. Decontamination chambers.

Aerosol generation chamber: Air was supplied to the unit by a blower with a variable output capable of delivering up to 200 cfm; for these tests, 100 cfm was employed. Before entering the generation chamber, the air passed through a 0.3  $\mu$  filter which removed microbial contamination. Six UV light tubes were installed inside the chambers for decontamination purposes. The Schoeffel Model 200A aerosol generator was used (Schoeffel Instrument Co., Hillsdale, N.J.). This self-contained unit generated a fine aerosol and dispensed 0.128-0.150 g of viral suspension min; this varied with the composition of the fluid. The aerosol was directed into a 7-inch diameter tube leading to the UV chamber.



AEROSOL TEST UNIT

FIG. 1

UV test chamber: The UV-light chamber consisted of a 36 inch long cylindrical aluminum tube of 7 inch diameter with an inner surface highly reflective for UV radiation in the germicidal range (Fig. 2). Inside this chamber was a helical baffle system that produced turbulences and directed the air-flow in a cyclonic pattern to insure complete exposure of the airborne particles to the UV rays. The UV tube extended longitudinally through the core of the chamber. (This chamber was provided by the Union Carbide Corp., Linde Co., Linde-Robbins Aseptic Air Systems, Venice, Calif.). The UV tube was a GE germicidal lamp (G 36T6) with an output of 13.1 watts at a wavelength of 2537 Å. A radiation intensity of 4.85 watts/ft<sup>2</sup> at 2 inches was produced by this lamp. The air-flow velocity through the chamber was 400 ft/min, which gave an exposure time of about 0.6 sec.

Sampling chamber: The Andersen sampler proved to be more effective than all-glass-liquid impingers for the sampling of viral aerosols in these studies. The top of the sampler was placed even with the inside of the tube. Regular Andersen sampler petri dishes were filled with 27 ml of 2% agar. The agar was then covered with 0.3 ml of a 20% skim milk suspension; this formed a thin viscous film over the agar. After the aerosol sample was collected, the skim milk film was suspended in 3 ml of Hanks solution. This fluid was then assayed for viruses. The sampling efficiency with the skim milk film on agar was about twice that of plain agar or a skim milk film on a plastic surface.

The stage distribution of viral plaque-forming units (pfu) in the Andersen sampler is shown in Table 1. Listed are the four viruses tested. (Coxsackie, B-1 containing  $4 \times 10^7$  pfu/ml in Eagles medium + 0.7% serum protein; vaccinia, containing  $1 \times 10^8$  pfu/ml in a 1:20 dilution of CAM suspension; Sindbis, containing  $5 \times 10^6$  pfu/ml in chorioallantoic fluid; influenza, containing  $1 \times 10^7$  pfu/ml in chorioallantoic fluid). More viruses were impinged on the third and fourth stages than on the extreme stages. Some "slippage" occurred, but for these studies this was not considered significant. Based on the calculations of Andersen, evidence suggests that a majority of the viral particles are contained in droplet nuclei 2-4  $\mu$  in diameter.

Viral assay: All viral titrations were done by tissue culture plaquing methods using disposable plastic flasks (Falcon Plastic Co., Los Angeles, Calif.).



FIG. 2. Cutaway of ultraviolet chamber, showing helix and ultraviolet tube

TABLE 1. Stage distribution of viral aerosols collected in the Andersen sampler

Virus	Plaque-forming units per stage of sampler					
	1	2	3	4	5	6
Coxsackie B-1	10,500	17,750	21,750	26,750	20,250	11,250
Vaccinia	8,775	30,480	42,930	141,750	49,500	1,785
Sindbis	1,830	4,000	15,750	14,100	8,850	840
Influenza A	150	1,200	4,200	3,300	450	--

The following plaquing systems were used: for Coxsackie B-1, Detroit-6 or Davis tissue culture monolayers were inoculated with 0.2 ml of skim milk-virus suspension. After viral adsorption, the cells were covered with 0.6% agar overlay containing tris buffer and 5% agamma calf serum. After 3 days incubation, the flasks were filled with 10% formalin, which fixed the cells and inactivated the virus. The overlay medium and formalin were poured off and the cell monolayer stained with crystal violet; this enabled direct counting of the plaques. For influenza A (WSN): plaques were produced on primary

chick embryo tissue culture monolayers using an overlay medium described by Hirst and Simpson which contained no serum. For Sindbis: plaques were produced on secondary, chick embryo tissue cultures, using tris buffer overlay. For vaccinia: Plaques were produced on secondary chick embryo tissue cultures; no overlay was used. Cells were fixed and stained after 36-hr incubation.

#### ULTRAVIOLET-LIGHT INACTIVATION

UV inactivation tests were carried out by first passing the viral aerosol through the lighted UV chamber, then repeating the procedure with the UV chamber light off. Each test lasted 10 min. Viral concentrations collected by the Andersen sampler with the light "on" were compared to concentrations collected with the light "off" and the percentage of inactivation determined.

#### RESULTS

The results are summarized in Table 2. The first column of figures represents the concentration of viral particles/ft<sup>3</sup> of aerosol. These figures are computed from the amount of virus contained in the viral suspension times the volume of fluid dispensed/min divided by the ft<sup>3</sup> of air passed through the chamber/min.

The next two columns list the number of viral pfu collected with the UV light off and on. The percentage of viral inactivation is seen in the last column. A value of 100% is obtained for vaccinia and influenza, 99.95% for Coxsackie B-1 and 99.6% for Sindbis virus. The lower figure for Sindbis might be attributed to a precipitate that formed in the viral suspension during this test.

TABLE 2. Inactivation of viral aerosols during passage at 100 ft<sup>3</sup>/min through a helical baffled UV light radiation chamber

Virus	Concentration of viral aerosol in pfu/ft <sup>3</sup>	Pfu collected/ft <sup>3</sup> of aerosol sampled		% of virus inactivated by UV light
		With UV off	With UV on	
Coxsackie B-1	57,200	10,755	5	99.95
Influenza A (WSN)	14,500	920	0	100
Sindbis	7,500	4,537	1	99.60
Vaccinia	128,000	27,522	0	100

#### CONCLUSION

The concentrations of virus passed through the UV chamber in these tests were many times greater than that which would be encountered under most conditions. The results indicate that the helical baffled UV chamber would be effective in inactivating virtually all airborne viruses passing through it. Because of the large volume of air handled by this unit, it readily lends itself to experimental or clinical conditions where the air must be relatively free of microorganisms.

#### DISCUSSION

VFIF<sup>\*</sup>: You mentioned, I believe, that there is high efficiency with the Andersen sampler -- higher than with the all-glass impinger. Could you elaborate on that? Could you show an extra slide?

Jensen: I hesitate to show this slide (Table 3) because the figures represent rather limited data, and the irregularities may be a result of my techniques. Using aerosolized T<sub>2</sub> bacteriophage, we were unable to collect any virus with the 12.5 liter glass-impinger; evidently there was a shearing effect with this sampler. With a midget impinger we could pick up a few particles; the Andersen sampler collected more than 909 phage particles/ft<sup>3</sup> as determined by Andersen's positive hole conversion table. Next, using aerosolized vaccinia virus, 1,066 pfu were collected with 12.5 liter glass impinger as compared to zero with the midget impinger and 27,500 pfu with the Andersen sampler. At this point in our study we decided to use only the Andersen sampler; the results of the Coxsackie tests had not been completed at that time. When these results were obtained, it was found that we had a greater efficiency with the 12.5 liter impinger: 27,500 pfu as compared to 8,000 and 10,775 pfu for the midget impinger and the Andersen sampler, respectively.

TABLE 3. Effectiveness of various sampling devices for collecting viruses in aerosol suspensions

Virus	Pfu of virus collected/ft <sup>3</sup> of aerosol sampled with		
	Liquid Impinger	Midget Liquid Impinger	Andersen Sampler
T <sub>2</sub> Bacteriophage	0	2	909
Vaccinia	1,066	0	27,500
Coxsackie B-1	27,500	8,000	10,755

\* VFIF = Voice from the floor

VFTE: I must say I am rather surprised that more of your particles fall on stage 3 or 4. If most of your particles fall on stage 3 and 4, it would not mean that the principal numbers, namely numbers of particles, would be in the 2-4  $\mu$  range, would it?

Jensen: Yes, it would.

VFTE: You must have many viral particles per droplet nuclei.

Jensen: Specifications on the aerosol generator, using a 5% saline solution, stated that most of the particles were in a 1.5  $\mu$  range or below, and about 70% by volume would fall into the 2-4  $\mu$  range. We haven't as yet determined the actual particle size in our aerosols; based on the specifications of the aerosol generator and the distribution in the Andersen sampler, we do find some discrepancy.

VFTE: Well, from these results can you estimate in different experiments the probable number of viral particles per drop of nucleus?

Jensen: I haven't tried this.

VFTE: The other question I have is this: Are you sure that this is UV and not ozone?

Jensen: The lamps used in these studies have been constructed so as to minimize ozone production. Ozone concentrations from four of these lamps burning in a small room for 1 hr would be less than 0.0005 ppm, which is far below the typical atmospheric oxidant levels in Los Angeles (0.02-0.13 ppm).

Goldberg: Did you compare the spiral with, say, the absence of spiral or was there perhaps one turbulating disk?

Jensen: No, this is the only unit we have tested to date.

Schulman: What was the relative humidity (RH), or did you determine RH inside the chamber?

Jensen: Yes, we monitored RH; depending upon the test it ranged between 62 and 66%. We made no effort to control the humidity. The time of exposure of the aerosol was only about 1 sec from the time of generation until sampling. The amount of fluid aerosolized would alter the RH inside the chamber by less than 1%. The effect of RH on UV inactivation of viruses has not been determined. This could be a critical factor.

Harper: I merely want to make the observation that one should not write off the passing of 1 sec as being unimportant -- one can get quite stringent death in that period of time; it is dependent on RH. So one should not say it is just 1 sec and so the RH does not matter.

VFTE: I wondered how long you thought this thing would work --

until it got dirty-- and then it wouldn't be as effective perhaps.

Jensen: We ran many milliliters of fluid through this unit and I became concerned about this possibility. But throughout these tests the lamp was not cleaned and we still had the same degree of inactivation. In normal operation, the UV unit would be used after a filter which would remove dust and the larger particles. In these studies approximately 100 ml of fluid were aerosolized directly into the UV chamber without prefiltration and we still had a high efficiency. The vaccinia tests were conducted last, so evidently there is a good safety margin.

VFTF: How effective is this with bacteria? Is it as effective?

Jensen: It is equally effective; tests have been conducted with various bacteria, including mycobacterium, and the inactivation rates were greater than 99%.

VFTF: I want to comment on this: I think GE quotes an effective life of around 1,000 hr or that sort before the glass solarizes, then you don't get enough UV to be effective anymore.

Jensen: The manufacturer lists the effective life as approximately 7,500 hr. There is an initial 100 hr period of greater intensity; these tests were conducted after the initial 100 hr of operation.

Goldberg: You might mention that against a natural bacterial air flora you would expect, say in a hospital, the reduction to be between 50 and 90%, depending on humidity. As you all know, the hospital organism is quite tough, it is embedded in dirt, so to speak, and against that type of organism your kill is not as effective.

Jensen: As I stated earlier, in this type of installation pre-filters would be used with the UV unit which would remove the large particles.

VFTF: Is there any effective temperature change in this situation with all of this intense radiation? Is there any increase in temperature during the run of the unit?

Jensen: This has not been determined; there is possibly a slight increase. I had thought there might be a reduction in particle size in tests with the lamp on due to increased heat, but the distribution in the Andersen sampler of the few particles that did come through was similar to that obtained with the lamp off; i.e. largest amounts on stages 3 and 4. The amount of impaction or heat inactivation obtained within the helical baffle unit, if any, has not yet been determined.

## MODELS FOR DEAGGLOMERATION AND FRACTURE OF PARTICULATE SOLIDS

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The aerosol generation of particulate solids consists either of fracturing larger particles and creating an air dispersion of their fragments, or of separating preformed particles held together in aggregates by surface contact or other forces. In the first case, new surface area is created, requiring in general larger expenditures of energy and applications of larger stresses. Concomitant with the exposure of fresh surface to the gaseous environment, opportunity for catalyzed chemical reactions is afforded, which could shift the powder into a non-equilibrium condition. Because of the relatively high energy and stress requirements for increasing surface area, aerosols of powders are usually generated from a presized product, prepared either by grinding or spray drying. Special techniques do exist, however, for the preparation of certain dry materials that make possible the stable aerosol generation of non-presized powders, through reduction of stress required in the creation of new surface by alteration of powder properties.

In general, however, any particulate solid aerosol generation process involves both the creation of fresh surface, or grinding, and the separation of basic presized particles held together as aggregates, or deagglomeration. The relative extent of grinding and deagglomeration depends upon the energy available in the generating system as well as its compressible flow characteristics, the particle-size distribution

of the pre-dispersed powder, the design of the aerosol generating mechanism, and the intrinsic physical properties of the solid medium comprising a particle.

The purpose of this paper will be to present models for grinding, and for particulate solid aerosol generation involving compressible fluid dynamics, in an attempt to correlate properties of the generating systems and the pre-dispersed powder, hopefully leading to a prediction of the initially generated aerosol size distribution. Modification of the initial aerosol distribution by coagulation, preferential sedimentation or diffusion, etc., will not be discussed.

#### AERODYNAMIC DEAGGLOMERATION OF PARTICULATE SOLIDS

##### A. Conformation of an aggregate

In the discussion which follows, it will be assumed that the basic particles comprising the powder are homogeneous in size, a basic particle being defined as the smallest volumetric subdivision of the powder possible without the creation of fresh surface. This assumption does not strictly apply to most powders dealt with experimentally, but if geometric standard deviations of the distributions are small the assumption does not introduce any serious error. A proper statistic, such as Sauter Mean diameter, average volume diameter, area mean diameter, etc., can be chosen to approximate an equivalent "homogeneous size" for a given application.

It will be further assumed that the basic particles are initially separated from each other, i.e. no aggregates are initially present, and that they are in random motion with respect to each such as applies to a turbulent gas suspension or to a mechanically stirred array. Random motion will produce collisions, collisions will produce aggregates, and a steady state will be reached when deagglomerating forces brought to bear on an aggregate are equal to forces of cohesion holding the aggregate together. Aggregates consisting of various numbers of basic particles can be formed in two limiting ways:

##### 1. Type I

Type I results from random collisions between basic particles simultaneously in every volume element of the array, and continuing collisions between resulting aggregates, etc. Non-uniform packing of basic particles results.

2. Type 2

Type 2 is caused by the initial formation of an agglomerate by collision of two basic particles, and successive build-up of this agglomerate by successive collisions between it and individual basic particles. Uniform packing of basic particles results.

A schematic representation of agglomerate formation in both models, assuming collisions between two particles for illustration, is given in Fig. 1

If  $\bar{P}_c$  is the probability of a single collision involving two particles, taken as equal for both models, then the probability of forming an agglomerate consisting of  $N_i$  basic particles for each type is:

$$\bar{P}_{(N_i)_1} = \bar{P}_c \left( \frac{\log_{10} N_i}{0.301} \right) \quad (1)$$

where  $N_i = 2^i$

$i$  = array index

$$\bar{P}_{(N_i)_2} = \bar{P}_c \left( N_i - 1 \right) \quad (2)$$

the ratio of probabilities of forming an agglomerate of type 1 to type 2 consisting of the same number of particles is then

$$R = \frac{\bar{P}_{(N_i)_1}}{\bar{P}_{(N_i)_2}} = \bar{P}_c \left( \frac{\log N_i}{0.301} + 1 - N_i \right) \quad (3)$$

Since  $\bar{P}_c < 1$ , it is seen that aggregates formed by the mechanism of type 1 are much more probable than those of type 2. For example, for  $\bar{P}_c = 0.5$  and  $N_i = 8$ , then  $R = 16$ ; or  $\bar{P}_c = 0.2$ ,  $N_i = 8$  then  $R = 625$ . As  $\bar{P}_c$  decreases, or  $N_i$  increases,  $R$  increases.

Hence, the conformation of what might be considered a most probable or representative agglomerate will be that determined by probabilities associated with type 1, having:

(1) a quasi-spherical shape, due to the spherical symmetry of random motion of colliding particles.

(2) five sub-agglomerate components, on the average being the

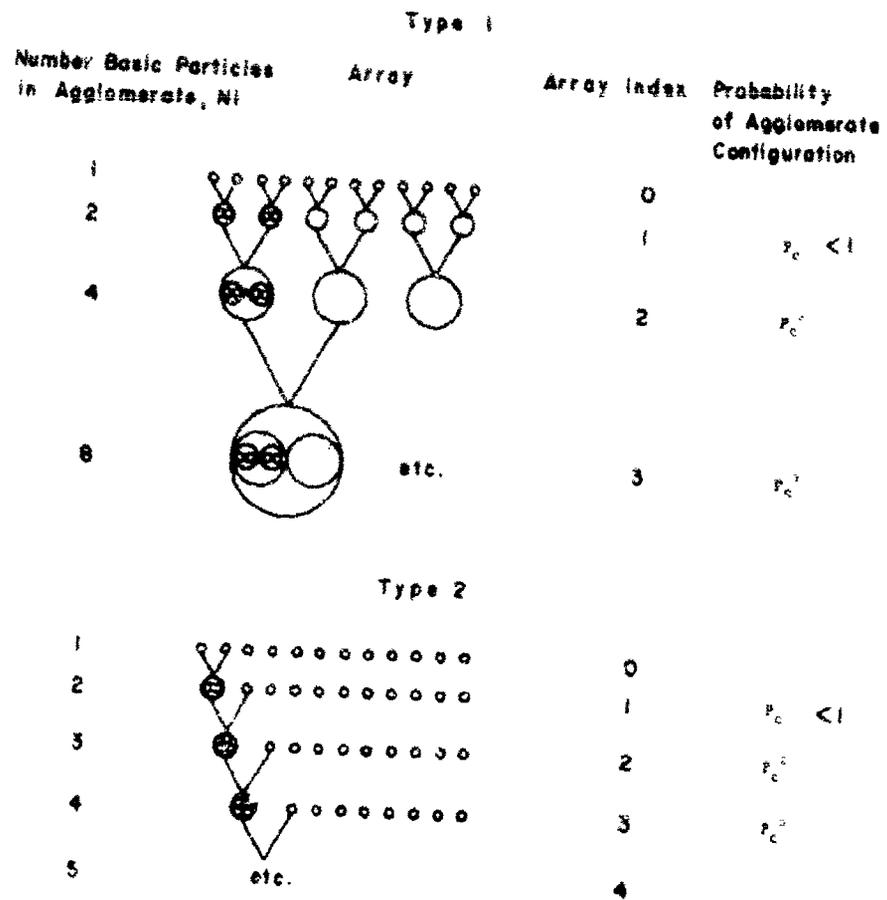


Figure 1. Limiting Types of Agglomerate Formation.  
(Two-Particle Collision Assumed.)

smallest number of colliding particles defining the spherical symmetry. Each of these component sub-agglomerates will consist of five smaller sub-agglomerates, etc., ending with a sub-agglomerate consisting of five basic particles, which would correspond to the first aggregate formed in the initially dispersed random array.

A schematic diagram of this hypothetical characteristic aggregate is shown in Fig. 2.

The largest characteristic aggregate encountered in a bed of pre-dispersed powder will be that determined by the magnitude and efficiency of application of the disrupting stress during the powder's history, schematically represented in Fig. 2. The process of aerosol generation then can be considered as a sequential process of breaking an agglomerate of type 1 into its component sub-agglomerates, etc., until either stable sub-agglomerate or basic particles are dispersed. An important property of the sub-agglomerate hypothesis is the decrease in numbers of contacts per unit area between component particles as the agglomerate increases in size, i.e. as the agglomerate gets larger, its shear strength or tensile strength gets smaller. This would not be the case for agglomerates of type 2 formation.

#### B. Disruption of agglomerates by aerodynamic drag

In general, mechanical forces capable of deagglomerating particles arise in seven ways in a compressible flow system:

1. Impact of particles with fluid flow boundary walls due to vorticity of the particles' motion. This could result from high-order turbulence in the fluid flow system, or centrifugal force applied to a particle as a result of its motion in a laminar vortex. The average velocity of the particle in this system would be in general parallel to the impact surface.
2. Impact of particles with each other.
3. Centrifugal disruption of a particle due to rotation about its own axis.
4. Direct impact with a fixed bounding surface, not requiring vorticity, where the average velocity vector of the particle intersects the impact surface.
5. Friction drag, or shear, resulting from velocity gradients.

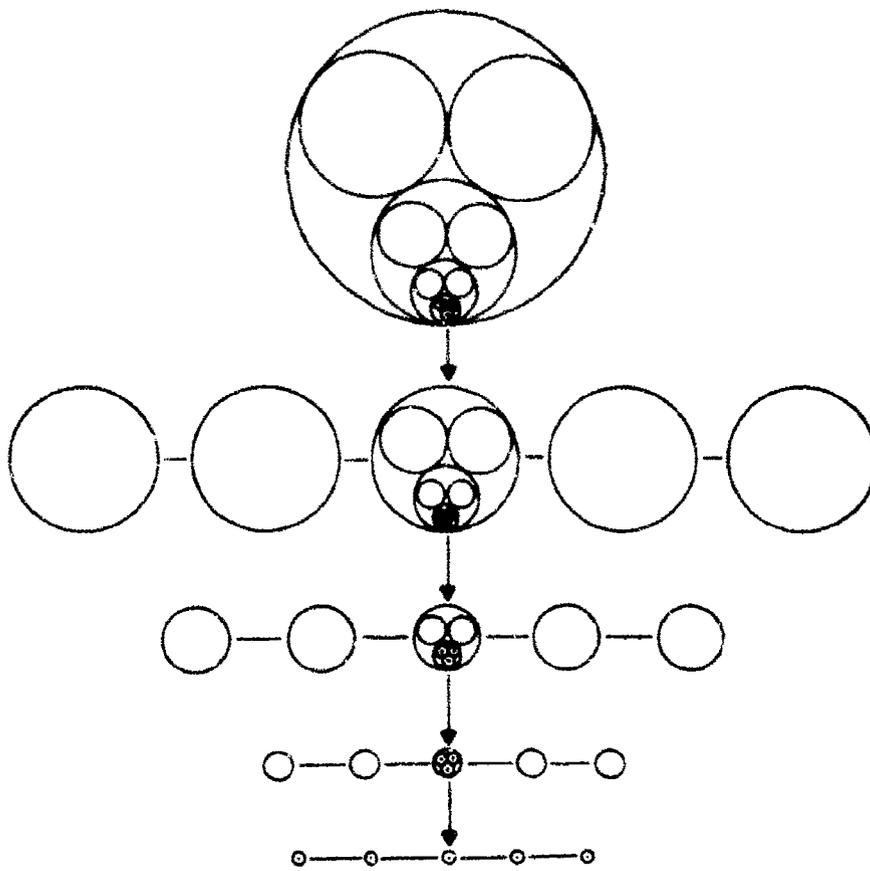


Figure 2. Cross-Section Schematic of "Probable" Quasi-Spherical Agglomerate and Mechanism of Deagglomeration.

in a viscous fluid system; and form drag, or pressure.

6. Inertia, causing preferential acceleration by drag forces acting unevenly over particles in contact and composing an aggregate.

7. Explosive decompression of an agglomerate containing trapped air in its voids if surface pressure is rapidly reduced. This effect can theoretically exist for a spherical agglomerate under drag in a free stream.

For the interaction of a free, unbounded gas stream and a particle, mechanisms (5), (6) and (7) would predominate. Drag includes both a pressure stress and a shear stress, and for a sphere in steady laminar flow of a viscous incompressible medium, or for a compressible medium at Mach  $\leq 0.3$ , these stresses are normal to each other and normal and parallel to the surface respectively<sup>(1)</sup>. The resultant drag force under these conditions is parallel to the direction of motion, and is given by Stokes' law for spheres with radii  $> 1 \mu$ . (The Cunningham correction factor to account for slip is required for radii  $< 1 \mu$ .)

$$D_s = 6\pi\eta U r \quad (4)$$

$U$  = relative stream-particle velocity

$r$  = radius of sphere, or characteristic dimension

$\eta$  = viscosity of medium

Stokes' equation holds for laminar flow, which, for a sphere, requires a Reynolds number,  $Re \leq 1$

$$Re = \frac{\rho U r}{\eta} \leq 1 \quad (5)$$

$\rho$  = density of medium

A general expression for the drag or resultant force on a particle of any shape in either incompressible or compressible media for either turbulent or laminar flow has been formulated in terms of a drag coefficient. Drag acts on a particle in direction of relative motion of the medium with respect to the particle.

$$D = \frac{C_D \rho U^2 S}{2} \quad (6)$$

$U$  = free stream velocity

$S$  = projected area of body normal to stream

$C_D$  = drag coefficient

$\rho$  = density of medium at particle surface

The drag coefficient is a function of shape and Reynolds number, and for irregular shapes is empirically determined<sup>(2)</sup>. A function relating  $C_D$  and  $R_e$  for spheres over the range of  $0 \leq R_e \leq 2000$  has been derived by Langmuir<sup>(3)</sup> with average deviations from experimentally determined values of less than 4%.

$$C_D R_e / 24 = 1 + 0.197 R_e^{0.63} + 2.6 \times 10^{-4} R_e^{1.38} \quad (6)A$$

If a particle is free to conform to an external pressure stress over its surface, its shape will be altered if the resultant stress is not uniform in magnitude and direction over every region of the surface. It can be shown that particles obeying Stokes' law are not distorted. However, particles accelerated in compressible media do experience non-uniform as well as non-symmetrical pressure stresses, and aggregates of particulate solids or liquid droplets will deform during acceleration if the magnitude of the stress exceeds the tensile stress or surface tension pressure of the respective particles.

For a sphere under acceleration in an inviscid fluid at rest at infinity, the surface pressure distribution as derived from a velocity potential can be shown to be<sup>(4)</sup>

$$\frac{P - P_0}{\rho} = \frac{1}{2} r \cos \theta \frac{dU}{dt} + \frac{1}{8} U^2 (9 \cos^2 \theta - 5) \quad (7)$$

where  $r$  = radius of sphere  
 $\theta$  = angle measured positively from flow axis from stagnation point  
 $U$  = velocity of sphere in inertial frame of reference  
 $P$  = pressure on surface  
 $P_0$  = stagnation pressure  
 $t$  = time

Since the  $\cos^2 \theta$  term is symmetrical about the axis of motion, the pressure distribution contributing to drag is:

$$\frac{P - P_0}{\rho} = \frac{1}{2} r \cos \theta \frac{dU}{dt} \quad (8)$$

Equation 8 is plotted in Fig. 3 for the case  $r = 12 \mu$  and an initial Mach 0.1 relative particle free stream velocity at atmospheric pressure. At Mach 0.1, compressibility effects are negligible. From Newton's second law and Equation 6

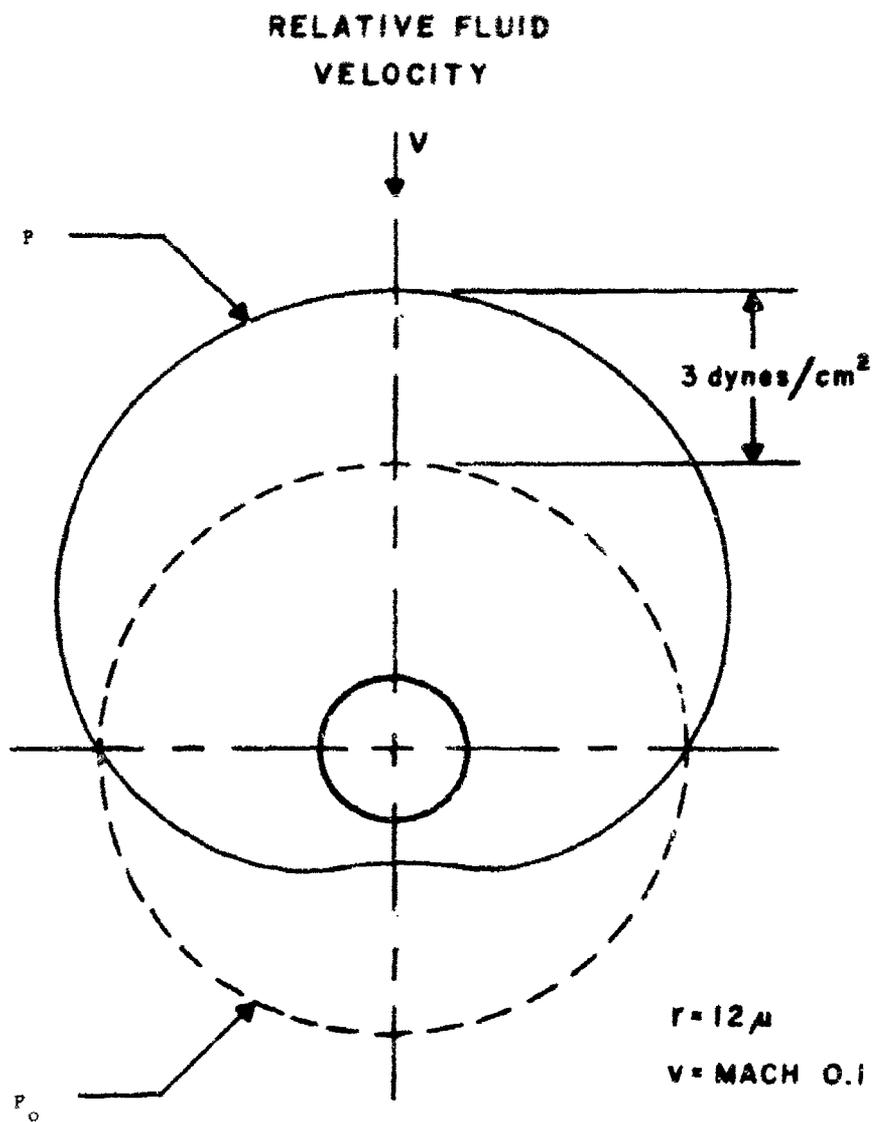


Figure 3. Initial Acceleration Pressure Distribution  
Potential Flow.

$$\frac{dU}{dt} = \frac{D}{\frac{4}{3} \pi r^3 \rho_p} = \frac{\frac{1}{2} \rho C_D U^2 \pi r^2}{\rho_p \frac{4}{3} \pi r^3} \quad (9)$$

$\rho_p$  = particle density

The radial pressure distribution of Equation 8 is asymmetrical and non-uniform, thereby contributing to acceleration of the particle in an inertial frame and, to some extent, to flattening of a particle in the direction of relative fluid-particle motion due to unequal acceleration of its fore and aft surfaces.

The second term in Equation 8, however, for the conditions of flow defined above, is greater than Equation 8 by orders of magnitude, and, although not contributing to drag because of its symmetry, does have the effect of producing greater particle distortion in a body-fixed frame of reference. This "deforming" pressure distribution is plotted in Fig. 4. It is seen that the minimum pressure is sub-atmospheric at  $\theta \geq 90^\circ$ .

A rough estimate of the rate of deformation of flattening for the case under consideration can be made by the following considerations: Consider a unit area on the surface at  $\theta$ . Then acceleration of the surface of an incompressible inviscid sphere in a body-fixed frame is given by

$$(P - P_0) = \frac{\rho U^2}{8} (9 \cos^2 \theta - 5) = \rho_s \frac{d^2 X}{dt^2} \quad (10)$$

$\rho_s$  = surface density of particle

X = radial distance moved by surface element,  $\theta$  constant

giving

$$X_\theta = \frac{\rho U^2}{\rho_s 8} (9 \cos^2 \theta - 5) t^2 \quad (11)$$

but

$$X_\theta = r_0 - r$$

$r_0$  = initial spherical radius

$r$  = distorted radius

Hence, for  $\theta = 0^\circ$  and  $X_\theta = r_0$ , then  $t = 4.5 \times 10^{-4}$  sec. Or, for  $\theta = 90^\circ$  and  $t = 4.5 \times 10^{-4}$  sec, then  $r_\theta = 90^\circ = \frac{9}{4} r_0$  or the sphere tends to develop into a crenated disc with a 125% increase in radius. The

RELATIVE FLUID  
VELOCITY

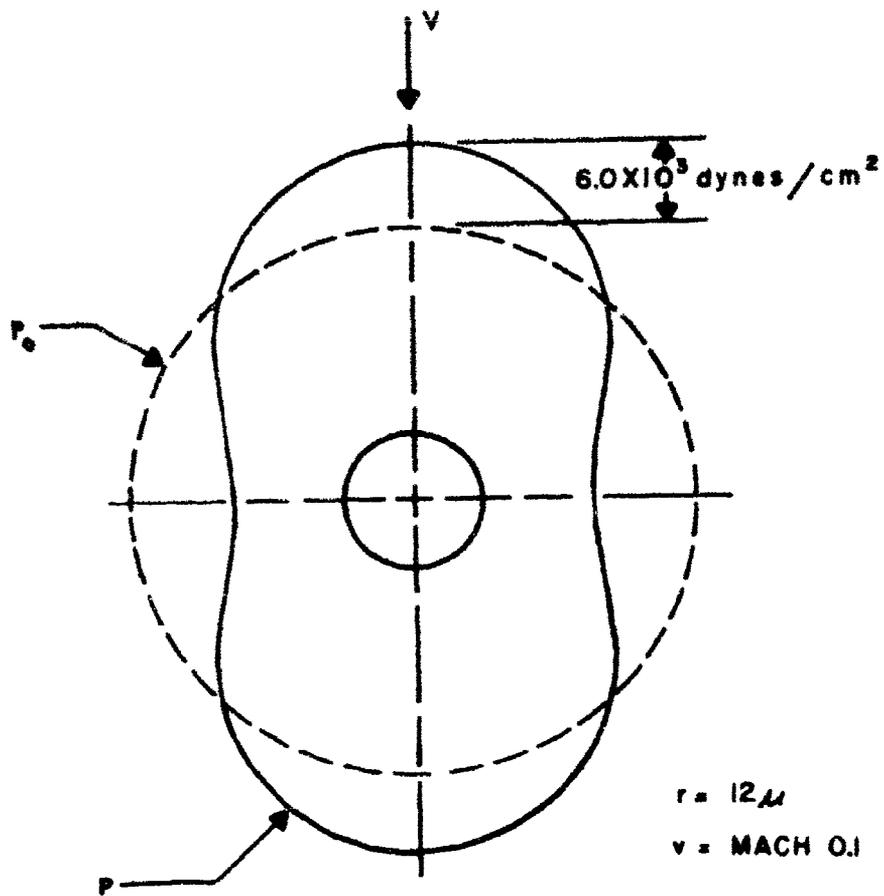


Figure 4. Initial Deforming Pressure Distribution.

pressure distribution will approach that over a disc with its plane normal to flow. This pressure distribution over the leading surface is given by<sup>(5)</sup>

$$P_r = P_0 - \frac{\rho a^2 r^2}{2} \quad (12)$$

where

$r$  = radius vector

$P_0$  = stagnation pressure

$a$  = constant

Cavitation will tend to develop behind the sphere as it distorts, producing an asymmetrical pressure distribution, and thus will further increase the drag. Hence, when deformation of the sphere begins, disruption will result if the applied stress is maintained.

The aerodynamic pressure stresses and viscous shear stress would then probably play the following sequential roles in the deagglomerating process of a spherical aggregate:

(1) Deformation or flattening of the sphere by normal components of stresses, Fig. 5a.

(2) Further deformation due to normal aerodynamic pressure stress on the resulting concave surface, causing continued radial separation of the aggregate with respect to its center of mass. As the sphere flattens, a high non-symmetrical pressure distribution occurs that will further accelerate the particle. If the pressure stress, greatest at the stagnation point, exceeds the tensile strength of the aggregate, it will begin tearing through the axis of symmetry, Fig. 5b.

(3) The tangential shear stress due to gradients in the boundary layer will produce an additional radial component of acceleration of the surface, Fig. 5c. For a laminar boundary layer ( $Re < 3000$  over a disc, this shear stress is given by<sup>(6)</sup>

$$\tau_0 = 0.332 \left( \frac{\eta \rho u^3}{r} \right)^{1/2} \quad (13)$$

(4) The inertial force of the aggregate acts through its center of mass in a body-fixed frame, Fig. 5d.

Hence, during acceleration of the aggregate, a couple is produced with radial components. The net effect is to increase the axial stress parallel to flow, with radial acceleration away from the center of mass. The shear stress on the surface produces additional relative

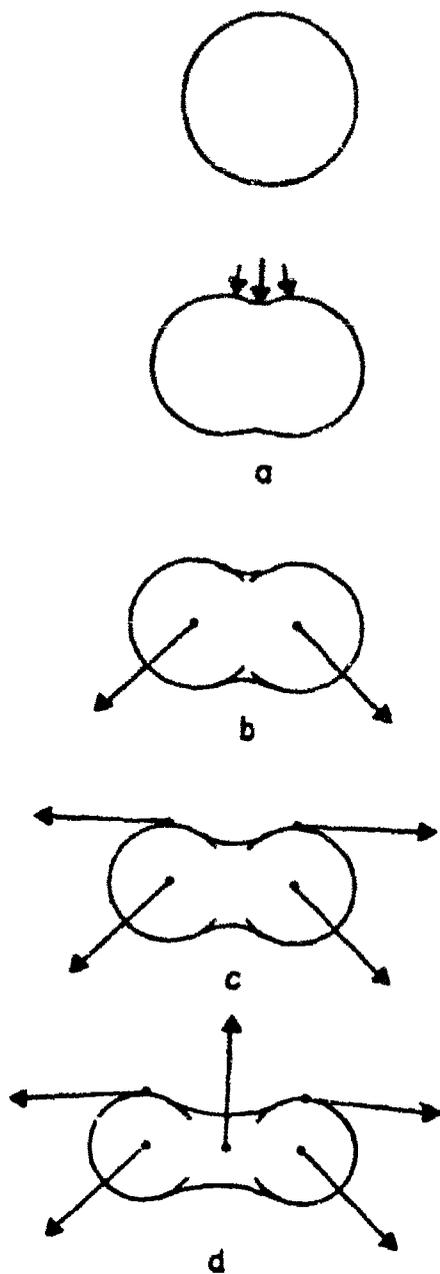


Figure 5. Schematic Diagram of Breakup Process of Quasi-Spherical Aggregate.

motion of the surface with respect to the interior of the aggregate, which moves with a radial component because of the off-axis normal pressure stress. Deagglomeration results from this relative motion of the component parts, or sub-aggregates, away from each other.

Another possible factor contributing to deagglomeration is "cavitation" in the agglomerate voids when the static pressure on the surface falls below the ambient pressure inside. A maximum pressure gradient for the potential flow case considered would occur at  $\theta = 90^\circ$ , leading to an "explosive decompression" radially and normal to the relative stream velocity.

## THEORETICAL AEROSOL SIZE DISTRIBUTIONS

### A. Mathematical form

#### 1. Drag-Induced Deagglomeration

It was shown above that an agglomerate consisting of small uniformly sized basic particles with non-uniform packing is more probable than one with uniform packing. Further, from symmetry consideration of random collisions, a quasi-spherically shaped agglomerate consisting of five sub-agglomerates would be the most probable non-uniform array. Hence, an aggregate is considered to consist of sub-agglomerates, each in turn of sub-agglomerates, etc., and the deagglomeration process to consist of breakup of a particle into its composing sub-agglomerates in a single-cycle, step-wise process.

An aerosol generated from a mass of uniformly sized basic particles will have an upper bound on its size distribution, i.e., a largest size will exist, and all aggregates larger than this size will have been deagglomerated. Deformation of an agglomerate will probably take place when an applied aerodynamic "deforming" stress exceeds the minimum tensile stress of the aggregate, and once deformation occurs, deagglomeration follows. A measure of the probability of deformation and therefore of deagglomeration of an aggregate into its sub-aggregates can be expressed by:

$$\bar{P} = K \frac{D}{F_c} \quad (14)$$

$\bar{P}$  = probability of deagglomeration

D = available deagglomerating force, assumed constant for a given process

$F_c$  = minimum cohesive force holding aggregate together

K = factor of proportionality, constant for a given process

For aerodynamic drag,

$$D = \frac{C_D \rho U^2 \pi d^2}{8} \quad (6)$$

d = diameter of spherical agglomerate

Also

$$F_c = \beta f_c \quad (15)$$

$\beta$  = number of contacts between sub-agglomerates in plane through center of agglomerate

$f_c$  = force per contact between sub-agglomerates

But

$$\beta = (n-2)3 \quad (16)$$

n = number of sub-agglomerates in each agglomerate  
(n = 5 as a probable value)

And for osculating spherical sub-agglomerates where point contact is assumed

$$f_c = f_{cb} \quad (17)$$

$f_{cb}$  = force per contact between two basic particles

Therefore

$$F_c = (n-2)3 f_{cb} \quad (18)$$

Hence

$$\bar{P} = \frac{K C_D \rho U^2}{\frac{8(n-2)3 f_{cb}}{\pi d^2}} \quad (19)$$

For all particles equal to or larger than the upper bound on the aerosol size distribution, which have been broken down by the application of the deagglomerating stress

$$\bar{P} = \frac{K C_D \rho U^2}{\frac{8(n-2)3 f_{cb}}{\pi d_u^2}} = 1 \quad (20)$$

$d_u$  = upper bound, or smallest sized aggregate with a probability of deagglomeration of unity

And for a given deagglomerating process, characterized by a constant drag force

$$K = \frac{8(n-2)3 t_{ch}}{\pi C_D \rho U^2 d_\mu^2} \quad (21)$$

K can be taken as a relative measure of the efficiency with which available specific energy in the gas stream is utilized in effecting application of the deagglomeration stress.

Combining Equations 19 and 21, the probability of deagglomerating any other aggregate of diameter  $d_i < d_\mu$ , into its composing sub-aggregates, is given by

$$\bar{P}_i = \frac{d_i^2}{d_\mu^2} \quad (22)$$

$d_i$  = diameter of aerosol agglomerates

$i$  = index denoting stage of successive agglomerate breakdown, 0, 1, 2 ---

But

$$d_\mu = d_0 + \epsilon \quad (23)$$

where

$d_0$  = representative number weighted diameter of largest particle in aerosol, after deagglomeration process is complete

$\epsilon$  = arbitrarily small number

For

$$\epsilon = 0.005 d_\mu$$

$$\bar{P}_i = \frac{0.99 d_i^2}{d_0^2} \quad (24)$$

But for  $n = 5$

$$d_{i+1} = \frac{d_i}{2} \quad (25)$$

Therefore

$$\bar{P}_i = \frac{0.99}{2^{2i}} \quad (26)$$

The number-size distribution of a stable aerosol, after the dissemination process is complete, can be computed on the basis of Equations 26, 25, and 23. A series expression with histogram class intervals, rather than a continuous distribution function, results. Random fluctuations about mean values for each class interval would physically result in a continuous distribution, however.

The number of particles in each size fraction of the generated aerosol is computed from the difference between the number entering a size class (from the size class immediately preceding it in the direction of increasing size) and the number leaving it as a result of the next successive stage in the deagglomeration process.

$$\eta_0 = N_0 (1 - \bar{P}_0) \quad (27)$$

$$i = 0$$

$\eta_0$  = number particles in zero<sup>th</sup> class corresponding to size  $d_0$

$N_0$  = total number of particles entering class with  $d = d_0$

Similarly

$$\eta_1 = (N_0 n \bar{P}_0 - N_0 n \bar{P}_0 \bar{P}_1) \quad (28)$$

$$i = 1$$

$n$  = representative number of sub-agglomerates in an aggregate

$n$  constitutes the average number of pieces into which a particle will break as a result of a single stage in the deagglomeration process, taken as 6 in the agglomerate model. Hence, the general expression for the number of particles in the generated aerosol, as a function of the deagglomeration stage, is given by

$$\eta_i = N_0 n^i \bar{P}_{1-1} \bar{P}_{1-2} \dots \bar{P}_0 (1 - \bar{P}_i) \quad (29)$$

or, using Equation 26 for  $\bar{P}_i$ ,

$$\eta_i = N_0 n^i \bar{P}_0^i 2^{-i^2} (2^i - \bar{P}_0 2^{-i}) \quad (30)$$

The average diameter of an agglomerate associate with each class,  $i$ , in Equation 30 is obtained from Equation 26.

$$d_i = \frac{d_0}{2^i} \quad (31)$$

$$i = 0, 1, 2 \dots$$

$d_0$  is determined by the physical parameters of the deagglomeration process from Equations 16, 20, 23

$$d_0 = \left( \frac{8 \beta f_{cb}}{K \pi C_D \rho U^2} \right)^{1/2} \quad (32)$$

A continuous cumulative number-size distribution can be constructed by

summing over  $i$  and associating  $\eta_i$  with sizes of particles having an upper class value of  $d_i$ . Number-size distributions for the case of  $n = 5$  are shown in Fig. 6 with various values of  $d_0$  as a parameter. A quasi-log normal distribution results with an average standard deviation of  $1.60 \pm 0.1$ . The slope is invariant with the value assumed for  $n$ .

The distribution is of course bounded by  $d_0$  and  $d_5$ , where  $d_5 =$  diameter of the basic particle. This precludes an explicit log-normal distribution form as mathematically inferred by Kolmogoroff<sup>(7)</sup> for particles resulting from grinding. It differs significantly from that of Martin<sup>(8)</sup> and subsequently derived by Dallavalle, who found the number-size distribution function in the case of fracture to follow the compound interest law.\*

The transformation from a cumulative number-size distribution to a cumulative weight distribution can be accomplished in two ways. Constant particle density is assumed.

1. Assume a quasi-log normal fit, and use an average value of  $\bar{\sigma}_g$  for the two straight-line portions of the curve:

Then

$$d_c = d_m e^{-3 \ln \bar{\sigma}_g} \quad d_c = \text{number median diameter} \quad (33)$$

$$\bar{\sigma}_g = 1.60 \pm 0.10 \quad (34)$$

or

$$d_m = 1.95 d_c \quad d_m = \text{mass median diameter} \quad (35)$$

2. Make no assumptions concerning the theoretical form of the distribution, and compute the distribution by

$$f_{(m)}_i = f_{(n)}_i d_i^3 \quad (36)$$

The increase in slope of the cumulative number-size distribution between  $\frac{d_0}{2}$  and  $d_0$  is due to the averaging effect of the model, which precludes non-integral changes in agglomerate sizes. For this large size class the series summation does not adequately reflect a continuum. Consequently, method number 1 for transforming the number distribution

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\* Georgia Institute of Technology, Atlanta, Georgia. "Studies and investigations of agglomeration and deagglomeration of solid particles," by C. Orr and J. M. Dallavalle. Semifinal Report. 30 June 1956. Contract DA-18-065-404-CML-88.

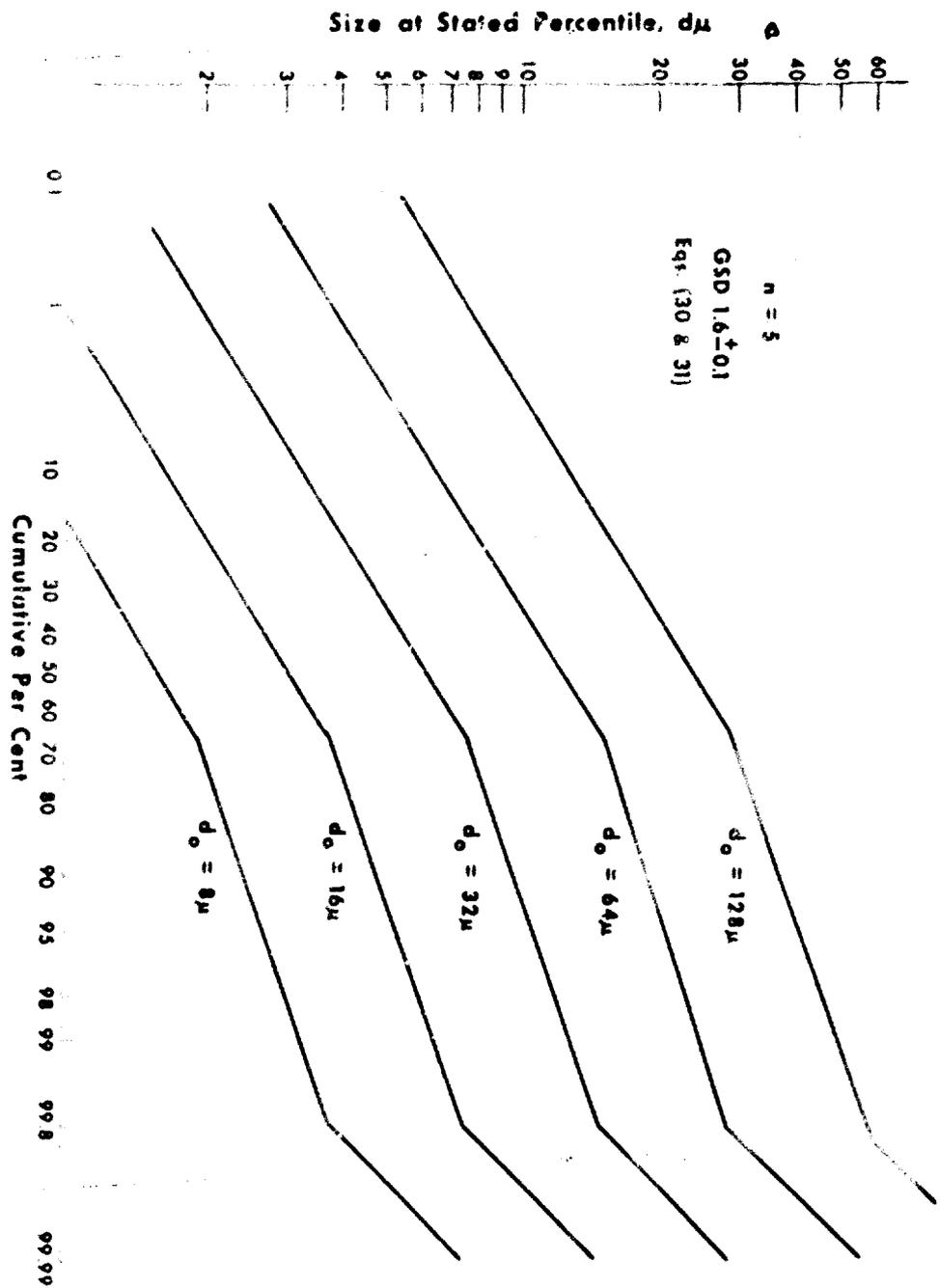


Figure 9. Theoretical Cumulative Number-Size Distributions of Dry Aerosols with Maximum Representative Size,  $d_0$ , as Parameter.

to a mass-weighted distribution is the more accurate. Variations of agglomerate density with size have been neglected in the transformation.  $d_0$  corresponds to the 97% in the mass transformed distribution.

Hence, in summary:

For number-size distributions

$$d_c = 0.21 d_0$$

$$\sigma_g = 1.60 \pm 0.1$$

$d_0$  = corresponding to the 99.99 percentile on a logarithmic probability plot

For mass-size distributions

$$d_m = 0.42 d_0$$

$$\sigma_g = 1.60 \pm 0.01$$

$d_0$  = corresponding to the 97.0 percentile

A plot relating the median size and the size corresponding to the 15 percentile to the representative largest size present in the aerosol,  $d_0$ , is given in Fig. 7 for both number- and mass-weighted distributions. The vertical lines on the mass distribution reflect the spread of values between the two methods of computing the transformation. The curve corresponds to method 1.

## 2. Size Distribution of Particles by Impact Grinding

The size reduction of basic particles by impaction grinding under conditions of turbulent flow can be considered to consist of two independent mechanisms, both associated with a probability, and the probability of breakup can be computed on the basis of the product of probabilities of each mechanism occurring. The mechanisms are: impact with a surface as a result of a particle leaving a stream line, and fracture as a result of the impact.

Hence

$$\bar{P}_B = \bar{P}_1 \bar{P}_f \quad (37)$$

$\bar{P}_B$  = probability of breakup

$\bar{P}_1$  = probability of impact

$\bar{P}_f$  = probability of fracture

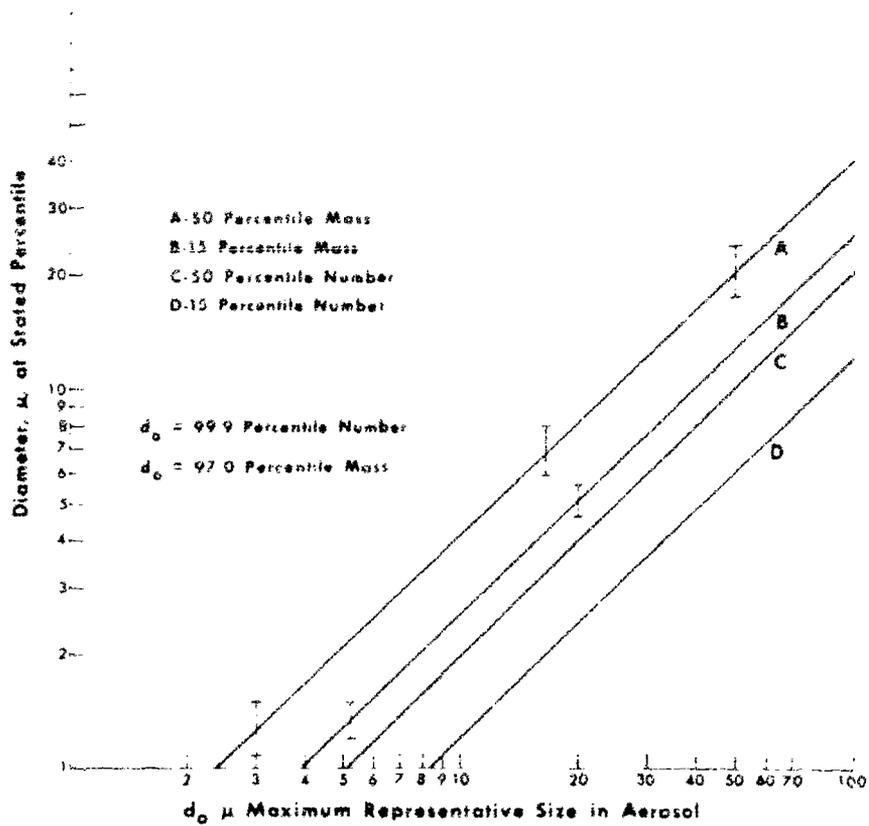


Figure 7. Theoretical Number and Mass Size Distributions of Dry Aerosols Determined from Maximum Representative Size,  $d_0$  CSD  $1.6 \pm 0.1$ .

The probability of a particle leaving a curved stream line and colliding with a bounding surface is proportional to the centrifugal force acting on the particle due to its inertia and the shear drag opposing radial motion

$$\bar{P}_1 = \bar{K} \frac{\frac{\pi}{6} \rho_p d^3 U^2}{R} / 3\pi\eta U d \quad (38)$$

or

$$\bar{P}_1 = \frac{K' \rho_p U d^2}{18 \eta R} \quad (39)$$

R = average radius of curvature of stream lines at impact surface

K' = constant of proportionality

The probability of fracture upon impact can be considered proportional to the specific energy available for fracture work and inversely proportional to the specific energy required for fracture. Particles undergoing plastic deformation would tend not to fracture.

$$\bar{P}_f = \frac{K'' \frac{1}{2} \rho_p U^2}{S^2 2E} \quad (40)$$

S = critical compressive stress of particle

E = modulus of elasticity of particle

Hence, probability of breakup under impact, using Equation 37

$$\bar{P}_B = \frac{K' \rho_p U d^2 K'' \rho_p U^2 E}{18 \eta R S^2} \quad (41)$$

Or for a given system

$$\bar{P}_B = K''' d^2 \quad (42)$$

K''' = constant

Data have been obtained by Epstein<sup>(9)</sup> in support of Equation 42 for the impact-breakage probability being proportional to  $d^2$ , in contradiction to a basic assumption in his derivation of lognormal distribution for breakage of solids

Applying the same reasoning used in the derivation of Equation 30, where, in place of an upper characteristic aggregate size an initial uniform basic particle size exists, an identical distribution of

numbers of particles as a function of stage in the breakup process is obtained. Sizes are quasi-log normally distributed, if the sizes related to successive stages are in a constant ratio.

For

$$\frac{d_i}{d_{i+1}} = 2, \text{ then } \sigma_g = 1.6 \pm 0.1$$

The geometric standard deviation,  $\sigma_g$ , depends on the ratio of sizes but is independent of  $n$ , the number of fragments resulting from a single fracture. For spheres,  $n = 4$ , or  $6$ ; for flat platelets,  $n = 2$ ,

$$\text{if } \frac{d_i}{d_{i+1}} = 2$$

## B. Experimental observations

### 1. Aerodynamic Deagglomeration

A major difficulty in determining aerosol properties is the effect of the measuring device on the parameter of interest. This is particularly true of devices that collect a "representative sample" of the aerosol, such as the Cascade impactor<sup>(10)</sup> for example. Light-scattering techniques, such as those of Dimmick<sup>(11)</sup> and O'Konski<sup>(12)</sup>, though they do not require the collection of representative samples and hence do not disturb the system being measured, nonetheless require careful interpretation, involving a knowledge of the interaction of the containing chamber with the aerosols, as well as interpolation of light-scattering phenomena. A further complication is the inherent instability of an aerosol due to collision processes involving the suspended particles, i.e. random collisions due to turbulence or Brownian motion<sup>(13)</sup>

One system that tends to minimize sampling and interpretation problems, and that is suited for aerodynamic aerosol generation studies, has been found to be the Sharples Micromerograph<sup>(14,15)</sup>. Designed for small quantities of material (< 50 mg) it measures a cumulative mass-Stokes' drag diameter distribution of the entire aerosol, and has been found satisfactory, with careful handling, for the determination of particle sizes  $\geq 1 \mu$  d. It operates on the principle of aerodynamic drag, subjecting a small mass of powder to a steep-fronted sonic air blast at controllable stagnation pressures. The particulate solids entrained in the sonic flow pass through an expanding conical orifice or nozzles of special design, and the steady-state aerosol is measured

by a continuous cumulative weighing of particles that have settled according to Stokes' Law, Fig. 8.

One series of studies on the effect of gas density on breakup\* with the Micromerograph used as the test powder Serratia marcescens, a ground, freeze-dried vegetative microorganism contained in an inert crystalline-like matrix. It had a mass median diameter,  $d_m$ , = 4.1  $\mu$  and a geometric standard deviation of = 1.6.

Similar studies, using capillary tubes as dispersing nozzles, were conducted by Orr and Dallavalle<sup>(16)</sup> with 3.3  $\mu$  MMD S. marcescens product. The basic size distributions, though not conforming to that strictly required of the model, i.e. homogeneity of size, small median diameters and geometric standard deviations. The characteristic basic particle size for the heterogeneous distributions would correspond to the average volume diameter. The mass-size distribution data obtained from both these studies, for a range of stagnation pressures of 50 psig to 400 psig, have been compared with the aerodynamic aerosol generation model presented above, in an attempt to correlate compressible flow and particulate solid properties with the aerosol size distribution as follows:

On the basis of the model, the absolute size distribution is defined in terms of  $d_o$ , the representative largest agglomerate present in the aerosol. This value in turn is related to the particle properties and compressible flow properties by Equation 32.

$$d_o = \left( \frac{8 \beta f_{cb}}{K \pi C_D \rho U^2} \right)^{1/2} \quad (32)$$

All quantities with the exception of K are either measured, calculated or inferred from the model.

a.  $d_o$ , maximum measured representative agglomerate size, corresponding to the 97 percentile on a mass weighted basis.

b.  $f_{cb}$ , the force of cohesion between sub-aggregates. Assuming point contact, this is equal to the force between basic particles. A minimum value for this force has been obtained from bulk tensile strength measurements of S. marcescens powder, assuming close packing

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\* Bures, M. G., J. S. Derr, unpublished data.

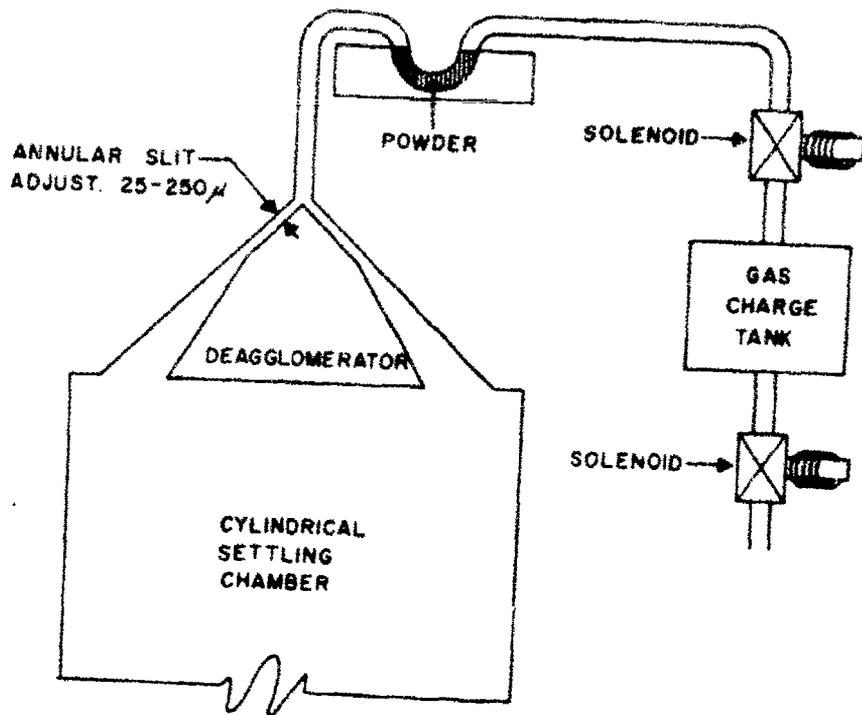


Figure 8. Schematic Diagram of the Micromerograph Deagglomeration Section.

of basic particles\*. Bulk tensile strengths are of the order of 2000 dynes/cm<sup>2</sup> for a packing fraction of ~ 0.20.

$$f_{cb} \geq 1.8 \times 10^{-4} \text{ dyne}$$

- c. U, gas velocity, sonic at the powder-gas interaction rate.
- d.  $\rho$ , gas density, computed for isotropic flow at Mach 1, for the various stagnation pressures.
- e.  $C_D$ , drag coefficient computed on the basis of Reynolds number, with  $d_0$  as the characteristic dimension. Values ranged from 0.38 to 0.50.
- f.  $\beta$ , number of contacts between sub-aggregates For  $n = 5$   $\beta = 9$ , assuming close packing.

Values of  $\beta/k$  were calculated using Equation 32, and a mean was determined for all experimental conditions, with the 95 confidence interval on the mean expressed.

$$\beta/k = 2.2 \times 10^4 (\pm 0.5 \times 10^4 \text{ 95\% C.I.}) \quad (43)$$

Using this value of  $\beta/k$ , theoretical mass-size distributions were computed using Equations 30, 31, 32 for the values of gas density used in the experiments. A mean value of  $C_D = 0.43$  was also used, and sonic flow assumed.

A comparison of the theoretical distribution with experimental average values is given in Table 1. The experimental geometric standard deviation is a mean of the 50/16 and 84/50 percentiles, as is the theoretical GSD. The agreement is close, considering the variety of conditions over which  $\beta/k$  was computed and the departure from experimental conditions of the model assumption of homogeneity of basic particle size. Generalization, however, cannot be inferred from the specific absolute value of  $\beta/k$  obtained from these experiments without additional work with other powders. As the mass median diameter of the basic heterogeneous particle size distribution increases, measured values of  $\beta/k$  for a given  $f_{cb}$  would increase, reflecting a larger apparent  $d_0$  than a homogeneous model would predict. What is perhaps of interest is the apparent constancy of the ratio  $\frac{f_{cb}\beta}{k} \approx 4$  under the conditions studied for the S. marcescens material.

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\* Derr, J.S., M. G. Bures, G. Gordon, unpublished data.

TABLE 1. Absolute mass-size distribution of *Serratia marcescens* powder-sonic deagglomeration model compared with experiment

Stagnation pressure		"4.1" <i>S. marcescens</i>	"3.3" <i>S. marcescens</i>	Theory
50 psig	$d_m^{\bar{x}}$	7.4 $\mu$	9.0 $\mu$	11.5 $\pm$ 3.2* $\mu$
	$\sigma_g$	1.61	1.75	1.60 $\pm$ 0.1
100 psig	$d_m^{\bar{x}}$	7.4	9.0	8.3 $\pm$ 2.0
	$\sigma_g$	1.68	1.67	1.60 $\pm$ 0.1
200 psig	$d_m^{\bar{x}}$	6.7	6.9	6.1 $\pm$ 1.5
	$\sigma_g$	1.60	1.71	1.60 $\pm$ 0.1
300 psig	$d_m^{\bar{x}}$	6.3	5.8	5.1 $\pm$ 1.4
	$\sigma_g$	1.58	1.62	1.60 $\pm$ 0.1
400 psig	$d_m^{\bar{x}}$	5.8	4.3	4.5 $\pm$ 1.1
	$\sigma_g$	1.51	1.54	1.60 $\pm$ 0.1

\* 95% confidence interval.

$\bar{x}$  Mass median diameter.

Nozzles: Conical deagglomerator, 3 tubes 1/2" L x 0.18", 0.08", 0.04" ID.

Values for both  $\beta$  and  $f_{cb}$  are minimal. Therefore,  $k^{-1}$  is maximal.

$$k^{-1} \leq 2.4 \times 10^3 \pm (0.6 \times 10^3)$$

$k^{-1}$  is analogous to the critical Weber number,  $We_c$ , for liquid droplet instability under aerodynamic drag<sup>(17,18)</sup>.

$$We_c = \frac{D \cdot r}{\sigma} \geq 3 \text{ to } 10 \quad (44)$$

D = drag pressure

r = drop radius

$\sigma$  = surface tension

The apparently high ratio of magnitude of  $K^{-1}/We_c$  may well be due to the uncertainty of the  $f_{cb}$  for which a minimum value has been measured. The presence of 30  $\mu$  d aggregates in the cleavage plane of the tensile strength measurements mentioned above would allow a 100-fold increase in  $f_{cb}$  and result in  $We = K^{-1}$ . Further work, however, is required to clarify this comparison with liquid droplet breakup.

Mass-size distributions determined by the Micromerograph technique for a variety of other powders have been compared with the shape predicted by the model, i.e. quasi-log normal distribution with a geometric standard deviation of  $1.6 \pm 0.1$ . These are shown in Fig. 9. For those powders with basic mass median diameters,  $d_m \leq \mu$  as determined either by optical sizing or with the Whitby Centrifuge technique (19,20), the agreement with prediction is good. Sodium fluorescein and sulphur aerosols, characterized by  $d_m > 14 \mu$  and basic  $\sigma_g \geq 2$ , depart from the model. Theoretical curves were fitted to the experimental ones at the median diameter.

For those powders conforming to the model,  $f_{cb}\beta/K$  was computed from Equation 32 for sonic flow. The results are given in Table 2. Interpretation of these data must wait further work on measurements of  $f_{cb}$ . It is interesting to note, however, that there is order of magnitude agreement for the various types of solids, and in the case of magnesium silicate close agreement in the values despite the large difference in stagnation pressure. Since  $f_{cb}$  and  $\beta$  are properties of the powder, and  $f_{cb}\beta/K$  approximately equal for the two pressures,  $K^{-1}$  should be equal and therefore independent of gas density. The same conclusion is reached in the absolute size distribution correlations discussed above for the *S. marcescens* powder. Hence, it may not be unlikely that  $K^{-1}$  has the same significance in solids aerosol dissemination that the critical Weber number,  $We_c$ , is for liquid aerodynamic breakup.

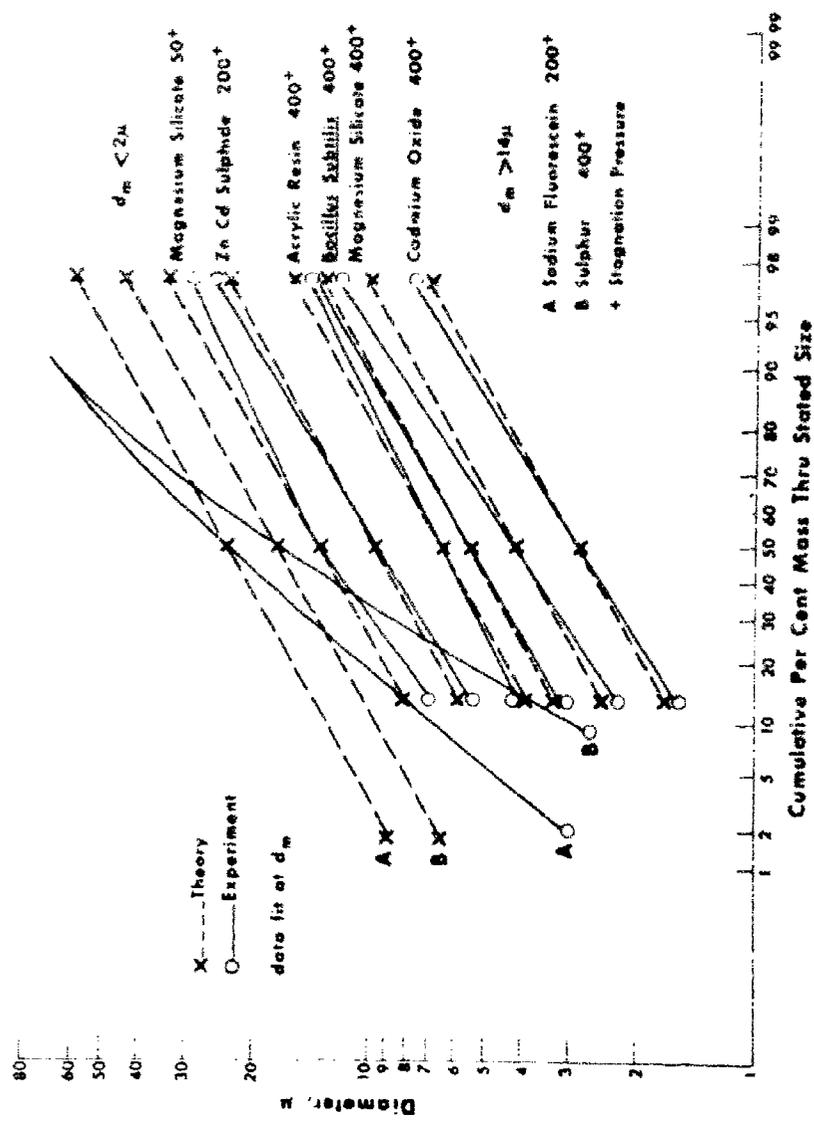


Figure 9. Mass-Size Distribution of Various Powders Sonic Deagglomeration by Micromerograph.

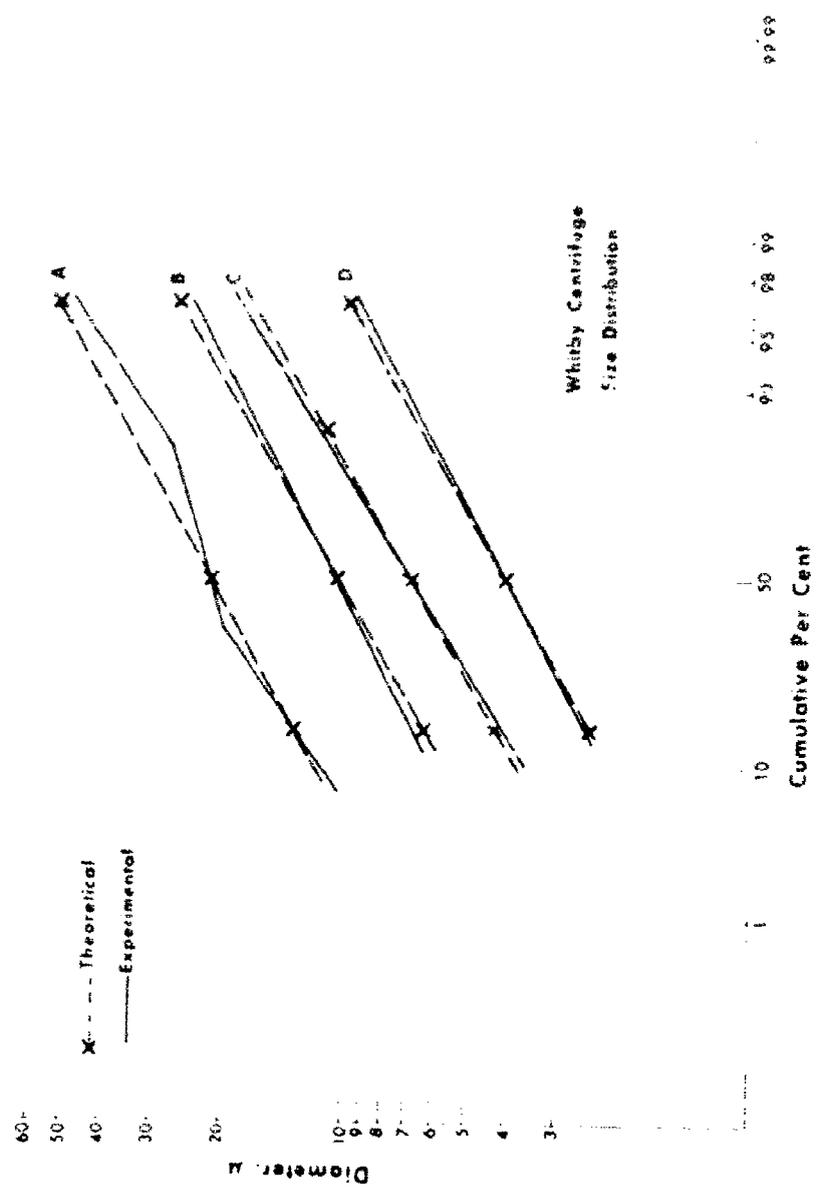


Figure 10. Mass-Size Distribution Freeze-Dried *S. marcescens*, Ground.

TABLE 2. Aerodynamic deagglomeration parameter,  $f_{cb} \beta K$ , for various powders-sonic flow

F powder	Stagnation pressure (psia)	Gas density ( $\text{gm cm}^{-3} \times 10^{-3}$ )	$d_o$ ( $\mu$ )	$C_D$	$\frac{f_{cb} \beta}{K}$ (dynes)
Magnesium silicate	50	2.84	31.0	0.43	4.9
Zinc-cadmium-sulphide*	200	9.5	24.0	.46	11.2
Acrylic resin (PT)*	400	13.9	15.0	.43	7.9
<u>Bacillus subtilis</u> *	400	13.9	13.5	.42	6.3
Magnesium silicate	400	13.9	11.0	.41	4.1
Cadmium oxide*	400	13.9	7.0	0.41	1.7

\* Reference (17).

## 2. Fluid Energy Mill Grinding

Grinding by a fluid energy mill is one effective way of reducing the basic particle size of solids. In a sense, a fluid energy mill can be considered a special type of solids aerosol generator, where high impact efficiency and shear rates are made possible through large energy inputs. The larger tensile or shear strength of basic particles as compared with basic particulate agglomerates requires increased energy and efficiency for grinding as compared with deagglomeration. For example, for S. marcescens powder the basic solid shear strength is of the order of  $10^7$  dynes  $\text{cm}^2$  as compared with  $2 \times 10^3$  dynes  $\text{cm}^2$  for the bulk powder tensile strength of  $4 \mu d_m$  product.

By controlling fluid energy grinder parameters, it is possible to reduce basic particle size in an empirically controlled way for a given material. Results of such a study with the freeze-dried S. marcescens powder are shown in Fig. 10<sup>(21)</sup>. This material, characterized by crystalline structure and a high bulk modulus of elasticity, is readily grindable. The basic particle mass-size distributions shown in the figure were determined by use of the Whitby centrifuge technique, representing that of the completely deagglomerated powder. Curve A resulted from an initial ball-milling operation, producing particles with platelet shape having a characteristic thickness of

5. Powders, whose distributions are shown in curves BC and D of Fig. 10, were obtained by the further fluid energy mill grinding of this ball-milled product, using different values of the grinder parameters. The experimental data are fitted to the modal predictions at the median value, and it is seen that logarithmic-normal mass-size distributions with a geometric standard deviation,  $\sigma_g$ , of  $1.6 \pm 0.1$  are obtained, as inferred by the discussion in the Section entitled "Theoretical Aerosol Size Distributions", Part A, 2.

#### DISCUSSION

Models leading to the prediction of initially generated particle size distributions of particulate solid aerosols and ground powders have been developed. For the aerosol case, a homogeneous distribution of basic particles is assumed, although powders with small median diameters and standard deviations afford working approximations. Data with such powders have been obtained for sonic deagglomeration, using the Micromerograph under a variety of compressible flow conditions. Under the conditions studied, data have been found to support the model predictions for the shape of the size distribution, i.e. quasi-log normal with  $\sigma_g$  of  $1.6 \pm 0.1$ .

For powders having median diameters large compared with aggregate sizes, and also widely heterogeneous,  $\sigma_g > 2$ , distributions do not conform to those of the model. Also, the distributions predicted by the model will not apply to aerosols that have undergone significant amounts of coagulation, sedimentation, or diffusion, after the initial processes of generation are complete. Further, if in the generation or grinding process, probabilities for interaction with breakup stress are weighted in favor of certain size fractions, the basic premise of equal access to the stress by all particles is contradicted, and the model would not apply. For example, if  $P_1$  is the probability that a particle subjected to a constant stress breaks up in a fixed time interval,  $\Delta t_1$ , then the probability that it will break up in  $\sum_1 \Delta t_1 = t_1$  is given by

$$\bar{P}_{1,t_1} = 1 - (1 - P_1)^{\frac{t_1}{\Delta t_1}} \quad (44)$$

Absolute size distribution predictions, based on particle properties and dynamic properties of the compressible flow disseminating system, were successful within the accuracy of model for the one powder tested and on a single scale of dissemination. In this study, there is indication that a generally applicable number,  $K^{-1}$ , may apply for aerodynamic breakup of solids, similar to the critical Weber number for liquid drop instability. An upper bound on the number was obtained. More research on basic particle contact forces and bulk tensile strengths of powders will be required to establish the validity of this concept, however. It may also be found that  $K^{-1}$  is a function of the compressible flow system, involving the length of time an aggregate of basic particles is subjected to the deagglomerating stress.

For friable solids, characterized by a quasi-crystalline structure, low shear stress, and high elastic modulus, grinding data have been obtained supporting the concept that grinding probabilities are proportional to the square of the particle diameter. In no sense can this be applied universally to all solids, however. For example, an increase in size of particles of Kaolinite (refined Devon China clay) has been observed during ball-milling<sup>(22,23)</sup>. Further, white blasting sand demonstrated an increase in particle size as a result of dry grinding by mortar and pestle<sup>(24)</sup>. For materials of these types, at least, a suitable grinding model would have to predict fusion of basic particles instead of fracture at some point in the grinding process.

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#### DISCUSSION

Wolochow: Would you make a comment on the problem of satellite production with the spinning disc?

Derr: Satellites were not predicted according to the theory of Lord Rayleigh, although he discussed them. They do exist, along with the main drops, in spinning disc atomization, and elimination of them poses a problem. Attempts have been made to utilize secondary air exhaust to isolate the satellites. Since they are smaller than the main drops they slow down faster, and consequently can be wafted out of the way by relatively slow velocity air stream.

Dimmick: Can you predict satellites from liquid suspensions?

Derr: No. This paper treats the breakup of solid particles. (Lord Rayleigh in his paper "On the Capillary Phenomena of Jets", *Proc. Roy. Soc.* 29, 1879, qualitatively discusses satellites on pp. 88, 89. A further discussion of satellites can be found in "Injection and Combustion of Liquid Fuels", Battelle Memorial Inst., March 1957, WADC Tech. Rept. 56-344, ASTIA Doc. AD 118142.) I might add, however, that droplet production with a "vibrating reed", which has a similar basic mechanism to that associated with a spinning disc involving the rupture of a filament, is free of satellites. In spinning disc atomization a filament of liquid moves from the disc edge under centrifugal acceleration, and when the product of its mass, square of tangential velocity, and inverse radius of rotation exceeds the product of surface tension and filament circumference, the filament breaks, producing the main spherical drop; the portion of the filament attached to the disc collapses, breaks at the disc, and forms a smaller droplet, or satellite. With the "vibrating reed" technique, a small wire dips into a reservoir of liquid, at a fixed frequency, forming a filament which breaks, producing the main drop. The filament does not break again at the reservoir, and no satellite is formed. (See "Study of Vibrating Reed in the Production of Small Droplets and Solid Particles of Uniform Size", Wolf, W. R., *Rev. Scientific Instruments* 32(10): 1124-1129, October 1961.)

VFIF\*: Is it possible that the ruptured filament producing the drop (with the vibrating reed) corresponds to the portion of the filament producing the satellite with the spinning disc, and that the primary particle, as associated with the spinning disc, is stuck on the end of the reed and does not come off?

Derr: Yes, that is because the reed is always wetted.

VFIF: And the vibrating reed utilizes satellites instead of primary particles, which go marching off as they please.

Thurston: In the context of your paper, is the treatment based on ground round particles? Are you assuming that ground particles are round, or can the distribution of shapes be random?

Derr: For purposes of ease of computation, Dr. Thurston, we assume spherical basic particles, although this is not necessary; it could be any small particle as long as it is characterized by the same average size.

Thurston: But if you have a random distribution of particles, you are going to have entirely different surface characteristics than those assuming perfect spherical particles.

Derr: Yes. The aggregates would certainly not be perfectly spherical, this we know; but on the average they might have quasi-spherical symmetry.

Thurston: What, if anything, has ever been done in actually making particles in the 1-5  $\mu$  range by spray-drying particles of that size using hot air for aerosol dissemination?

Derr: This has been done. Particles produced in this way would be the basic particles that I have assumed in the model, not the aggregates. Of course, these basic spray dried particles are beautifully spherical.

Thurston: How do they agglomerate? In a manner of polystyrene?

Derr: Polystyrene latex would be an example of this.

Thurston: Then it is a liquid, not a solid? You are making an emulsion then; a suspension?

Derr: Essentially. These are dried, and would be dispersed as a dry aerosol; but we have tried spray-dried S. marcescens and these particles also can be disseminated in the same way.

Thurston: Would there be any difference between polystyrene and a bacterial organism? In the one case you have a straight hydrocarbon

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\* VFIF = Voice from the Floor.

type of material; in the other case you have polypeptides, proteins, lipids, and so forth. There ought to be a lot of difference in hydrogen bonding and other intermolecular forces. Is there?

Derr: This difference in surface chemistry would bring out the differences in contact forces and energies between the basic particles, which are controlling parameters. There are differences among the different materials. For example, you saw an acrylic resin here which is an example of a plastic, and B. globigii which would be an example of a fairly smooth and quasi-spherical biological material.

Thurston: How does relative humidity (RH) come into your projection?

Derr: RH would essentially influence again the force of cohesion. RH will do two things. It would allow the possibility of the adsorption of a water monolayer at the interface between the two particles. If this layer should build up, it could contribute to a surface tension force of cohesion. This could happen because the curvature of the surface is negative, and the vapor pressure on the particle surface at the air-water interface would be lower than the ambient vapor pressure. So at any appreciable humidity you would get a condensed water layer. Now the second thing would be the influence of humidity on the matrix material of the basic particle. If this were hygroscopic and the particles were in contact, a fusion could take place which could exceed the area of fusion predicted by, for instance, van der Waals' attraction for perfect spheres. There would be diffusion of the material from one particle to another and actual bonding could occur. This has been noted.

Thurston: In other words, it would result in greater difficulty in deagglomeration?

Derr: Yes.

## A STUDY OF DYNAMIC AEROSOLS OF BACTERIA SUBJECTED TO RAPID CHANGES IN RELATIVE HUMIDITY

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Airborne microorganisms are often subjected to rapid fluctuations of relative humidity (RH) and temperature in their natural environment. The influence of such changes on the viability of airborne bacteria has not been extensively investigated. Results of studies conducted under conditions of unchanging RH have suggested that death is primarily attributable to alteration in cellular or molecular structure of proteins resulting from lehydration (Kethley, Fincher, and Cown, 1957; Ferry, Brown, and Dammon, 1958; Webb, 1960).

To study this problem we subjected aerosols of Sarcina lutea and Serratia marcescens to a sudden change in RH at a constant temperature and pressure in a Dual Aerosol Transport Apparatus (DATA), consisting of two of the units described by Leif and Hebert (1951). Airborne cells were first allowed to equilibrate at a given RH for about 5 min and were then either subjected to dilution with air at the same or at a different RH. The change in "rate" of death of bacteria was examined in the first (primary) condition and the second (diluted) condition.

### MATERIALS AND METHODS

#### Organisms and Preparation of Spray Suspensions.

Cells of S. marcescens, strain 8 UK, were grown in either modified Bunting's medium (Dimmick, Heckly, and Hollis, 1961) at 1/2 strength or in trypticase soy broth (BBL). Only third-pass cultures of cells cultivated 21-12 hr at 31 C on a rotary shaker were studied.

Usually, cell populations were chilled at 4 C for 15 min and immediately diluted in their respective growth medium to concentrations containing from  $2.8 \times 10^5$  to  $8.5 \times 10^6$  viable organisms/ml and then held at 4 C until they could be aerosolized. Suspensions of cells in the complex medium contained 0.1% silicone added to control foaming. Although cultures were atomized usually without removing spent medium constituents, in some experiments cells were washed three times in, and sprayed from, 0.1 M phosphate buffer pH 7.0. Unless otherwise noted, three aerosols were disseminated each day from a single spray suspension. The first aerosol contained populations of cells ordinarily atomized within 60 min after the growth period. The second and third aerosols contained cells aged at 4 C for approximately 3 hr and 5 hr, respectively.

Suspensions of *S. lutea* were produced from cultures grown in heart infusion broth (Difco). The incubation temperature, period of growth and general methods for preparing these suspensions were the same as above. Suspensions containing approximately equal numbers of the two species of bacteria were also aerosolized.

#### Description and Operation of the DATA.

The DATA (Fig. 1) was constructed by connecting short sections of stovepipe. The primary chamber consisted of a tube 6 inches in diameter and 45 ft long inserted approximately 2 ft into a second chamber 8 inches in diameter and of similar length. The two tubes were folded back by 90° bends at 30 and 60 ft. Mixing of the air stream was facilitated by a series of Stairmand baffles (Stairmand, 1951) spaced 22 inches apart on a plane normal to the axis of the tubes. A portion of the aerosol was withdrawn for viable assay and for measurement of physical decay at several sampling ports located at convenient intervals along the apparatus.

A bacterial aerosol at a controlled temperature (21.6 C) and desired RH was introduced into the primary chamber at 1.6 CFM, a flow rate that produced an air velocity of 8 linear ft/min. This input aerosol was diluted at the junction of the two ducts with air also at 21.6 C and at a second desired RH. The resultant linear flow through the secondary chamber was also 8 ft/min; this was accomplished by allowing the conditioned air to flow into the larger chamber at approximately 1.25 CFM and by withdrawing aerosol from the terminal end of

the secondary chamber at 2.85 CFM. The resulting dilution ratio (1.6/2.85) was 0.56. Mixing of the two air streams caused a dilution of the input air of approximately 56% and, in some experiments, a rapid change in RH without an appreciable change in temperature or in pressure. For example, mixing input air at 90% RH with secondary air at 15% RH produced effluent air at 56% RH. The theoretical transit time of the primary aerosols was 5.69 min and the time of the diluted aerosols was 5.56 min; the total transit time, therefore, was 11.25 min.

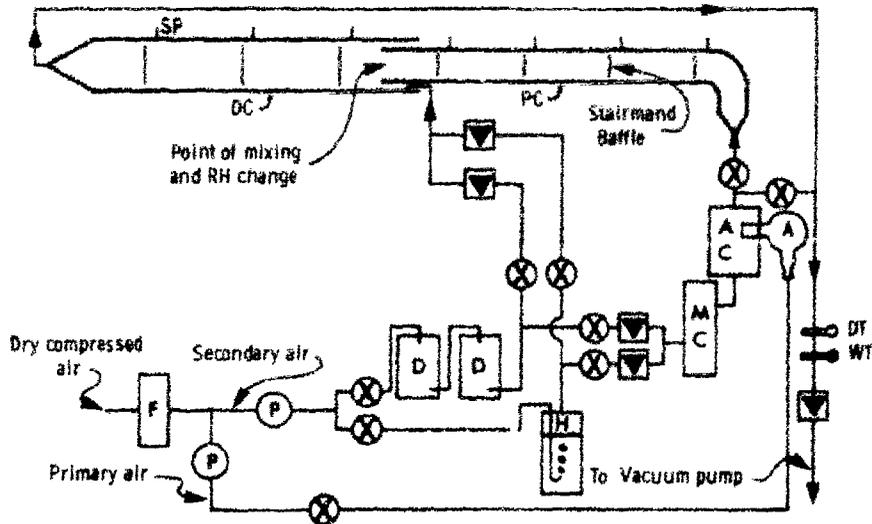


FIG. 1. Schematic diagram of the dynamic aerosol transport apparatus (DATA).

PC, primary aerosol chamber; DC, diluted aerosol chamber; A, reflux-type Wells atomizer; AC, atomization chamber; MC, mixing chamber for conditioning of air; D, Lectrodryer\*; P, pressure regulators; F, filter; SP, sampling ports; H, humidifying chamber; DT, dry-bulb thermometer; WT, wet-bulb thermometer;  $\otimes$ , valve;  $\square$ , rotometer.  
 (\* Registered trade mark)

#### Aerosol Dissemination and Assay.

Suspensions were aerosolized from a reflux-type Wells atomizer operated at 10 psi. Aerosols were generated for 15 min prior to sampling. Cells of *S. marcescens* were collected on blood agar base (Difco) plates by slit samplers operated at 4 liters/min, whereas tryptose agar (Difco) plates were employed to assay aerosols of *S. lutea*. Plates were incubated for 24 hr at 31 C and 48 hr at 35 C, respectively,

prior to counting the colonies. When both bacterial species were present in a given aerosol, duplicate samples were always collected; one on tryptose agar containing 0.15% phenylethyl alcohol and another on blood agar base. The alcohol selectively inhibited growth of S. marcescens, but had no effect upon growth of S. lutea.

Physical decay of the aerosols was monitored by the method of Dimmick, Hatch, and Ng (1958) with a forward-angle scatter aerosol monitor (Goldberg, 1961) and a Universal Photomultiplier Photometer, Model PH-200 (Eldorado Electronics Co., Berkeley).

The number of viable particles per liter of aerosol obtained at the first sampling station, which represented a 30 sec aged aerosol, was taken as 1.0. The viable numbers found at other stations were then expressed as surviving fractions and these decay rates (Kethley, Fincher and Cown, 1957) were labelled total decay. Similarly, the light-scatter readings observed at the first station were also regarded as 1.0. Subsequent readings were expressed as fractions and these rates were labelled physical decay.

#### RESULTS AND DISCUSSION

##### Preliminary study of aerosols of washed S. lutea and S. marcescens: 23% maximum RH shifts.

Six aerosols each of S. lutea and S. marcescens were examined. The physical decay constants of the primary ( $k_{1p}$ ) and the diluted ( $k_{2p}$ ) aerosols of washed cells of both species were of similar magnitude (Table 1), but occasionally some divergencies were noted. A similar relationship was apparent between the total decay constants  $k_{1t}$  and  $k_{2t}$ . These data indicate that after cells of either species had equilibrated to a given atmosphere, a change in the RH and dilution of the aerosol failed to alter appreciably the decay constants. However, differences between the overall physical and total decay rates were evident; obviously cellular death was occurring in the airborne droplet. The rate of total decay was greater with aerosols of washed S. marcescens than with aerosols of washed S. lutea. Moreover, washed cells of the former were so sensitive that the numbers of viable cells per liter of aerosol could not be determined accurately at the low humidity levels tested. The fact that cells of S. lutea demonstrated some biological decay is presumptive evidence that this organism cannot always be employed as a stable biological tracer.

TABLE 1. The effect of rapid changes in relative humidity (RH) on the death rate of airborne populations of washed Sarcina lutea and Serratia marcescens

Organisms	RH, %		Physical Decay <sup>a</sup>			Total Decay <sup>a</sup>		
	Primary Chamber	Secondary Chamber	k <sub>1p</sub>	k <sub>2p</sub>	Observed Dilution ratio	k <sub>1t</sub>	k <sub>2t</sub>	Observed Dilution ratio
<u>S. lutea</u>	22	37	.004	.005	.62	.014	.029	.55
	22	37	.009	.005	.70	.005	.022	.53
	49	49	.008	.008	.57	.016	.022	.53
	47	47	.008	.017	.56	.009	.004	.56
	93	70	.016	.014	.50	.030	.020	.58
	93	75	.008	.017	.49	.038	.030	.68
<u>S. marcescens</u>	27	36	.008	.002	.67	--	--	--
	28	35	.008	.004	.70	--	--	--
	47	48	.008	.005	.56	.136	.121	.33
	48	48	.006	.004	.57	.099	.129	.48
	93	70	.008	.011	.51	.032	.035	.51
	93	86	.008	.020	.54	.039	.024	.50

\* See text for method used in determining the values of the surviving fractions, N and N<sub>0</sub>. The decay rate constant, k, was determined by the method of Kethley (1957), where

$$k = \frac{\log N_0 - \log N_t}{t}$$

k<sub>1</sub> represents the decay rate constant for the primary aerosol and k<sub>2</sub> the constant for the secondary aerosol. N<sub>t</sub> is the surviving fraction at the first sampling station, which represented an aerosol age of 30 sec. t is the aerosol transit time in min.

Preliminary study of aerosols of unwashed S. marcescens grown in a minimal medium: 21% maximum RH shifts.

Fifteen aerosols of unwashed S. marcescens grown in the defined medium were studied at RH percentages changing from 24-36, 49-51, and 90-72. The patterns delineated for three typical aerosols are presented in Fig. 2. Neither the physical nor the total fall-out rates of diluted aerosols were basically altered from those of the primary aerosols, regardless of a change in the RH.

Since the dilution ratio of the air was always 0.56, any departure from this value, observed with respect to either the physical or biological measurements indicated that either the particle size or the biological behavior of the cells had changed. We have designated these as observed ratios

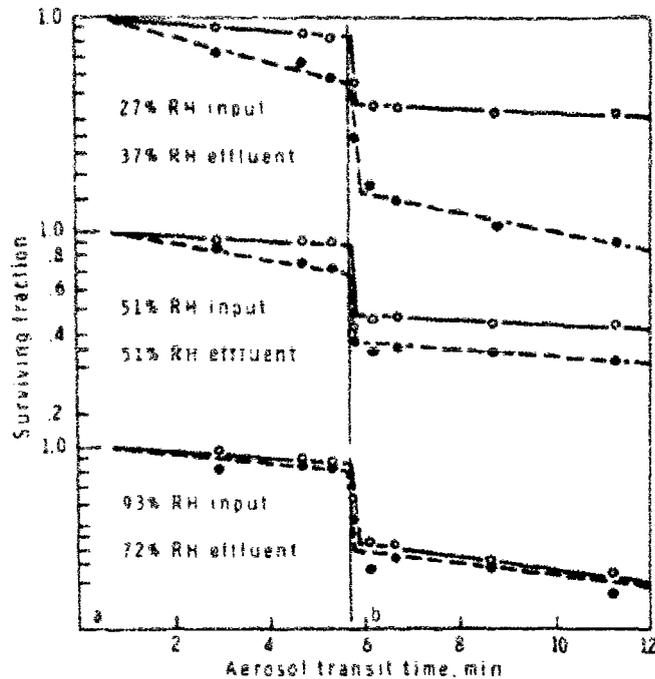


FIG. 2. The effect of rapid changes in relative humidity on airborne populations of *Serratia marcescens* grown in a chemically defined medium. Physical (o) and total (●) decay of primary (a) and diluted aerosols (b).

When primary aerosols, initially equilibrated at about 25% RH, were mixed suddenly with air at 50% RH so that the final RH values were about 36%, we calculated from the physical fall-out data that the average observed ratio of five aerosols was 0.60. The mean observed ratio, when no RH change occurred, was 0.57, and a mean observed ratio of 0.52 was found when the RH was changed from approximately 90 to 73%. Apparently, an increase in RH increased the particle size which resulted in an increase in the light scatter area of each particle. Conversely, a decrease in RH resulted in a decrease in the size of the aerosol particles. The mean observed ratios obtained from the light scatter data from all 89 aerosols included in this report (subjected either to an increase, no change, or a decrease in RH) was found to be 0.67, 0.59 and 0.51, respectively; differences between the means of the three groups of aerosols exceeded the 0.1% level of significance.

Moreover, we found that the observed biological ratios

$$R_B = \frac{\text{Number viable cells after dilution}}{\text{Number viable cells before dilution}}$$

of the 15 aerosols from unwashed cells also deviated from the calculated dilution ratio. When primary aerosols were subjected to an increase in RH, the average observed ratio was 0.48. It was 0.60 for the aerosols with no change in RH and 0.56 for aerosols subjected to decreased RH. These data provide evidence that death within the bacterial population had occurred as a result of the sudden change in the RH although the subsequent decay rates of the diluted aerosols were not markedly changed.

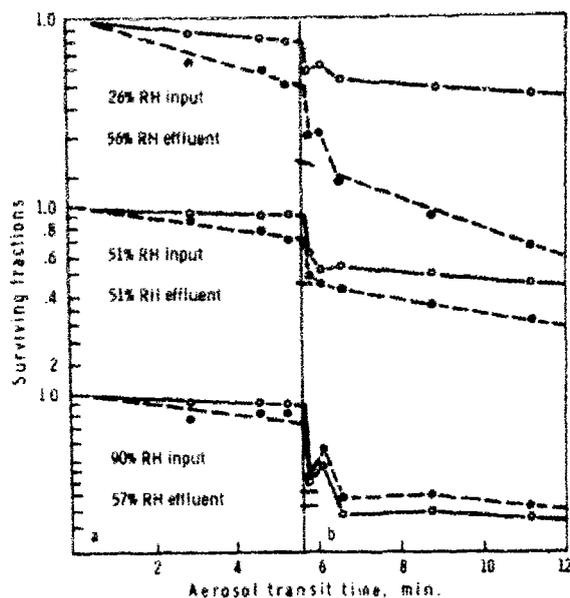


FIG. 3. The effect of rapid changes in relative humidity on airborne populations of *Serratia marcescens* grown in a chemically defined medium. Physical (o) and total (●) decay of primary (a) and diluted aerosols (b).

Study of aerosols of unwashed *S. marcescens* grown in a minimal medium: 33% maximum RH shifts.

In Fig. 3 data are presented for aerosols of unwashed *S. marcescens* initially equilibrated at a low, moderate or a high RH, as before, but the RH of the secondary air was conditioned to achieve a final moisture increase or decrease of approximately 30% RH. Again we observed that the physical decay slopes of the primary and diluted

aerosols were not demonstrably altered by this treatment. Similar results were apparent in the total survival patterns

Dunklin and Puck (1948) reported that *S. marcescens* was extremely sensitive to moisture when in the airborne state at RH values between 50 and 60%. We wondered whether an enhanced death rate would be demonstrable when airborne cells, equilibrated in the primary aerosols at intermediate humidities both above and below the 50% level, were allowed to cross over the RH level of 50%. In Fig. 4 we show the result of subjecting aerosols to a decrease from 60% to 40% RH. Also shown are data from aerosols subjected to an increase from 40 to 60% RH. In the control experiments, the aerosols were diluted with air at an RH of 51%. Again, there was no evidence of significant differences between the slopes of the primary or the diluted aerosols as a result of either an increase or decrease in RH.

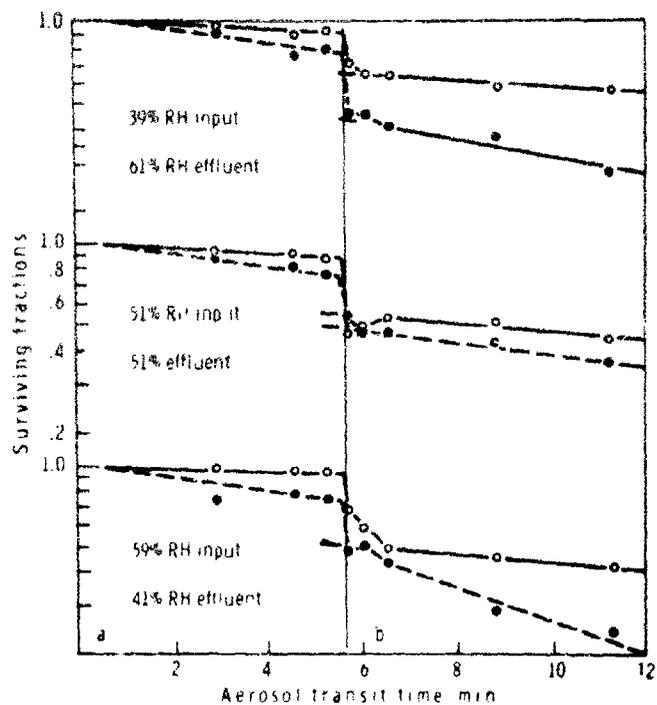


FIG. 4. The effect of rapid changes in relative humidity on airborne populations of *Serratia marcescens* grown in a chemically defined medium. Physical (○) and total (●) decay of primary (a) and diluted aerosols (b).

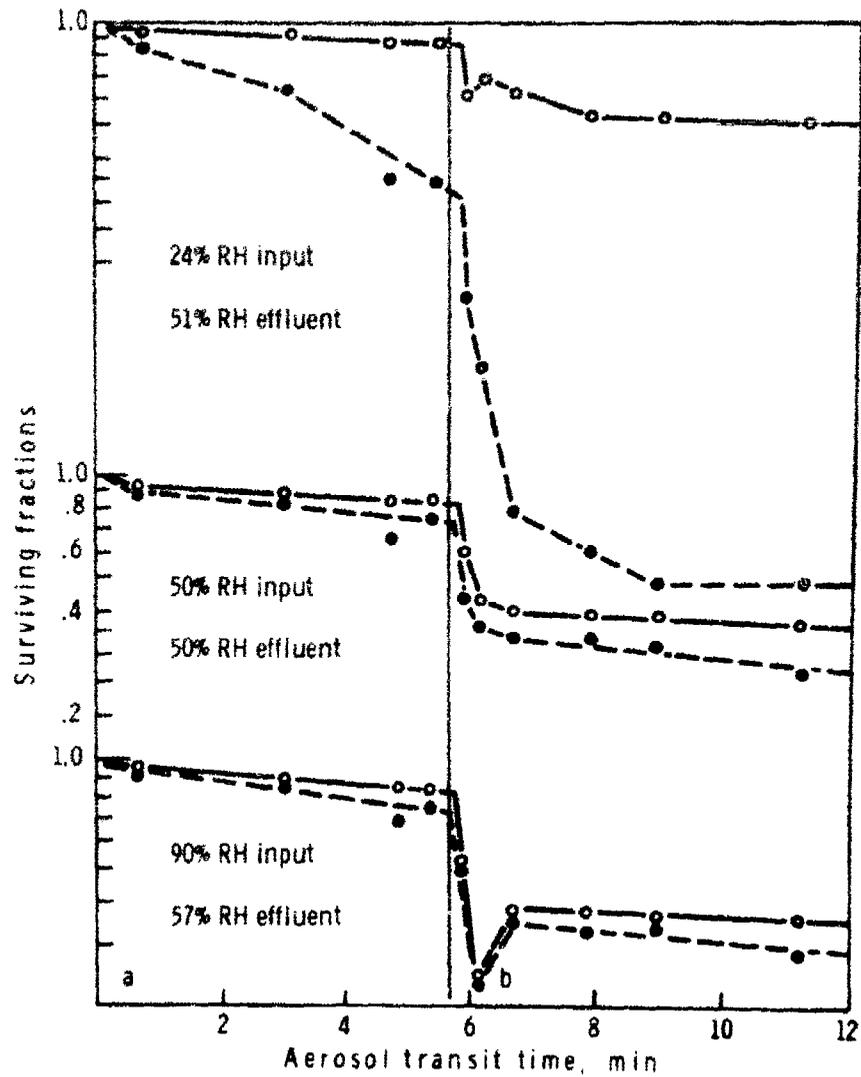


FIG. 5. The effect of rapid changes in relative humidity on airborne populations of *Serratia marcescens* grown in an enriched medium. Physical (o) and total (●) decay of primary (a) and diluted aerosols (b).

Study of aerosols of unwashed *S. marcescens* grown in a complex medium: 35% maximum RH shifts.

Willoughby (1962) demonstrated that the growth medium significantly influenced the survival patterns of airborne *S. marcescens*. In his studies, cells grown in a minimal medium showed an enhanced survival over cells grown in complex media. It was of interest to ascertain whether cells grown in, and dispersed from, medium containing either an abundant or a limited quantity of nutrients would behave in a similar way. We cultivated *S. marcescens* in a complex medium and aerosolized the cells by the usual procedure. In Fig. 5 we present the results of this study. Aerosols that were mixed with air at the same RH or with air at an RH of about 13%, resulting in a change from 90 to 57% RH, showed no real differences in either the physical or total decay patterns. But after a change in RH from 24 to 51%, the death rate increased. Also, immediately after the increase in RH, an altered pattern of survival as well as multiple slopes in the survival curves were seen. Apparently, some cells grown in a complex medium were killed in droplets that had increased in moisture content. But an enhanced rate of death did not occur when airborne *S. marcescens* grown in complex medium were subjected to an RH increase from 59 to 72% (Fig. 6).

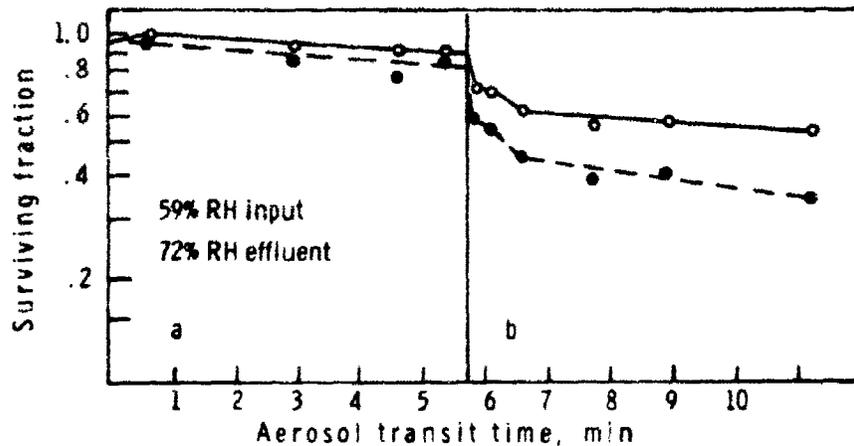


FIG 6. The effect of rapid changes in relative humidity on airborne *Serratia marcescens* grown in a chemically defined medium and aerosolized in an enriched medium. Physical (o) and total (●) decay of primary (a) and diluted aerosols (b)

Hence, "sorbed death" occurred only at certain RH levels. These data are at variance with those reported by Dunklin and Puck (1948), who believed that death of airborne bacteria resulted from cellular dehydration.

To determine whether the observed decrease in survival resulted from toxic constituents in the trypticase soy medium or from cells that were phenotypically different from those grown in the defined medium, we subjected populations of cells grown in the defined medium and sprayed in the complex medium to sudden changes in RH. The results indicated that significant differences in survival patterns of the aerosols diluted at constant RH, or with air at an RH less than that existing in the primary aerosol, did not occur. However, an increased rate of death was observed when cells were subjected to an RH that increased from less to more than 50% (Fig. 7). Physical decay curves of primary and diluted aerosols were essentially unaltered, whereas the biological or total decay curves showed that cells were extremely sensitive to an increase in RH in this range.

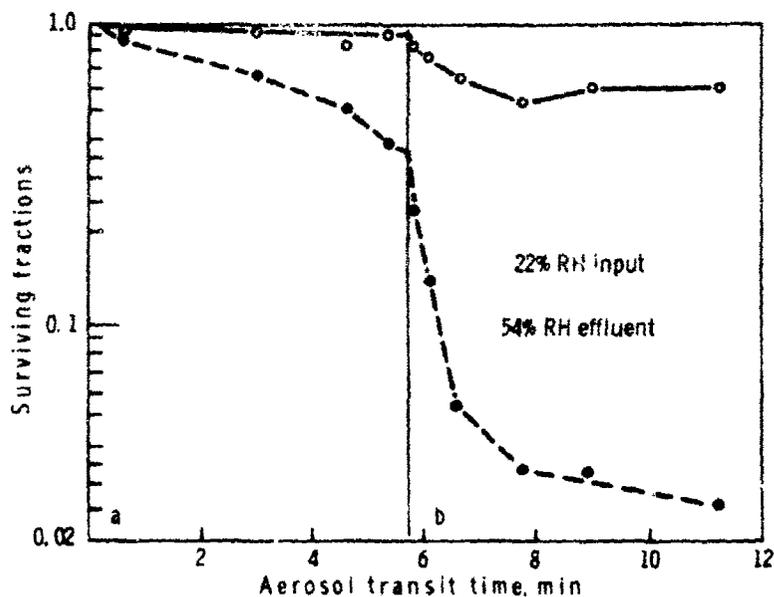


FIG. 7. The effect of rapid changes in relative humidity on airborne populations of *Serratia marcescens* grown in a chemically defined medium, and aerosolized in an enriched medium. Physical (o) and total (●) decay of primary (a) and diluted aerosols (b).

Although mechanisms for inactivation of cells in airborne droplets by sorption of water have not been adequately elucidated in this study, we have demonstrated that airborne droplets may increase in size by sorption of water; presumably, solids contained in the droplets could have dissolved as a result of the added moisture. Further, we suggest that these substances were not "toxic" when limited quantities of moisture were available. Instead, when the moisture content of the droplet increased, the cells' respiratory and endogenous mechanisms were stimulated into a more active state. Death from unbalanced growth could have occurred. The evidence indicates that the hygroscopic substances became "toxic" only to a portion of the cell population and that not all cellular activities were impeded. A similar phenomenon of "sorbed death" has been reported in freeze-dried S. marcescens by Monk and McCaffrey (1957) and by Monk, McCaffrey, and Davis (1957).

We are uncertain as to whether death of the cell occurred during the transition in the moisture content of the airborne droplet or whether death was a combination of this event and the result of the specific environment employed in the viability assay methods; that is, a kind of "sampling shock" to an already injured bacterium. We have previously observed that cells injured by heat are capable of cellular repair and reactivation when placed in a favorable growth environment (Dimmick, 1960), but the conditions for cellular rejuvenation are unknown. Present evidence indicates that these conditions are variable and highly dependent upon the physiological state of the injured cell. Moreover, we have some additional evidence that the phenomenon of "sorbed death" of cells, grown in the defined medium, suspended in the complex medium, and allowed to "age" at 4 C for varying periods of time before being atomized, was correlated with metabolic activity of the cells. If true, enhanced sensitivity of airborne bacteria atomized in the complex medium was not entirely a "toxic" phenomenon but a result of a changed cellular phenotypic expression.

#### ACKNOWLEDGMENTS

Acknowledgment is made to J. E. Hebert, HMCS, USN, for carrying out the aerosol experiments, with the technical assistance of Mr. Stephen Dunn, Mr. Douglas Wilcox, and H. A. Correa, HM2, USN.

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#### DISCUSSION

Morton: Well, the first question will come from myself. Before you publish your good and worthy technique, are you going to turn it onto other organisms? I confess here to a prejudice against S. marcescens as an experimental tool and I think that if you are not going on to pathogens, there are possibly other organisms that are better handled -- that are not so "idiosyncratic" in their reactions.

Hatch: Yes, we do anticipate investigating other organisms eventually. However, it is our thought at this time that we should first modify the present unit and perhaps extend it. We have envisioned adding a third parameter (i.e., changing the RH twice instead of only once). So I feel that these studies are just beginning and certainly other organisms should be looked at. Perhaps, with S. marcescens, we do have problems, particularly with atomization, and I think we are going to have to investigate these and other variables first.

Middlebrook: Did you make any observations on the rate at which the change influenced the time of the lag period or the initiation or modification in growth or the appearance of colonies under different conditions of treatment of aerosols?

Hatch: No. We are aware, however, that under stress conditions S. marcescens particularly will tend to form petite colonies which appear later than the others, and that we must also employ more than one type of media if we are actually going to find out how many cells are viable and that each step in the system must be characterized.

Levine: I wonder if you were to spray the complex medium without organisms, allow it to undergo whatever oxidation might be undergone, and then add it back to Serratia, does the complex medium, after being so aerosolized, become a toxic menstruum?

Hatch: We haven't done this. Are you inferring that respiratory activity is taking place in the airborne state?

Levine: No.

VFTF\*: But this is entirely possible, according to some work I've heard of.

Levine: Let me rephrase my statement. When you spray organisms in a complex medium and observe the biphasic decay, could it not be due to alterations in the medium? For example, I propose that if you took uninoculated, sterile medium, aerosolized it; that is, subjected it to a situation of gross oxidation among other things, and then added this to Serratia, it might be that by virtue of aerosolization this medium has become somewhat toxic.

Hatch: I don't think the spray medium per se would be toxic because excess death only occurred under certain situations. Our data indicated that the medium was not always "toxic". I think we must realize that we have a dynamic system wherein a bacterium is responding

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\* VFTF - Voice from the floor.

from moment to moment to its micro-environment and, at least from the data we have shown, it looks like the cell is changing physiologically, and that perhaps the "toxic" element only seems to be there because we are catching the cells in a given phase. However, to prove your point we would actually have to do experiments which we have not done.

Mallison: I wondered how you measured your RH. Was it with one of these newer sophisticated electronic gadgets that measures to within 1 or 2% RH?

Hatch: Only by wet-dry bulb.

Dimick: Dr. Morton, you mentioned your prejudice regarding S. marcescens. I would be interested in hearing a further comment on that.

Morton: This sounds like a counter-question. One of the things I recall is the very peculiar physical behavior of bacteria when examined in micro-droplets. May's experiments, for instance, may have been brought to your attention.

Harper: This is largely our problem at Porton. I think primarily it is associated with growing bacteria on solid medium. What happened was that May first observed this and it became apparent during collection and pre-impingement. It produced a sort of skin on the surface of the particles, particularly it was associated with S. marcescens, and the skin was extremely tough, and extremely light, and sat on the top of the fluid in the pre-impinger.

Silver: The point is, when we took deep-frozen (or whatever you call it) material from the deep-freeze process, this did not occur at all.

Hatch: I would like to put in a comment at this point. We recognize that S. marcescens is quite variable and very sensitive in many regards. We are not kidding ourselves. If we said, let us take something that is more stabilized, we would be saying, let us eliminate this complicated problem and just ignore that it exists. If we are going to understand the problem of bacterial behavior, in aerobiological systems, we ought to take a complicated microbial system such as S. marcescens, probe into mechanisms and the responses of cells during stress and actually try to find out, from the basic research viewpoint, what really happens and when it happens. I don't think we are going to gain insight into this problem until we recognize that it does exist and start working with complex and highly variable organisms, such as S. marcescens. Others, such as Pasteurella tularensis are variable and can also be troublesome in this regard. So, let's face the

problem and maybe we can learn more, rather than trying to pretend it doesn't exist.

Webb: I feel that there is a great deal of uniformity between the work that we are doing and the findings reported here. For example, we have observed a similar sensitivity of the cells and a similar pattern of events with respect to the humidity effect. In our own work we found that if the RH of the incoming air was higher than the aerosol itself, that one got a secondary kill due to the rehydration.

Hatch: Yes, Dr. Webb, I am aware of some of the work you have reported, particularly those studies in the Collison spray atomizer where the input, I believe, was about 5% RH and the output was about 72% RH. If you raised the input RH in the Collison to a higher value, this enhanced death phenomenon did not occur. I might mention that in our studies this enhanced death phenomenon only occurred with a step-up in moisture between about 40-60% RH; above 60% RH we don't observe it.

Webb: I found it also within the drum.

Hatch: Oh, yes, but you didn't elaborate.

Webb: When we studied this in the aerosol drum, we found that one got a maximum rehydration effect if one raised the humidity above 60% RH. The secondary kill was observed only if the RH was raised from about 50 or less to about 60%. But if the RH was raised from 60 to 70% for example, we did not observe this secondary kill phenomenon.

Silver: I am sure some of you are familiar with Druett's work which, in fact, we mentioned at an earlier symposium 2 yr ago at Detrick. He had only just started then, but he used a decompression cycle with a piston in a cylinder. He had a cycle rate of about, I think, 4/min, although this can be varied; and of course, he employed virtually instantaneous changes in RH on this sample. Initially he set up the aerosol in a static cylinder. He then plotted the decay biologically by using BC spore tracers and at an appropriate number of cycles he would stop the piston; he would normally operate from the lower to the higher level. The reason was that he wanted a fairly rapid decay in the first place, to give him a change of slope he could measure. He operated around 50% by compression-decompression cycle, then raised this up towards 100%. The first observation with spores indicated that it didn't matter what he did to the RH cycle; nothing affected the BC spores. That satisfied him regarding the tracer concept; it could be used to determine viability of the test organisms. He then examined a large number of Brucella species and at least one

strain of Escherichia coli, and he may have looked into something else, but I am not too sure of that. The prime observation was that there was no significant change in the death pattern until he obtained condensation on the particles near saturation. The moment that happened there was a change in the rate of decay; i.e., accelerated death. If you saw his results, I think you would be quite startled. Until he got condensation you could only notice the very fast changes in the rate of decay. More recently, he has added salts to the suspending fluid in an attempt to obtain condensation at RH significantly less than 90%. It is really not fair to speak for him because I haven't any data myself, but there is some evidence that if you have condensation, a very rapid change in the death rate will take place. He is trying not to extend his studies for longer periods.

Bismick: I have heard of some of his work, but I think unfortunately for all of us, it has not been published. I am wondering now what the effect of a change of pressure would have. As I recall, he does have a pressure differential added to his dilution. This is another variable we might study.

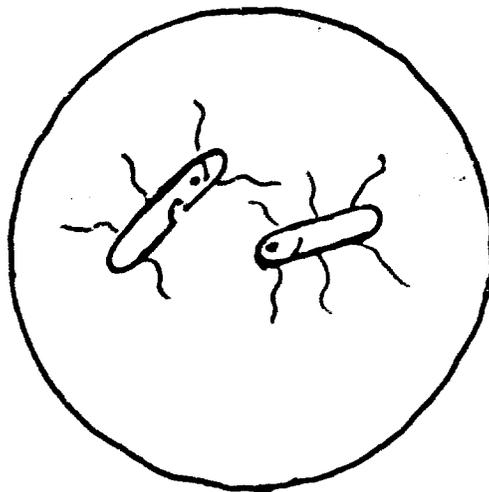
Wolfe: Some experiments have been done with pressure changes and I think the matter is ruled out because, unless condensation occurred, no change was noted. In some instances the air was virtually sterilized, except for BC spores, after condensation.

Hatch: Perhaps we should not overlook the possibility that there may be a practical application here. If we want to sterilize areas where there are infectious aerosols, perhaps we could investigate just that particular shift-up in RH needed to produce complete kill.

## SURVIVAL AND VIABILITY SECTION

Gardner Middlebrook, Moderator

An understanding of mechanisms leading toward survival and maintenance of infectivity in a microbial cell is fundamental to our eventual victory over respiratory disease. This section includes papers concerning theoretical studies of reactions of airborne bacteria to controlled, laboratory environments.



"Now remember, when he presses the stopwatch we each form triplets!"

## ADDITIVES TO INCREASE AEROSOL STABILITY

Leonard Zimmerman  
U.S. Army Biological Laboratories  
Fort Detrick, Frederick, Maryland

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Serratia marcescens is a gram-negative non-pathogenic bacterium that is widely used as a test organism. Suspension of this organism yields aerosols whose viable cell populations are inherently stable in atmospheres at high, but not at low, relative humidity (RH). Several attempted methods for improving the stability of aerosolized cells have proved unsuccessful. These included nutritional studies (growing the cells in various media), genetic studies (subculturing the survivors of aerosolization or some other stress) or mechanical processes (changing the aerosol-generating and aerosol-sampling devices).

We have added solutes to cell suspensions in the hope that, first, we might stabilize the viability of S. marcescens against aerosol exposure and, second, that we might achieve some insight into the question of why aerosolized cells die. Suspensions were all aerosolized at 47% RH, a very unfavorable humidity for the survival of S. marcescens.

1. One factor that had previously forestalled studies with additives was the conviction that added solutes might greatly accelerate the physical decay of aerosolized cells. To test this assumption, suspensions were prepared with progressively increasing concentrations of sucrose up to 32% and aerosolized. Maximum aerosol populations were obtained from suspensions containing 16% sucrose. Thus, the effects of added solutes in intensifying physical aerosol decay, it seemed, could safely be tolerated in practice.

2. Studies on other sugars showed that di- and tri-saccharides acted as aerosol stabilizers, but that monosaccharides did not. The differences between the effects of the mono- and oligo-saccharides were in the order of two magnitudes, with per cent recoveries varying from as low as 0.005 (4% glucose) to 8.7 (8% raffinose). High sugar concentrations did not impair the recovery levels. The monosaccharides were all penetrable into the cells, whereas the di- and tri-saccharides were not. The stabilizing effects of the latter sugars were attributed to their ability to plasmolyze aerosolized cells, thus possibly interrupting their metabolic activity. Laboratory studies have shown that the ability of *S. marcescens* to oxidize glucose is markedly impaired in the presence of plasmolytic concentrations of sucrose or NaCl.

3. Aerosol stability was also enhanced by salts of ascorbic, glucuronic, galacturonic and other organic acids. These acids were most effective in combination with multivalent cations ( $\text{Ca}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Sr}^{++}$ ) and much less so in the presence of monovalent ones ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Li}^+$ ). There were some inconsistencies noted: K and  $\text{NH}_4$  glucuronate and  $\text{NH}_4$  galacturonate were particularly ineffective. I would point out that  $\text{K}^+$  and  $\text{NH}_4^+$  are both essential metabolites; also  $\text{NH}_4^+$  easily forms complexes with polyvalent metal ions. These effects were also correlated with penetrability: multivalent cations were not penetrable into the cells and the monovalent ones were, up to a point. NaCl alone at concentrations up to 1% depressed aerosol stability, but at 5% and 10% levels aerosol stability began to improve. This reversal was attributed to the plasmolytic effects of the higher salt concentrations, creating an environment like that obtained with non-penetrable sugars. It seemed possible that these effects were caused by the displacement of  $\text{K}^+$  from the cells by  $\text{Na}^+$ ; but KCl and NaCl proved to have identical effects upon aerosol stability.

4. The stabilizing effects of plasmolytic salt solutions suggested that other methods for interruption of metabolic activity might enhance aerosol stability. Enzyme inhibitors were investigated as potential aerosol stabilizers and this work led to the discovery of another class of stabilizers: the metal-binding or chelating agents. These include such unrelated compounds as 8-hydroxyquinoline, sodium arsenite, thiourea, dipyridyl, phenanthroline, the dithiocarbamates, the hydrazides and dihydrazides, and kojic acid. One-tenth per cent

8-hydroxyquinoline provided the highest recovery after 2 min of aerosol time (56%) whereas 0.1% Na-dimethyldithiocarbamate yielded the highest recovery after 16 min (34%). The control, with no additive, was 26 and 7% recovery, respectively.

This class of compounds has the following characteristics: (1) they are effective in relatively low concentrations, 0.1% or less, (2) they are maximally effective only in the presence of solutes such as sugars capable of plasmolytic activity, (3) many of them are enzyme inhibitors, (4) in higher concentrations, some of them are toxic to stored cells, (5) some of them are toxic to cells aerosolized at high RH. It is also interesting to note that the adverse effects of  $\text{NH}_4$  salts on aerosol stability are completely eliminated in the presence of these metal-binders.

5. Using combinations of sugars, and metal-binders, we have made S. marcescens nearly as stable as spores of Bacillus subtilis var. niger (B. globigii). For example, adding raffinose, ascorbic acid and Na arsenite at pH 7 to S. marcescens, I found that the per cent recovery after 30 min aerosol time was 24%, compared to 25% for B. globigii.

The stability induced by additives, however, depends in great part upon the state of the cells. Growing cells are least aerosol stable in the log phase and most stable in the stationary phase of the growth cycle. Stationary-phase cells suspended in spent culture medium, however, were not made more aerosol-stable by the addition of solutes. If these cells are removed from the medium and resuspended in water they can be stabilized; if this suspension of cells in water is aerated for several hours, its pH rises and its oxygen uptake level falls to a very low level. Such starved or resting cells are maximally responsive to the potential stabilizing effects of added solutes.

#### DISCUSSION

Hatch: I have two questions: How did you get started on this path, and would you care to speculate a little more on possible death mechanisms?

Zimmerman: I set out to minimize the rate of death of aerosolized S. marcescens. The reasons for the success of my procedures, however, are not clear. I do have some speculations as to mechanisms by which aerosol stabilization may be induced.

The death of aerosolized cells probably does not result from generalized destruction or denaturation of cell substance. This must be true if the addition of a few simple chemicals to the cells can render them aerosol-stable.

All energy generated by the cells is ultimately used either for maintenance or for synthesis of new cells. Treatments that prevented the cells from securing maintenance energy would of course be lethal. All our stabilizing treatments, we think, keep the cells in a non-growing, maintenance-only state. The combined treatments of cell starvation with plasmolyzing solutes non-specifically reduces the amount of energy that the cell can generate. The metal-binding solutes, however, may owe their effectiveness to their enzyme-inhibiting properties; somehow, they push the resting cell deeper into the resting state.

A system recently suggested by Maaløe to account for the synchronous growth of bacteria may be of interest here. Maaløe postulates that the division cycle of normal cells appears to consist of two periods, one during which a complete round of DNA replication takes place, and one during which no DNA is made. Also, the inhibition of protein and RNA synthesis, by removal of nutrient or other means, does not interfere with the completion of a DNA replication presently in progress, but prevents the initiation of a new round of replication.

We visualize the possibility that all suspensions contain some sensitive cells and others that are insensitive to aerosol exposure at low RH. It is possible that the sensitive cells are those in the process of DNA manufacture. Our stabilizing treatments consist of a combination of starvation, addition of a plasmolyzing agent, and of a chelating agent. These treatments may combine to put and keep more and more of the bacterial population into a non-DNA-replicating state in which they are insensitive to aerosol exposure.

Dimmick: I'm fond of your approach; speculation is the grandmother of a well-formed hypothesis and intuition is probably the grandfather. I'm impressed by your findings since we have found much the same thing regarding culture age. You didn't state what medium you used and what growth temperature, and I wonder whether you think this might also change the response to aerosolization?

Zimmerman: We have checked on both of these factors. The cells used in all these tests were grown in aerated media containing 2% Bacto-Tryptose, 0.5% glucose and 0.004 M sodium phosphates. Batches

of cells with equally good stability were grown from aerated media containing either (1) 3% Trypticase Soy Broth (TSB) plus 2% glucose, or (2) a citrate-glucose-NH<sub>4</sub>Cl-salts chemically defined medium.

We also tested the relationship between aerosol stability of cells and temperature of growth. Cells inoculated into TSB + glucose media were grown at 25 C and at 37 C. The cultures grown at the 37 C were non-pigmented and yielded lower viable cell populations than the cultures grown at 25 C. The aerosol stability of the 37 C-grown cells, alone or in the presence of stabilizers, was poorer than the stability of the 25 C-grown cells. This observation was not explored; we were only looking for growth conditions which permitted the production of cells with maximal aerosol stability.

## BACTERIAL RESPONSES TO DESICCATION AND REHYDRATION

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The magnitude of microbial hazards believed to be associated with environmental microorganisms has frequently found expression in the great effort put forth to reduce their numbers in man's surroundings. This is particularly true in the case of staphylococci, where a multiplicity of sources may serve as potential reservoirs of infectious material.

In order to present an environmental hazard of major proportions, staphylococci must survive the fluctuating conditions of desiccation and rehydration found outside the host, withstand rehydration in host fluids and create those structures and products that are characteristically associated with their ability to initiate infectious disease processes in susceptible hosts. Effective indirect transmission thus implies survival of staphylococci in fully infective form under conditions operative in the external environment.

Over a period of years, experiments have been conducted in attempts to determine the role that desiccation and rehydration treatments play in governing the survival, infective capacity, and virulence of staphylococci. Earlier studies of this problem indicated that staphylococci, when dried as films and injected immediately after reconstitution in broth, produced fewer infections in mice challenged by the intramuscular, intracerebral or intravenous route than did similar doses of nondried organisms<sup>(1)</sup>. Other tests carried out at this time

pointed out that staphylococci surviving desiccation and rehydration procedures showed evidence of sublethal injury reflected by prolongation of the lag periods, greater sensitivity to reconstitution and storage in various fluids and slower rates of production of coagulase<sup>(2)</sup>.

In attempts to obtain more information regarding the responses of staphylococci to desiccation and rehydration, both aerosol and film drying techniques have been employed in subsequent experiments. Two broad lines of approach have been taken: these include studies of changes in permeability and changes in metabolism which are most likely to affect retention of characteristics related to the disease-producing capacity of staphylococci\*.

#### PERMEABILITY CHANGES

It is well recognized that control of permeability is lost in dead bacteria. It is also apparent that increases in permeability take place when bacteria are subjected to desiccation-rehydration procedures<sup>(3)</sup>. Consequently, measurement of the magnitude of leakage of substances from bacterial cells provides some measure of their death. It is considered likely that organisms surviving such stress treatments would also show indications of increases in permeability. Indeed, additional leakage of material from Escherichia coli has been found when the survival capacity of the organisms was enhanced by drying the cells with a protective additive<sup>(4)</sup>. Evidently, the loss of such materials is not necessarily associated with death of the organisms. Typical ultraviolet adsorption spectra of released leakage materials from washed preparations of nondried staphylococci (Strain H2) were compared to the greater leakage obtained with the same cell mass in dried preparations (Fig. 1). Organisms were derived from early log and stationary phase cultures, film dried for 20 hr at a relative humidity of 45% and reconstituted in distilled water. Microbial suspensions were centrifuged and the supernatant fluid containing materials lost from the cells was scanned at wave lengths of 230-300 mμ. Organisms derived

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\* The strains of Staphylococcus aureus used in this study are pathogenic in mice. Strain 8074 is a weak alpha toxin producer and is less virulent in mice than Strain H2. Strain H2 was originally isolated from a human abscess in our laboratory, while Strain 8074 was obtained from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario. Strain H2 withstands desiccation and rehydration stresses much better than Strain 8074.

from log phase cultures were more permeable than those obtained from the stationary phase of growth, although no appreciable differences in viable counts of the samples were found.

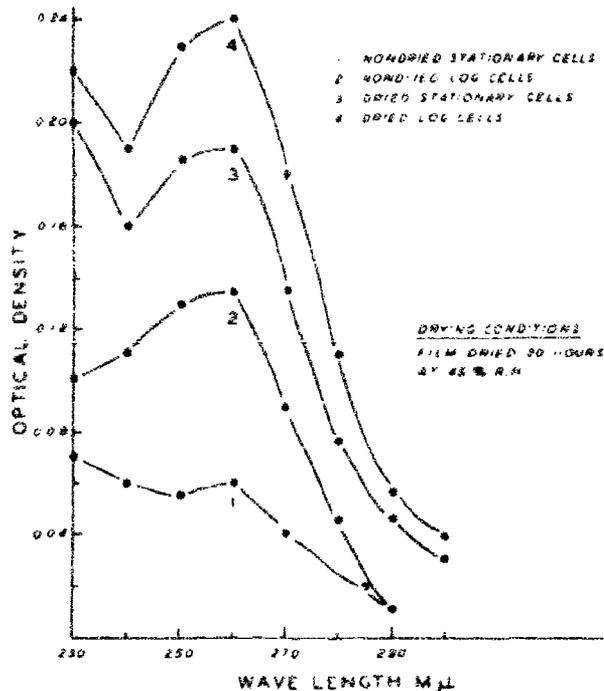


FIG. 1. Ultraviolet spectrum of materials released from Staphylococcus aureus

Since the leakage materials have not been characterized to date, it is not known whether the same materials are lost from the cells before drying as those noted after drying. However, present qualitative tests show indications of amino acids, phosphates, protein and ribonucleic acid compounds in the leakage materials. It is possible that the prolongation of lag previously noted might be due in part to the time required to replace or synthesize some of these lost cell substances. Since leakage does reflect increased cell permeability, one must also suspect increased access of substances outside the cell to certain areas within the cell. For example, the entry of p-nitrophenyl phosphate into organisms previously dried and reconstituted is enhanced, because greater measurable acid phosphatase activity occurs in dried staphylococci (Strain H2) than in the same cell mass of nondried

organisms (Fig. 2). However, it is not known at this time whether permeability increases reflect only physical changes or whether energy transport mechanisms of the organism are also affected. Furthermore, it is possible that critical spatial relationships of certain structures and chemical substances might be changed, thereby influencing selective permeability in bacteria subjected to desiccation and rehydration procedures.

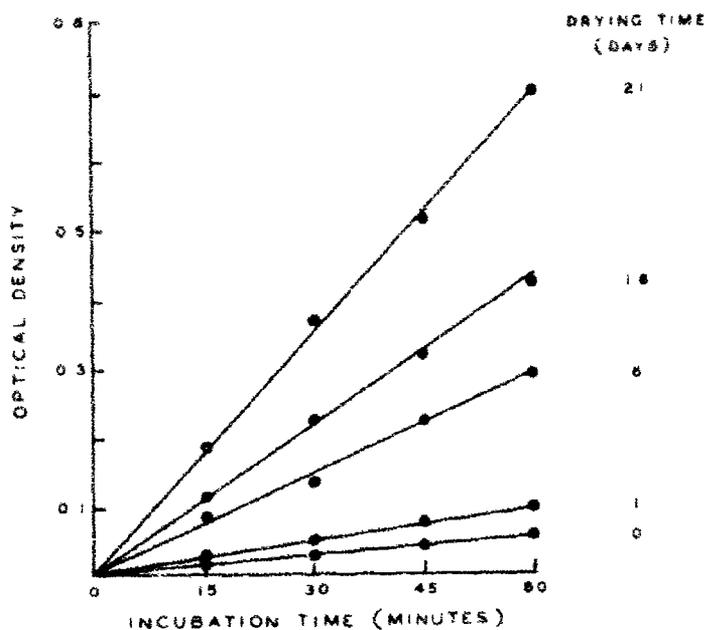


FIG. 2. The effect of desiccation-rehydration on acid phosphatase activity

It is reasonable to assume that the infective potential of surviving pathogenic bacterial would be affected by their past drying history, since increased permeability might allow entry of some potential antimicrobial host substances normally excluded from vital sites in the cell. Although one of the characteristics of coagulase positive staphylococci is their resistance to bactericidal components in blood or serum<sup>(5)</sup>, increased permeability of staphylococci, stored as aerosols or film preparations, might explain the subsequent decreased ability of such cells to survive in human serum, blood or plasma containing staphylococcal antibodies<sup>(2,6)</sup>. Bactericidal activity was

abolished when complement was inactivated by heat or zymosan and no bactericidal activity was noted in nondried preparations<sup>(6)</sup>. These results suggested that the mere entry of nonbactericidal materials to cell sites was not related to death of the bacteria. Under these conditions cellular resistance also appears to be more efficient, as evidenced by faster clearance of aged aerosols of staphylococci (Strain H2) from mouse lungs (Fig. 3).

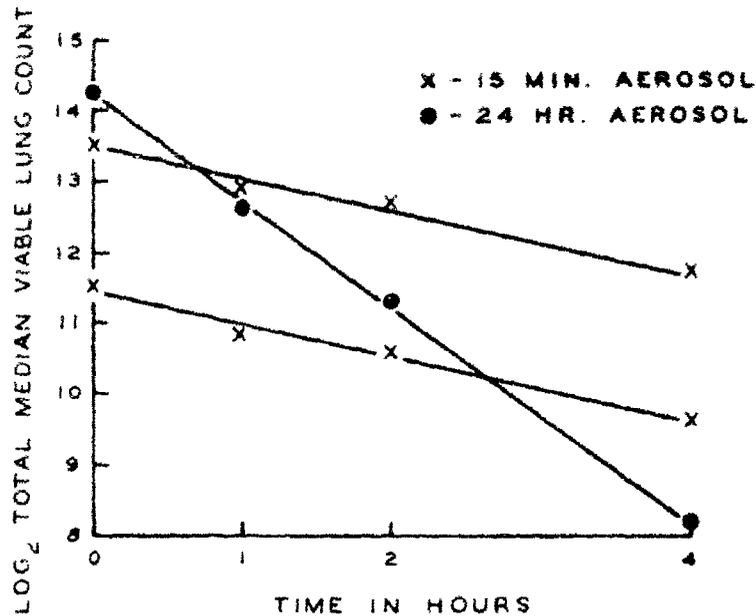


FIG. 3. Decreases in viable count of staphylococci in mouse lungs with young and aged aerosols

Although increases in permeability of staphylococci due to drying stress treatments have been demonstrated, it appears that permeability returned to normal values if metabolism was allowed to take place. This might account, in part, for the fact that incubation in broth within the lag period before intravenous injection increased the ability of previously dried cells to survive the initial residence in mouse tissues<sup>(6)</sup>.

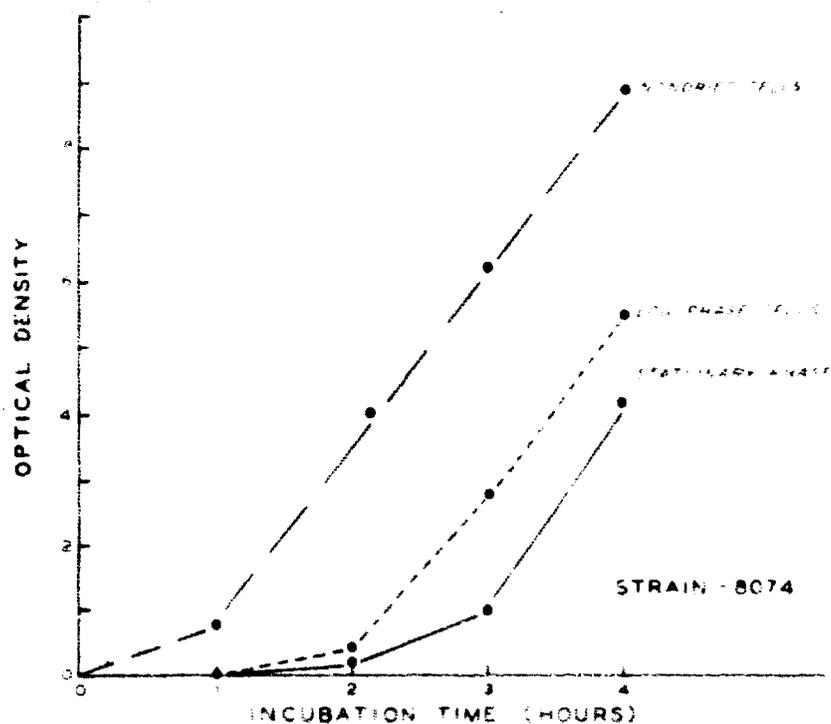
#### GROWTH RATES AND METABOLISM

The likelihood of success in the identification of the processes involved in reduction of viability, infectivity and virulence of

Staphylococcus aureus by desiccation procedures\* points out the need for studies of growth patterns and of the related broad physiological and biochemical changes which occur when the organisms are reconstituted in growth media. The study of changes in the ribonucleic acid (RNA) and protein synthesis of dried and nondried staphylococci in relation to cell growth, infectivity and virulence might yield useful information.

Previous studies have indicated that the lag period of staphylococci progressively increased with increases in drying time<sup>(2)</sup>. These lag measurements were concerned with the time required to double the initial viable count when the cells were incubated in broth. In addition, excellent regeneration capacity was noted, for it was found that once the log phase was reached, the minimum generation time in broth was constant, regardless of the previous drying history of the cells.

Subsequent optical density measurements of growth of both log and stationary phase cultures of the two strains of staphylococci used in this study (Strain H2 and 8074) have shown that the true lag phase is also inhibited by desiccation-rehydration stress, particularly in Strain 8074 (Fig. 4,5).



**FIG. 4.** The effect of desiccation-rehydration on growth of Staphylococcus aureus

\* For growth, protein and ribonucleic acid measurements used in this study, film samples of staphylococci were dried for 24 hr at a temperature of 80 F and a relative humidity of 40%.

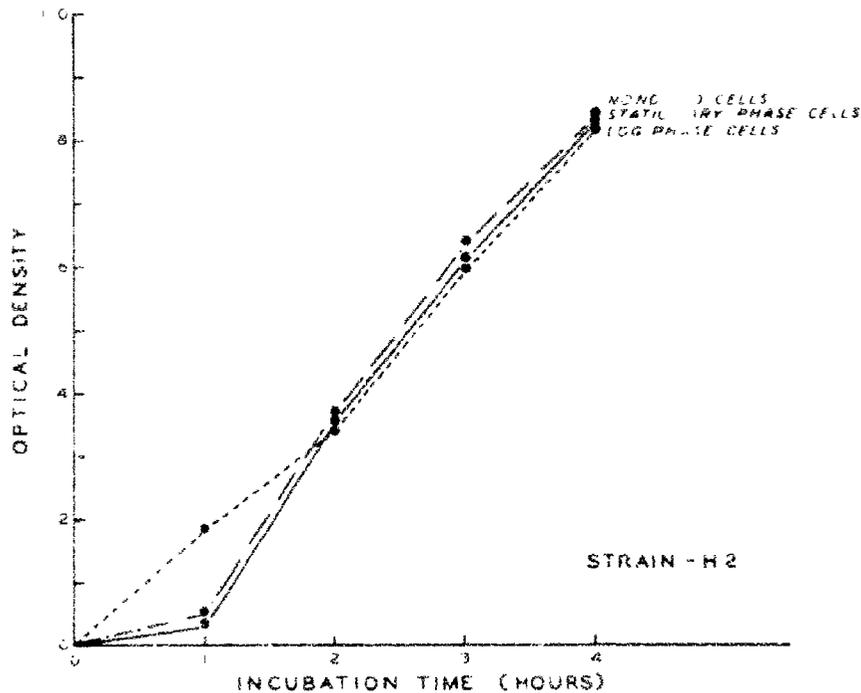


FIG. 5. The effect of desiccation-rehydration on growth of Staphylococcus aureus

Some metabolic function is apparently not operating initially at normal efficiency in the stress organisms, indicating that a repair process is required for the cells injured to a sublethal degree.

Because of the previous finding that coagulase synthesis was slower in staphylococci dried and reconstituted in plasma<sup>(2)</sup>, and because incubation in broth within the lag period appeared to be necessary to raise infectivity and virulence levels<sup>(6)</sup>, broad measurements of metabolic activity were considered important. Therefore, protein and RNA synthesis of dried and fresh staphylococci was followed in aerated Heart Infusion Broth (HIB) cultures at 37 C. The results indicated that the initial rate of protein synthesis was slower in cells previously subjected to desiccation-rehydration procedures (Fig. 6,7). Apparently coagulase synthesis and possibly toxin synthesis occurred at a slower initial rate because there was a general decrease in the rate of protein syntheses, although this does not rule out additional more specific effects on the mechanisms governing coagulase production.

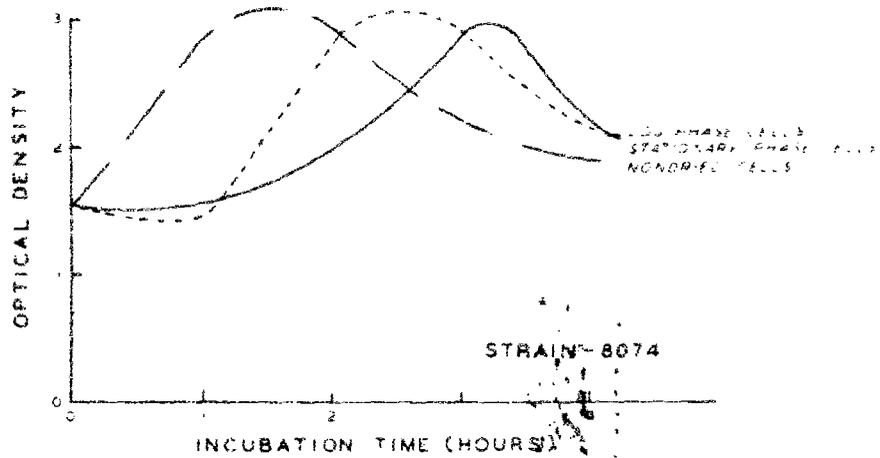


FIG. 6. The effect of desiccation-rehydration on protein synthesis.

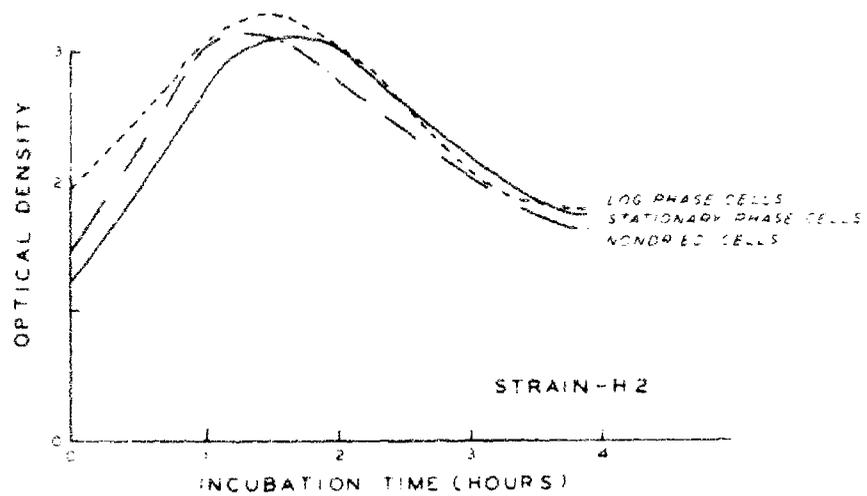
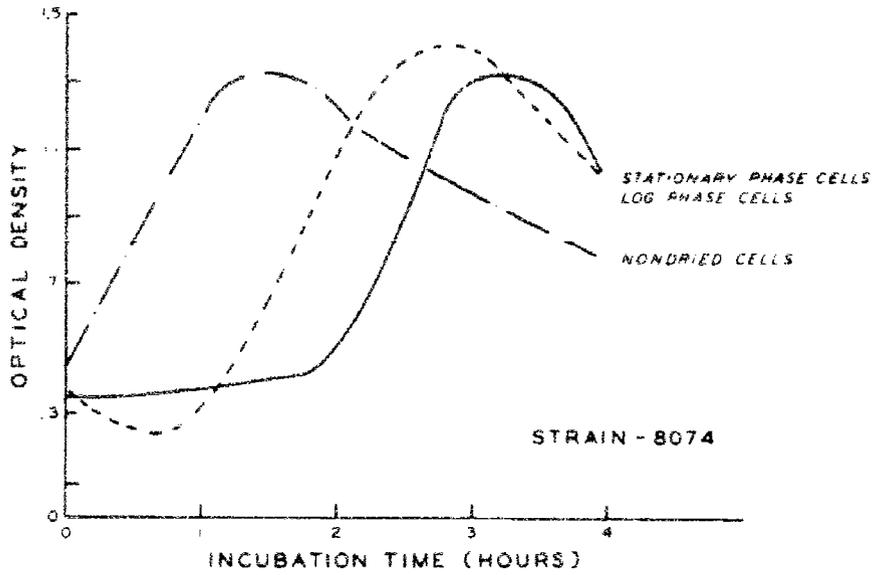


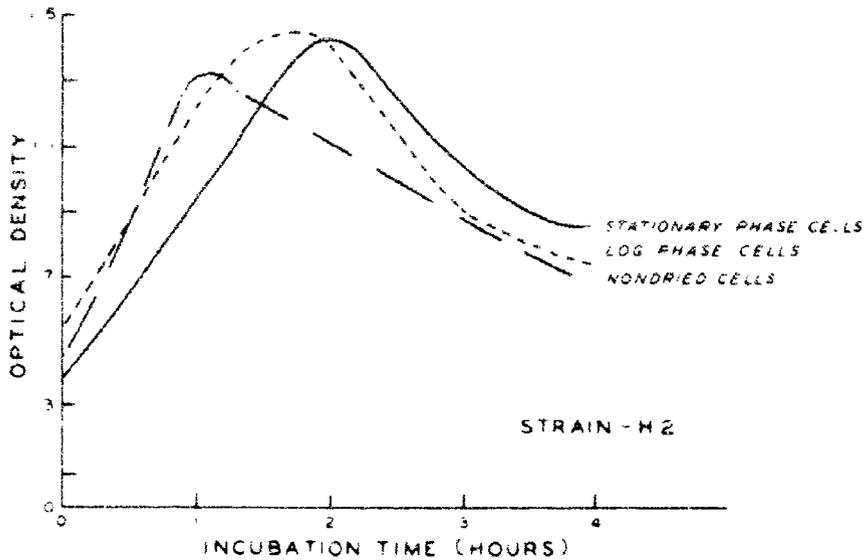
FIG. 7. The effect of desiccation-rehydration on protein synthesis.

In addition, results showed that the synthesis of RNA was also affected, providing a partial explanation for the reduced initial synthesis of protein (Fig. 8,9). Rates of synthesis of protein and RNA returned to normal values more quickly in log phase cells than in stationary phase cells, although log phase organisms are generally considered to be more sensitive to desiccation and rehydration than organisms taken

from the stationary phase of growth.



**FIG. 8.** The effect of desiccation-rehydration on ribonucleic acid synthesis



**FIG. 9.** The effect of desiccation-rehydration on ribonucleic acid synthesis

When one considers that RNA tends to be an unstable chemical compound, that  $Mg^{++}$  is required to stabilize ribosomal RNA, that the absence of only one component of the amino acid pool is required to stop protein as well as RNA synthesis, and that the stress treatments would require loss or inactivation of only one type of vital chemical compound to affect both RNA and protein synthesis, it does not seem too surprising that desiccation and rehydration might well influence these important reactions to some degree. It is unlikely that lack of nutrients in HIB can be the cause of the slower rates of synthesis of both RNA and protein. Rather, it seems more likely that metabolic replacement of substance(s), inactivated by desiccation-rehydration stresses or lost by leakage due to increased permeability, may be necessary in order to utilize these nutrients in normal fashion. However, it is not known at the present time which exact sites and specific reactions of the intracellular turnover systems are affected. Regardless of the exact mechanism involved, the broad reaction systems of staphylococci affected by desiccation-rehydration treatments are reflected in the prolongation of the lag. As a matter of fact, the well-known relationship of growth measurements of nondried bacteria as an index of protein and RNA synthesis also appears to be operative in the case of staphylococci subjected to desiccation and rehydration stresses.

It is clear that the present studies do not provide information regarding the possible particular replacement substance(s) or the specific repair area required so that protein and RNA synthesis can take place in normal fashion. Consequently, the studies outlined here must be considered preliminary, since experiments dealing with intracellular turnover are necessary to obtain more detailed information regarding the exact mechanisms inhibited by stress procedures associated with desiccation and rehydration. Information concerning such turnover in microorganisms has been reviewed recently<sup>(7)</sup> and can serve as a useful guide in designing experiments necessary to gain a better understanding of the functional relationships of the biochemical events in cells after various stress treatments.

#### ACKNOWLEDGMENT

It is a pleasure to acknowledge the technical assistance of Mr. Donald Lust during the course of this investigation.

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#### DISCUSSION

Middlebrook: We need more of this kind of information in the field of aerobiology and I am delighted, personally, to see that you are working with S. aureus whether you got positive information or not regarding virulence (whatever that is). When you showed those last slides, were those per unit weight of bacterial cells?

Maltman: Yes. It would be fundamentally that. They were based on a standard optical density.

Middlebrook: Apparently some multiplication was going on during incubation; did not the turbidity values or assays change during this time?

Maltman: Yes.

Middlebrook: Would it have to be unit weight of the bacterial cells present?

Maltman: Yes.

Middlebrook: So you can't standardize by weight alone, you must take into account fluctuations in counting.

Maltman: It is true that you cannot standardize, strictly

speaking, by any single method since growth and cell division occur during incubation. This is the reason that a constant optical density of cell suspension was used, since it bears a practical relationship to unit weight, although this relationship would certainly not be absolute.

VFTF\*: Do I understand correctly -- log phase cells are particularly sensitive to desiccation stresses but they recover fast in growth medium?

Maltman: Yes. We pointed that out; such cells are more sensitive, but they do recover more quickly than other cells. This might provide a useful technique for basic studies. We don't exactly know how this applies yet, but it could be tied to the fact that the protein and RNA concentrations in these young log phase cells were approximately twice as high as they were in cells taken from the stationary phase cultures.

Webb: We have been carrying on similar studies with other organisms, not staphylococci; but with the sort of organisms that are sensitive to aerosolization; in other words, those that lose viability whereas staphylococci don't. But the phenomenon you mentioned here is exactly the same in both types. We measured the same release of material from cells -- and when we protect them with compounds, such as inositol or glucose (despite the earlier paper, glucose has been shown for the last 20 years to be protective for aerosolized organisms), you get even more release of this material; yet cells survive better; also you get the same inhibition of other RNA-protein synthesis mechanisms and, as you know from earlier papers of mine, we also get the same increase in sensitivities of certain enzymes, not phosphatases, but carboxylases and the like, and there is a very dramatic increase of such activities.

Maltman: These leakage products may be important in terms of growth, even though they might not be important in terms of surviving numbers. By equating the magnitude of the greatest amounts of leakage, when cells are dried in different ways, with growth rate, one should observe the greatest slow-down in initiation of growth. These experiments are in progress.

Shinefield: I just wondered whether you have used other parameters to determine virulence (whatever that is, again) in your dry and non-dry organisms.

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\* VFTF = Voice from the floor.

Maltman: I am not sure I understand you. We used intramuscular, intravenous and intracerebral challenge.

Shinefield: To mice?

Maltman: To mice.

Shinefield: Have you examined numbers of organisms necessary to produce lesions on suture materials because a mouse is not particularly . . . . (interrupted)

Maltman: No, nothing like this.

Shinefield: Since a mouse is such a bad animal for staphylococci, some of the more subtle changes might be made more manifest with other animals.

Maltman: Perhaps; we have not performed this type of test in other animals.

Eichenwald: Basically, what you are trying to suggest is the fact that virulence of staphylococci is a particularly difficult concept because organisms that cause human disease and those staphylococci that do not cause human disease are indistinguishable in animals.

Maltman: Yes. For example, if you allow staphylococci which have had a drying history (where you can show infectivity has decreased) to metabolize within a lag period, then infectivity will increase so one suspects that some of these cells do get into host sites where they can incubate and increase their metabolic activity in order to return toward "normal" capabilities.

Middlebrook: I think what you say is quite true, at least within the experimental design. Dr. Maltman has shown differences which may or may not be relevant to our clinical problems. It may or may not be relevant to those properties of staphylococci which are important in human virulence -- but still, they are observations and I think that working in this area will be profitable. When we know more about other infective properties of Staphylococcus aureus, perhaps this type of investigation could be broadened; at least you did see differences in the disappearance of culturable bacterial cells from the lung depending on pre-treatment of the organisms.

Maltman: Not only from lungs, but from any system the effective in vivo numbers are reduced.

## SURVIVAL OF LYOPHILIZED BACTERIA DURING STORAGE

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I believe that lyophilization, or rather 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 are in direct contact with the atmosphere. Thus, with the exception of irradiation studies, we can subject lyophilized organisms to much the same treatment as the airborne cells. Of course, lyophilization differs considerably from aerosolization, one difference being that during drying of cells in micro-drops they are subjected to a rather severe stress due to the surface tension of water as it evaporates from the liquid phase. However, one might use this difference to evaluate the effect of surface tension on aerosolized microorganisms.

Another advantage of using lyophilized materials is that such bacteria can be subjected to a wider range of variables than when they are in the airborne state. These conditions are not necessarily practical in aerobiology, but by using extreme conditions, the effects of certain factors can be more easily demonstrated. In large chambers, such as are required to contain aerosols, it is difficult to reduce the humidity to near zero, whereas this can easily be achieved using lyophilized materials. Small containers of lyophilized organisms are also easily subjected to either extremely low or extremely high temperatures.

It is easy to obtain precise samples of lyophilized organisms, since we need not be concerned with physical loss, and large samples

are readily available for physical or chemical analysis.

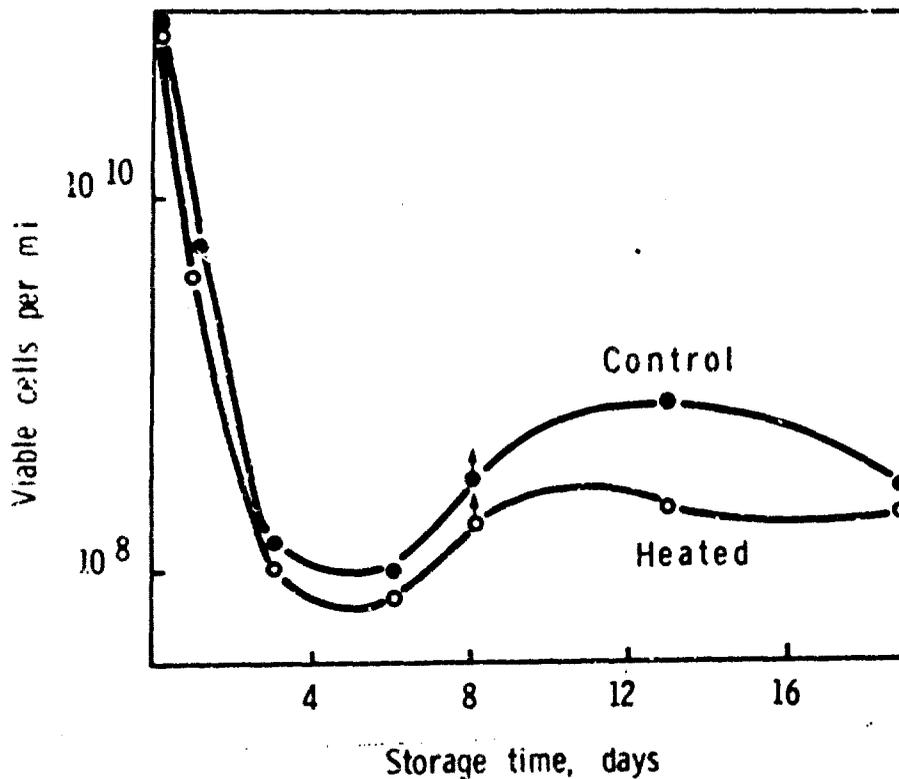


FIG. 1. Viability of lyophilized *Serratia marcescens* held at room temperature in air. The 8-day samples contained too many cells to count in the highest dilution tested. Heating was at 52 C for 10 min before drying.

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It has been generally assumed that freeze-dried organisms are dormant, but I would like to present some data suggesting that this is not necessarily so. Usually the number of viable cells decreases during storage of dried preparations, but as is shown in Fig. 1, we have observed instances of recuperation in which the number of viable cells appeared to increase during the storage period. In this instance, the number of *Serratia marcescens* cells that formed colonies increased at least 2-fold between the 4th and 12th day of storage of the lyophilized preparations. Figure 2 shows a similar recuperation of dry *Escherichia coli*. This experiment was designed to determine what effect the method of growing the organisms had on their subsequent survival in the lyophilized preparations. Those grown on solid medium survived better when stored in vacuum than those grown on liquid cultures.

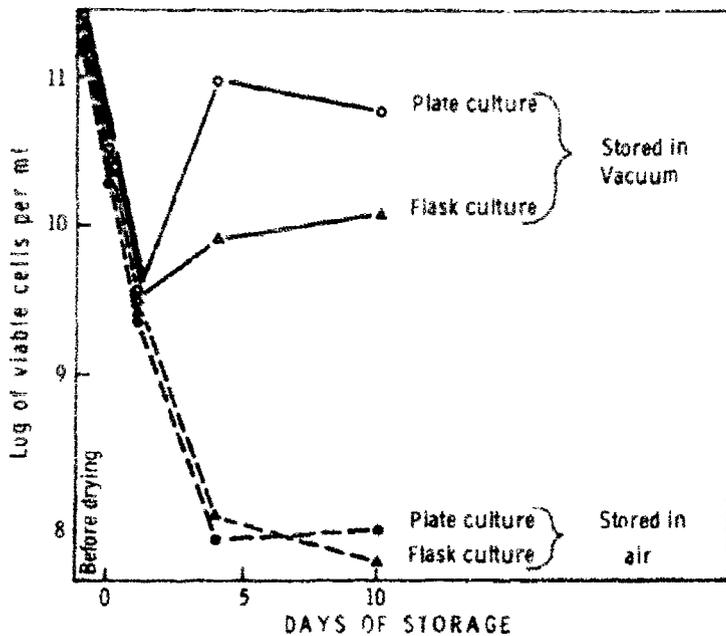


FIG. 2. An instance of recuperation during storage of freeze-dried *Escherichia coli* grown on nutrient agar and in broth.

When stored in air, survival was poor, and there was no significant difference between the two types of cultures. There seems to be little doubt that there was recuperation between the 1st and 5th day of storage in vacuum, especially of the plate culture. Perhaps the cells in some way adapted to their new environment during this period so that, when reconstituted and plated, they were better able to grow and form colonies. It is conceivable that immediately after being dried their metabolic balance was disturbed and that, when placed in a liquid environment some of the cells simply were caught off-balance and perished. So the sudden change of their environment from gas to liquid may have constituted a severe shock, various rehydration procedures were tried. It was surprising to find that with *S. marcescens*, more cells survived a rapid rehydration (direct addition of distilled water) than a slow rehydration of exposure to water vapor. In some experiments, over 100 times as many cells produced colonies when water was added directly as when the cells were first exposed to 100% relative humidity (RH) at 20 C before addition of the water. We observed the

greatest effect of high RH in those preparations subjected to additional drying by heating to 100 C at 10-20  $\mu$  Hg for 1 hr. It is indeed difficult to determine whether or not cells are dead in dry preparations because at present we have no criteria, other than growth or colony production, for determining viability.

In an attempt to demonstrate that dry bacteria have some metabolic activity, Dr. Dimmick used a micro-respirometer to measure the gaseous uptake of dry S. marcescens. At low moisture levels, the rate was so slow that it took weeks to obtain measurable uptake. Nevertheless, significant gaseous uptake (presumably oxygen, since KOH was used as an adsorbant) was demonstrated and furthermore, the rate of oxygen consumption increased with increasing humidity.

Free-radical production by dry bacteria over a period of days (Reckly, Dimmick and Windle, 1963) also is evidence of some kind of activity, because free-radicals have been demonstrated to be intermediates in many metabolic systems. More significantly, both free-radical production by lyophilized S. marcescens and death were greater in the absence of lactose than when lactose had been added to the medium before drying. There was a correlation between loss of viability and free-radical concentration, but not in direct proportion. In no instance have we demonstrated free-radical production by organisms that were killed by mercury salts or heating before lyophilization. A correlation between free-radical production and loss of viability has been shown for several microorganisms in addition to S. marcescens; e.g., Sarcina lutea, Micrococcus radiodurans, baker's yeast, E. coli, and Streptococcus lactis. Under vacuum, viability was maintained and minimal production of free-radical was observed, whereas in air the same preparations died rapidly and free-radical concentration increased rapidly. To-date, free-radical production was minimal under all conditions in which survival of organisms was high.

Figure 3 summarizes a more extensive experiment using S. marcescens. There was no significant free-radical production in cells stored in vacuum, whereas the free-radical concentration increased rapidly in preparations exposed to air. Samples represented by lines A, C, and E did not contain lactose. The others were dried from an 0.5% lactose solution. The Electron Paramagnetic Resonance (EPR) spectrometer signal developed at approximately the same rate in the samples left open to

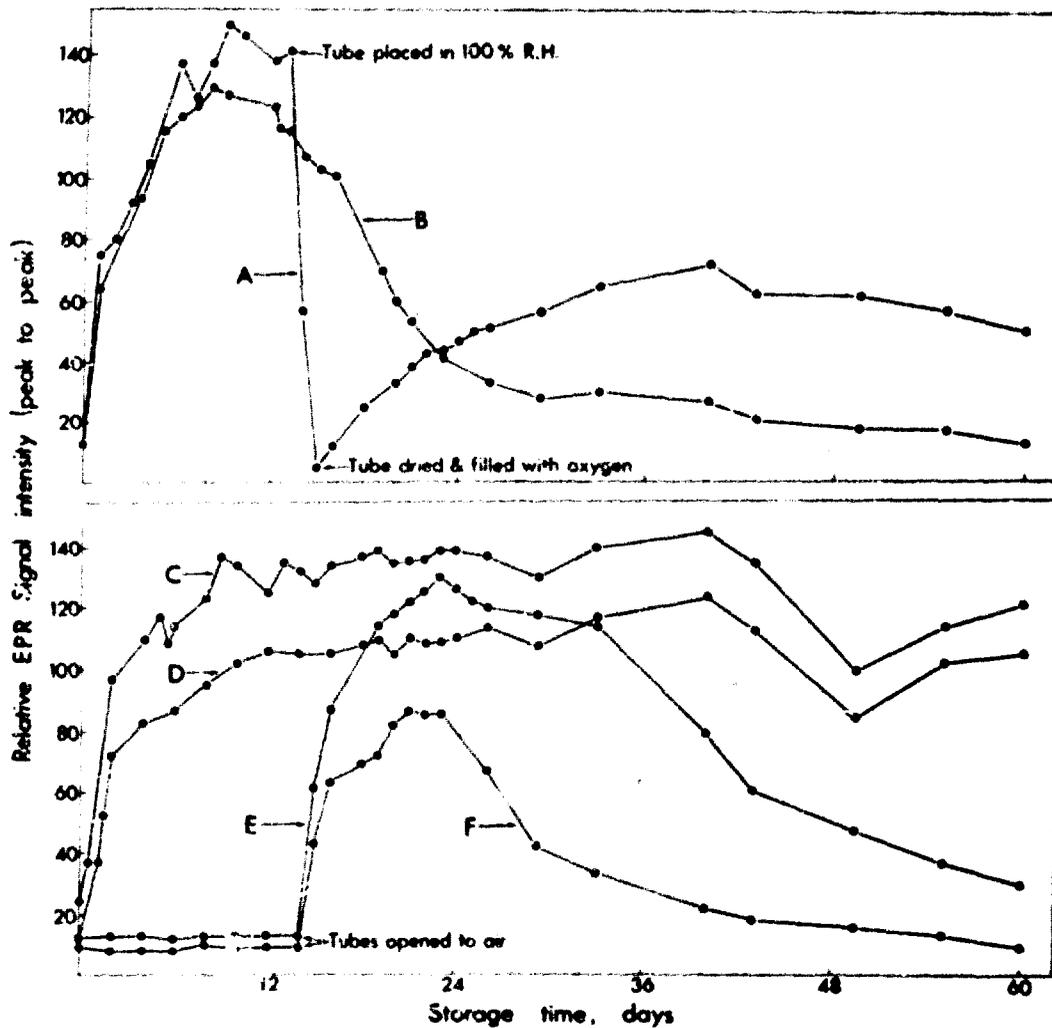


FIG. 3. Effects of lactose, air, and moisture on free radical formation by lyophilized *Serratia marcescens*. A: cells suspended in buffer, dried, and packed in vacuum, then opened to room air for 13 days. B: cells suspended in buffer containing 0.5% lactose, packed in vacuum, then opened. C: same as sample A, but cells packed and sealed in air. D: same as sample B, but packed and sealed in air. E: same as sample A, but not opened until after 14 days. F: same as sample B, but not opened until after 14 days.

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the air (lines A and B) as in those that were sealed immediately after filling with air (lines C and D). Although no signal developed in preparations sealed in vacuum (lines E and F), free-radical production was comparable to that in the other tubes as soon as air was admitted. Note that the rate of production was comparable. When sample A was placed in a humidified jar, the signal intensity returned to virtually zero within 48 hr. Moisture uptake was probably responsible for the slow decay of the EPR signal in those samples open to air (Lines B, E, and F). In this experiment, viability was maintained better in the presence of lactose, but even with lactose, 99% of the cells were no longer viable after 12 days' storage in air. It is evident that the presence or absence of an EPR signal per se is not necessarily significant; rather it is the change in signal intensity that is important. If there is no signal, all cells may be either dead or alive. If the signal is increasing, cells are probably dying, but the decreasing signal does not signify recuperation.

Recently Blois et al. (1961) described an EPR signal in lyophilized nucleoprotein. Since the signal he described as at  $g = 2$ , it could be due to the same free radical we have observed in our lyophilized organisms. They did not indicate how the signal developed or the exact conditions of preparing and storing their preparations.

Figure 4 shows some kinetics of the lactose protection. In this experiment, a 1% lactose solution was added to each of two S. marcescens cultures, 6 and 12 hr old. At frequent intervals, after adding the sugar, samples were removed, frozen rapidly and dried. Maximal protection was obtained during the 1st minute. The horizontal lines above each curve represent the number of viable cells before lyophilization (100% survival level). The curves remind me of the over-shoot of a recorder with insufficient damping. I hesitate to interpret these results, but it does seem to be more involved than a simple physical protection of the cells by the added sugar. Perhaps the cells are reacting to the change of environment and have overcompensated in some manner.

The next three figures pertain to an experiment in which cultures of various ages were lyophilized after either slow or fast freezing. As a slight digression, I'd like to consider the interpretation of "death curves" such as these. According to a common concept, a plot of the number of viable cells vs. time of storage, such as in Fig. 5,

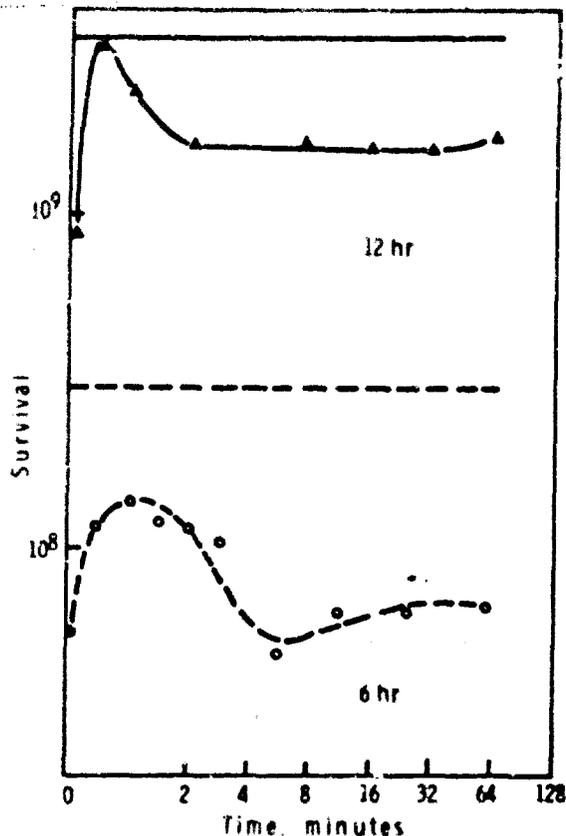


FIG. 4. Recovery (24 hr storage) of freeze-dried *Serratia marcescens* as a function of time after the addition of a final concentration of 1% lactose and before freezing. Horizontal lines represent 100% recovery level.

represents a death rate. Implicit in the rate concept is the assumption that all of the cells have an equal probability of surviving and if the slope of the curve changes, as most of these do, the culture must consist of two or more populations. I believe that most populations are indeed heterogeneous, and perhaps it would be more realistic to consider the death curves as representing the cumulative results from a distribution of cells having various survival capabilities. Thus, in comparing the 21-hr and 24-hr cultures, we find that the 21-hr culture contained fewer cells capable of surviving 8 days than did the 24-hr culture. Considering only the solid line, initial slopes of both the 21- and the 24-hr-old cultures were comparable.

In Fig. 5, the dotted lines with the large marker represent the survival of lyophilized cultures rapidly frozen by immersing small vials containing 1 ml of culture in a dry-ice and solvent bath. The

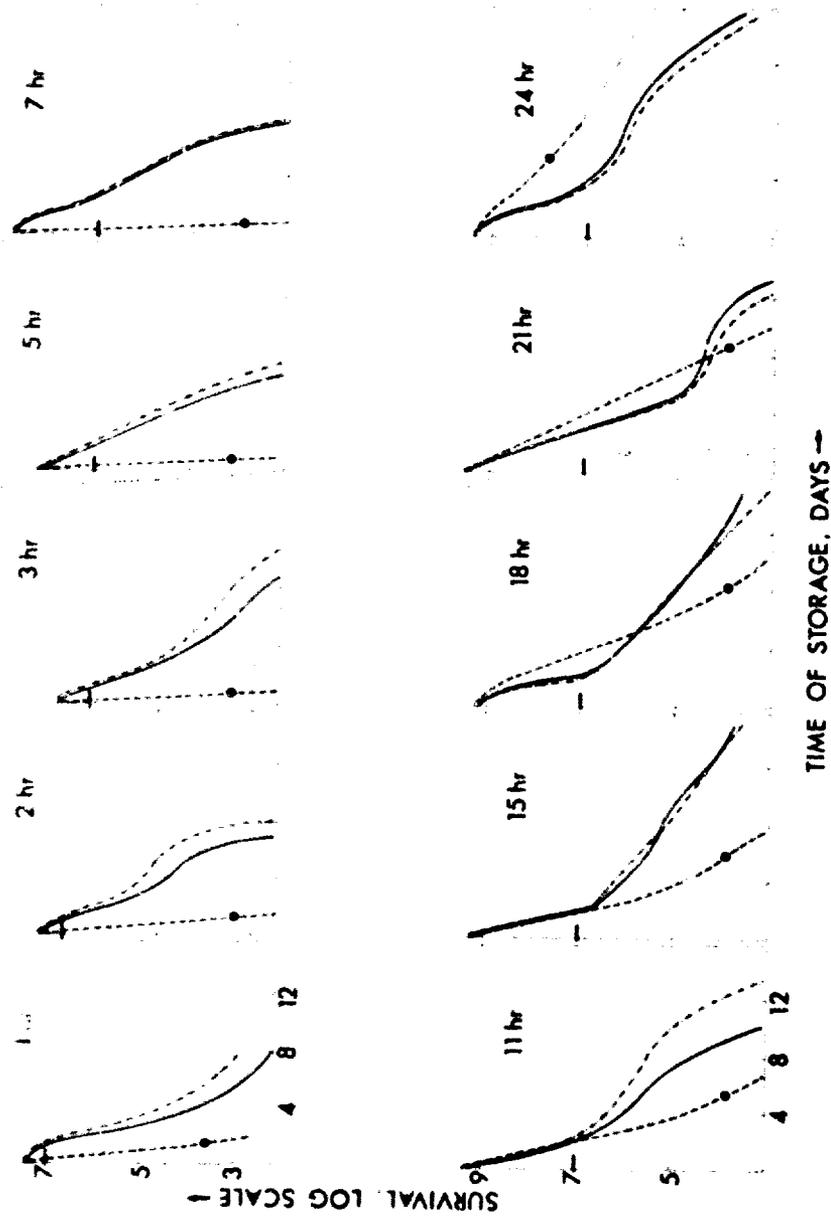
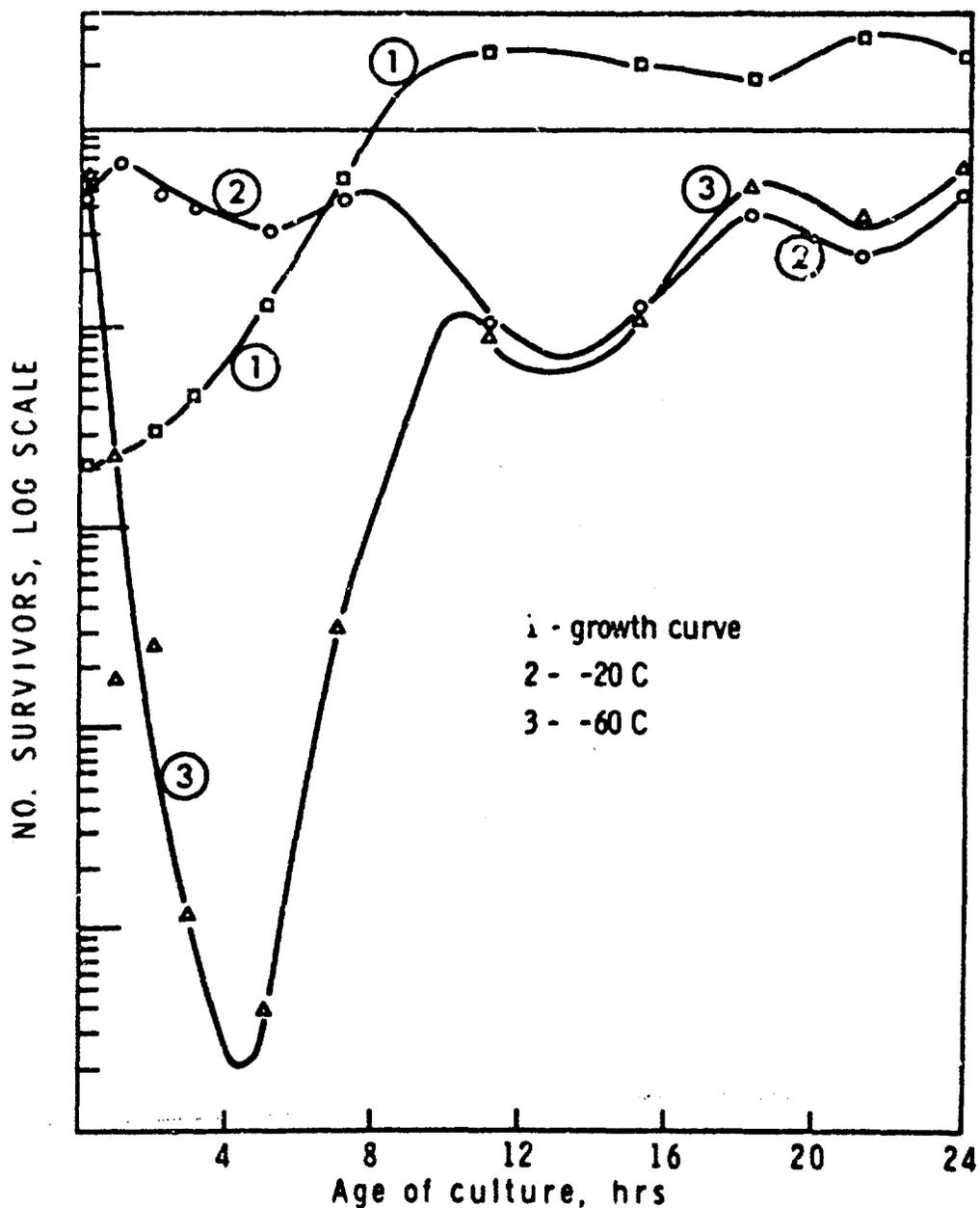


FIG. 5. Survivor patterns of freeze-dried *Serratia marcescens* stored at 21 C as a function of age of culture, when plated on two different media. The broken lines indicate viability when plated on trypticase soy and the solid line indicates apparent viability on chemically defined medium. The lines marked with a dot represent cultures frozen at  $-60^{\circ}\text{C}$ ; the others were frozen at  $-20^{\circ}\text{C}$ .

other two curves represent cultures frozen slowly, i.e. in a 5 ml bottle placed in a -20 C freezer. You can see how the patterns change from the young cultures, which are very sensitive to rapid freezing, to the older, mature cultures that survive rapid freezing better than slow freezing.



**FIG. 6.** Two-day storage survival of freeze-dried *Serratia marcescens* frozen at two temperatures as a function of age of culture. Horizontal line represents 100% recovery.

Figure 6 summarizes the results of this experiment in terms of

number of viable cells after 2 days' storage as a function of age of a culture at the time of freezing. For reference, the growth curve is shown as line 1. Line 2 represents the preparations slowly frozen and dried. The extreme sensitivity of 4-hr-old culture to freeze-drying is evident. The solid line across the top of the slide (Fig. 6) indicates the 100% survival level.

All of the assays in this experiment were made on two media: a simple, chemically defined medium (Bunting's) and a complex medium (trypticase-soy agar), because it was suspected that some of the cells not producing colonies were not dead, but failed to grow because they had been injured and had lost ability to synthesize some vital constituent. Figure 7 summarizes the results of this aspect of the experiment. Except for line 1, which is the growth curve for the culture as a reference, the lines represent the ratio of number of cells on Bunting's vs. trypticase-soy agar medium. Thus a ratio greater than 1 indicates that more colonies developed on Bunting's than on the complex medium. The left portion of the figure pertains to the cultures frozen slowly and the right side to cultures frozen rapidly. Data for lines 2, 3 and 4 were obtained after 2, 5 and 8 days' storage respectively. Since there were instances, such as the very young cultures after 8 days' storage, in which ten times as many cells grew on the complex medium than on the Bunting's, the choice of assay medium markedly influenced the results obtained. In general, the most accurate estimate of viability is obtained with the complex medium. Note that in only a relatively few instances did the count on Bunting's agar exceed that on complex medium. These fluctuations are significant, but I doubt if identical results would be obtained on repeating this experiment, especially with regard to the precise age, or extent of differences, because there are so many variables which affect the behavior of the organisms. The cyclic nature of the various curves obtained in these studies is obvious, but again we have no explanation, since the fluctuations do not seem to be correlated with the growth curve.

In view of all of these facts, together with many of similar implications already in the literature, I have difficulty in understanding why aerobiologists seem to deal with the problem of airborne bacteria as if cells were somehow not living systems - dynamic, responsive and each unique. Only as we begin to obtain a complete picture of the

behavior of microorganisms and their reactions to the environment can be hope to understand airborne organisms. It is reasonable to suggest that more attention than in the past should be given to details of what my colleague and I currently term "microbial systematics" in studying the various survival capacities of microorganisms.

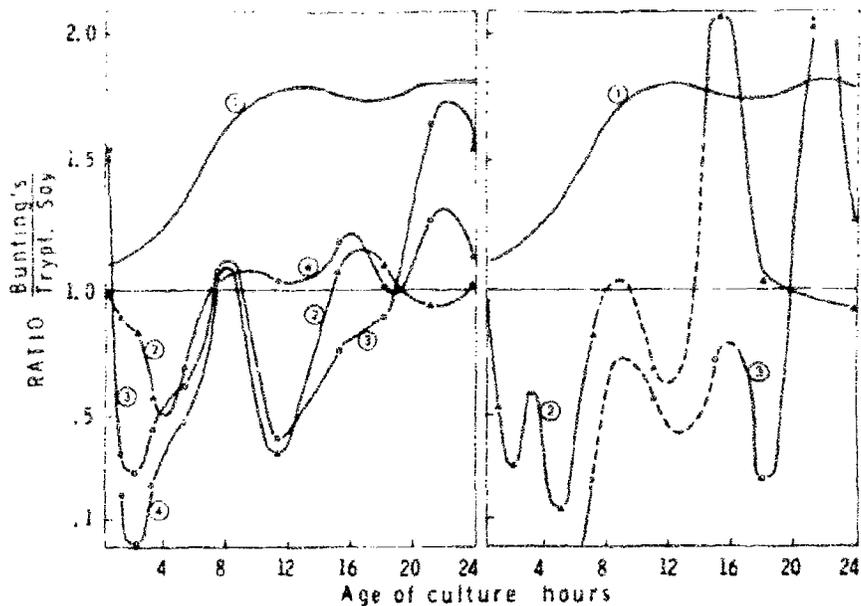


FIG. 7. Change of plating ratio of two sampling media as a function of the age of culture of freeze-dried *Serratia marcescens*, time of storage and freezing temperature. Left, -20 C; right, -60 C. Curve 1 is the growth curve for comparison. Curves 2, 3, and 4 are 2, 4, and 8 days' storage, respectively.

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## DISCUSSION

Morton: Dr. Heckly, I don't understand this slide (Fig. 5) in which organisms of various age were frozen either rapidly or slowly before lyophilization, particularly the 1 hr, 2 hr, 3 hr legend.

Heckly: These are the ages of the cultures before lyophilization; that is, if you take very young cultures, the number of cells that survive to 8 days is low. The dotted lines with the large dot represent those cultures which were rapidly frozen by immersion in a dry ice methylocellulosolve mixture. The others were slowly frozen by placing 5 ml of culture in a relatively large bottle in a -20 C deep-freeze. One obvious thing is that the pattern changes, particularly as we progress from the 11-hr to the 24-hr culture.

Morton: Does the media used to culture the organisms in the first place affect viability?

Heckly: Of course, I would expect it to do so, but Bunting's was used as a medium in all of these experiments.

Morton: What if you had put an additive, such as skimmed milk, in your medium?

Heckly: If lactose is added just before drying, such as described in the previous paper this morning, survival would be improved. We haven't explored the effect of cysteine or sucrose on free radical production as widely as we have the effect of these things on viability. We have just now obtained our own EPR machine and look forward to exploring this problem further.

Morton: Would you tell me a little bit about free radicals and how they are measured? (Question passed to Dr. Dimmick.)

Dimmick: When there is an unpaired electron in a molecule, that molecule is known as a free radical. It is what, in your early chemistry, you might have been taught could not possibly exist; i.e., a broken covalent bond. These are highly reactive portions of molecules that have had electrons added to them in some way or another, they are in a high-energy state; for example, most peroxides are free radicals. The device used to measure them is not too important from our aspect, and I'm not sure I could explain it, anyway. The instrument most commonly used is built by Varian and can detect about  $10^{11}$  molecules, or spins. The important thing is that often, when an enzyme catalyzes

a reaction, there appears to be a moment when it has split the substrate molecule apart and has not quite put it together again in some other form. These two fragments are, in many instances, free radicals. They are highly reactive, so they don't last very long, especially if moisture is present. This is why we commonly don't find them in a moist state for any length of time. But in the dried state, they are more or less stabilized and this allows us to measure them.

Heckly: Apparently there is no predominating theory of why free radicals are produced in the dried state. We may have just blocked one part of an enzyme mechanism, so that you have an accumulation of those particular free radicals. This is what we think right now. There is usually a gradual decay, so that most of these free radical curves that I have shown probably represent an equilibrium condition. Of course, we could be observing a simple oxidation, but it is difficult to explain why this does not happen with dead cells.

## VIABILITY OF MICROORGANISMS USING A SUSPENDED DROPLET TECHNIQUE

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For some time now we have been looking into ways and means of studying the survival properties of organisms subjected to atmospheric stresses but free from the mechanical and associated stresses normally encountered in conventional aerosol techniques involving spraying and sampling. Admittedly, from a practical viewpoint, the type of equipment in common laboratory use represents a reasonable approximation to both the nature and sequence of the numerous stresses imposed on the organism, in that the cycle of suspension - atomization - equilibration - equilibrium - collection - suspension is rarely short-circuited. The main drawback of the system is the difficulty in isolating the various stages of the cycle and hence the possible interplay between these stages. This hinders interpretation of the results. If we wish to understand what is happening to an organism subjected on to atmospheric stresses, then it is necessary to devise a different type of apparatus so that mechanical stresses can be eliminated or minimized. The need for such apparatus is 2-fold; first, we wish to determine the extent to which viability is lost in the airborne phase and not merely measure the difference between unsprayed material and that recovered from the sampler; second, we believe that the function of chemical substances governing the survival of the organism is most likely to be recognized by these means. In practice, we have to admit that the airborne phase cannot be isolated because a bio-assay for viability inevitably results in physical and chemical stressing of the organisms.

but, as you will see, at least some control can be exercised in the recovery and assessment phases.

We have examined two ways of producing unstressed droplets containing bacterial cells. One has so far proved unsuccessful, but the other has been in regular use for 2 yr or more and has been employed in producing the experimental results I shall be presenting later. The unsuccessful method I will briefly describe because we badly need a facility of this type, and it might stimulate some ideas. Essentially, drops of predetermined size, but within the size range usually associated with aerosol work, were produced by a vibrating reed. The drops could equilibrate in the top section of a vertical tube and would be maintained buoyant by an upward-moving conditioned airstream. After the requisite holding period the particles would be allowed to fall under gravity into a suitable collector. Considerable difficulties were experienced in stabilizing the column because of sensitivity to thermal and electrostatic forces. Dr. Cox spent much time in combating these difficulties, but it became evident that it required full-time investigation by a small team, and the problem is now to be tackled by a University as an extra-mural research item.

The other method, which we call the fibre technique, is quite different and deals with single drops of a much larger size. It was intended that the two methods should be complementary rather than alternatives, the fibre method handling drops larger than, say, 50  $\mu$  diameter, and the column method from 50  $\mu$  downwards. The fibre technique, since it employs single drops, is usually concerned with sizes of 100  $\mu$  or more in order to contain a sufficient number of organisms to give reliable and reproducible results. In any case, the smallness of the drop is limited by surface tension effects and it is this size factor which is the most criticizable feature of the technique; if particle size is important in determining viability, then this method will not accurately portray the behavior of the small particles which constitute the normal aerosol.

The essence of the technique is to deliver from a microsyringe a small quantity of mixed suspension containing the test organism and a spore tracer. Using a micromanipulator, the fluid is transferred to a fine glass fibre lying across an orifice through which saturated air is passing (Fig. 1). The figure shows at the top the droplet formation, followed by stages of evaporation to solidification at the bottom.

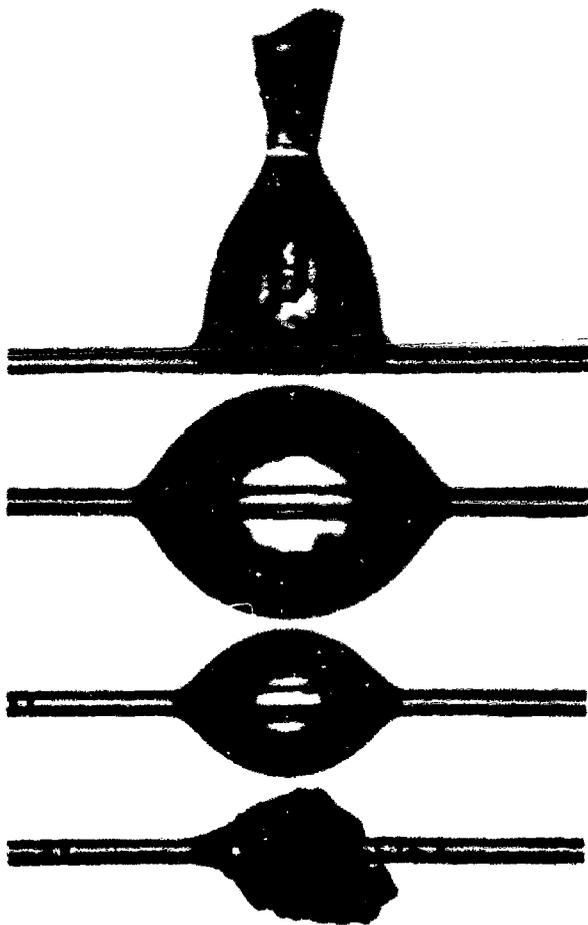


FIG. 1. Stages of evaporation of a droplet on a glass fibre.

In practice, a plastic box fitted with a lid with six orifices was used so that up to six fibres could be charged in any one experiment (Fig. 2). The air supply was so arranged that saturated air could be replaced by conditioned air at a selected rate depending upon the air velocity chosen. It was thus possible to control the evaporation rate of the suspended drop. After exposure, the fibre could be removed by forceps, dropped into collecting fluid, and the contents assessed. The drops could be observed optically by mounting the plastic box on the moveable stage of a microscope and it proved a valuable feature of the technique (Fig. 3).

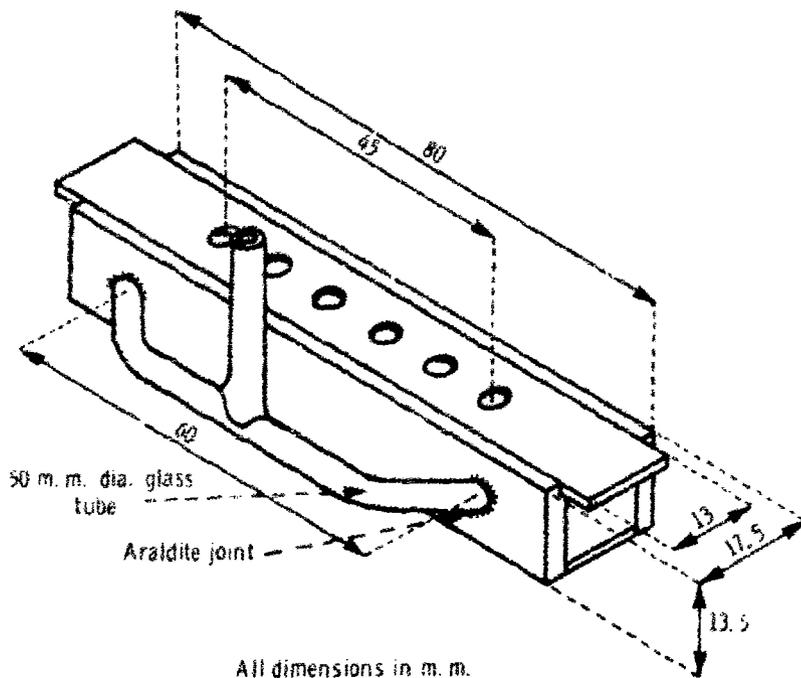
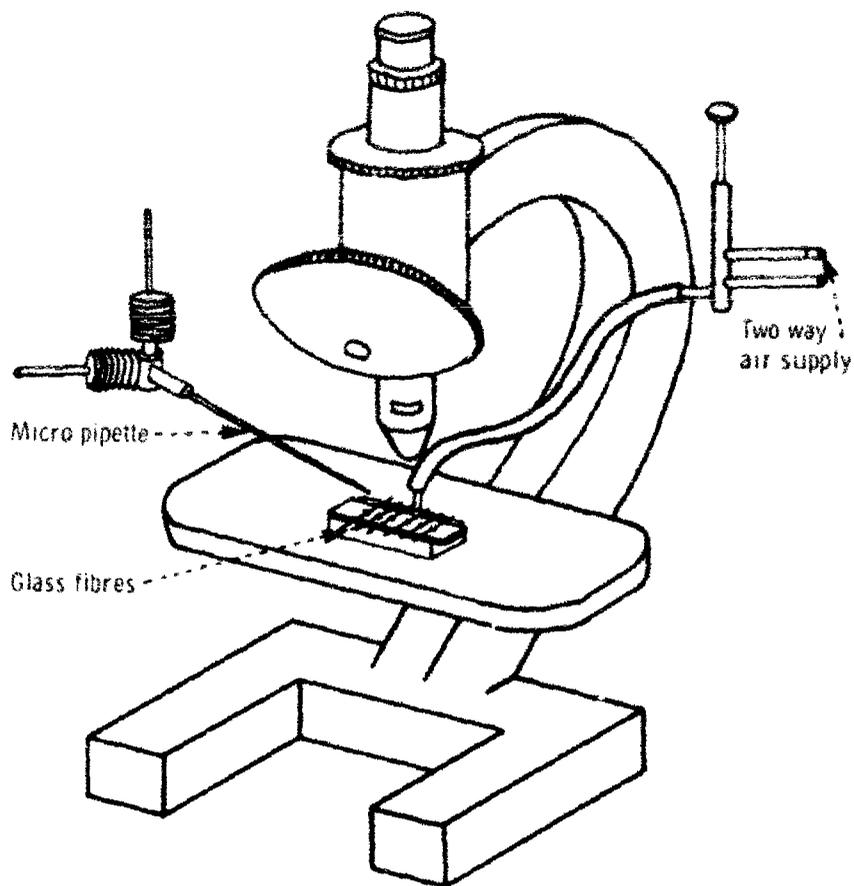


FIG. 2. Plastic box used to hold glass fibres in conditioned air.

The fibre method has been used in a number of ways:

1. Correlation of the behavior of solutes on evaporation with survival.
2. Measurement on the influence of rates of evaporation and rehydration on survival.
3. Comparison of survival on the fibre and in aerosols.

Dealing first with behavior of solutes on evaporation, it is clear that nearly all solutions supersaturate heavily. Crystallization from the metastable state is markedly variable; some solutes refuse to crystallize under any conditions; others readily crystallize but only non-reproducibly either in terms of the relative humidity (RH) or the time lag at any particular humidity; other factors such as batch-to-batch variations are also noticeable. Substances which crystallize appear to be poor protectors; many inorganic and organic salts come into this category. However, crystallization alone is not the controlling factor for it is linked with the rehydration process and this can best be demonstrated by example.



**FIG. 3.** Arrangement of fibres and box on microscope stage.

For a long time we were puzzled by the high death rate of a number of bacterial species when sprayed from solutions of mannitol compared with other hexitols or sugars. Mannitol, unlike these other compounds, can crystallize on evaporation, often at a high RH. Occasionally it is possible to create successive droplets at a given humidity, only one of which crystallizes. Table 1 shows how the different end-states affect survival.

TABLE 1. Survival of Escherichia coli 163 at 70% relative humidity in 1 M mannitol

Holding Time 16 min			
Collecting Fluid - Phosphate Buffer			
Initial diameter (μ)	Equilibrated diameter (μ)	Rehydrated diameter (μ)	Viability %
160	75*		5
155	75*	130	10
160	80		23
160	80	140	89

\* Crystallization.

You will notice a substantially higher level of survival when the mannitol fails to crystallize, but perhaps more important is the influence of controlled rehydration. This is achieved by exposing the drop to saturated air until swelling ceases before dropping into collecting fluid. The increase in viability is small for the crystallized drop but large for the supersaturated one. It is likely that a similar result could have been achieved by using a collecting fluid of high osmotic pressure instead of re-wetting the drop and collecting in phosphate buffer. The inference is not only that the act of crystallization permits further evaporation to take place, desiccating the cell to a greater degree, but also that rehydration occurs at a different rate from the supersaturated cell and physically stresses the cell accordingly. However, you will see in a minute that there is no simple rule concerning the influence of rehydration rate. Aminocaproic acid is another substance that crystallizes in a variable manner and gives results similar to mannitol.

Turning now to rate of evaporation and rehydration, the fibre technique has been used to examine the way in which controlled evaporation and rehydration affect survival. So far as evaporation is concerned, we have not discovered any significant effect on survival. Of course, we should remember that although the evaporation rate may be similar to that occurring in fine aerosols, the time taken by these relatively large drops to come to equilibrium is very much longer -- in this sense the comparison is poor. Rehydration, however, presents a very different picture.

TABLE 2. Influence of rehydration in viability. Survival of *Escherichia coli* 163 in various fluids at 50% RH  
Holding Time 30 min

Collecting Fluid	Spray Fluid*								
	Sucrose 0.2 M	SNP	Raffinose		10% Dextran	5% dextran + sucrose			
			0.3 M	0.4 M		1 M	1.5 M	2 M	4-5 M
Buffer	15	66	24	71	16	25	60	56,50	25,33
Buffer (rehyd.)	47	3 $\frac{1}{2}$	49	69	16	47	60	90,48	61,33
2 M Sucrose	53	--	67	--	--	--	--	56,98	--

Numbers are % viability.

\* All spray fluids contain buffer.

$\frac{1}{2}$  Three determinations, holding time 15 min.

You will see from Table 2 that controlling the rehydration either by exposing the drop to saturated air before collecting or by using a collecting fluid of high osmotic pressure can have an important effect on the result. The Table unfortunately gives the impression of lack of reproducibility, but this is largely traceable to media preparation (I am showing results collected over a period of time); under well controlled conditions a group of results shows better agreement. Nevertheless, fluctuation in response to controlled rehydration is quite common from batch-to-batch. The so-called osmotic effect whereby death of the cell is attributed to an imbalance between the response of the cell wall and the rate of inflow of water, is not reproducible with any regularity. Such transient hypertonic states often can be prevented by controlling the rate of rehydration. I think it not unreasonable to assume that the physical properties of the cell wall vary and that we need not look further for the cause of death in these particular circumstances. Where controlled rehydration produces the reverse result, namely that survival decreases, an alternative reason is needed. The example given in Table 2 is provided by cells suspended in supernatant fluid but other samples exist -- phosphate buffer can show the same phenomenon, but at high humidity. Perhaps the reason here is that prolonged exposure to high concentrations (possibly critical concentrations) results in death due to chemical or metabolic activity rather than just physical stresses. There is a measure of evidence that lethal zones occur in conditions of high humidity and that the

death rates in these zones can be very high.

Finally, it is interesting to compare survival on the fibre with that in aerosols. In broad terms, agreement is quite good although the bias is for higher survival on the fibre. In fact, of the large number of chemical substances examined, only glycerol and erythritol gave rise to consistent differences in survival, the better survival again being obtained on the fibre.

It is not easy to explain the reason for the behavior obtained with glycerol and erythritol, especially since ethylene glycol does not exhibit the same property. It is conceivable that since the interval required to evaporate a large drop is greater than for a small drop, more glycerol or erythritol penetrates the cell when the fibre technique is employed and perhaps maintains the characteristic shape of the cell better than in fine aerosols. The higher stresses one might expect to find as a result of osmotic phenomena are overcome, to a degree, in the fibre technique by the slow rate of rehydration when the fibre is dropped into collecting fluid and the cells dispersed by hand-shaking. That ethylene glycol does not fit the pattern is more readily explained by the fact that cells will not tolerate very high concentrations of glycol in liquid suspension, whereas they live for quite long periods in, say, 10 M glycerol or saturated erythritol solution.

#### DISCUSSION

Morton: What do you think about droplet populations? I mean, anything from one per droplet up to very large numbers of organisms. What effect does this have?

Silver: I can't completely answer that. Certainly in some of the other work that I did, I used a variable population, but not deliberately. We appreciate, of course, that in order to get an answer which is meaningful, you must start off with pretty reasonable numbers.

Morton: Yes, but you do still obviously have latitude here. You can study a very wide range and still make a practical experiment out of it.

Silver: My observation is that we have not studied this, other than semi-accidentally, and inasmuch as we have, we have noticed no difference.

Shon: In view of the rapid evaporation of extracellular water that could ensue when a droplet is disseminated at a reasonably low RH, and the attendant concentrating of solute peripheral to the cell, and in view of the almost instantaneous plasmolysis that occurs when cells are exposed to hypertonic solutions, is there any reason to believe that these cells would be anything other than completely plasmolyzed?

Silver: Well, suppose you start off with a solute concentration which is, shall we say, below the plasmolytic level. To begin with, at least, they are certainly not plasmolyzed.

VFTF\*: One would have to stipulate that this is just below the plasmolytic level at the time cells are aerosolized. This could be an extremely dilute solution which will fairly rapidly achieve a concentrated or saturated level.

Silver: Yes, but everything else is concentrating, too; water is leaving the system.

Shon: Well, this is plasmolysis, is it not?

Silver: I'll shift my ground in a minute and ask you what you mean by plasmolysis. It is not plasmolysis to me; to me plasmolysis is the separation of the plasma membrane from the cell wall.

Shon: No, that is only one manifestation of plasmolysis.

Silver: It is the one you normally see. It is the one you normally are able to recognize.

Middlebrook: I think it is very important, because I can't understand why a cell, any cell, can survive under these conditions. Even with distilled water, substances come out of the cell, into the droplet. At a RH of, say, 50%, the rate of evaporation is enormous, and I wonder if any cooling would take place. Does anyone know anything about the thermodynamics? Is there a period of tremendous cooling here?

Goldberg: Your observation about an enhanced recovery due to the change of rate of hydration is interesting. When you measure ability to colonize, you can't relate it back to particular steps -- let's say, the aerosol step, or the droplet step, where you were relating it back to a multiple-step process; the statement that the cell was alive or dead, so to speak, can't really be made. It is all too often that you see a high oversimplification of these multiple-step processes in terms of the drop containing live or dead cells at a particular time. We don't know where to assign the death process yet!

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\* VFTF = Voice from the floor.

Silver: I quite agree, but there is no alternative. We have to experiment and try to draw conclusions. This is relevant to what Dr. Morton said yesterday when he was reviewing procedure\* -- when he was drawing on the blackboard criticizing, justly, the way we go about it. I mean, he knows the problem as well as anybody else; he could just as easily defend what we do. If we want to find out in detail what is happening to an organism, this is the sort of approach we've got to maintain, in a way more refined, even, than we have been doing it.

Levine: I suppose I should know the answer to it, but why, as evaporation proceeds far enough, does not sucrose crystallize in a manner analogous to mannitol?

Silver: Well, because it is a property of soluble substances.

Levine: Surely the sucrose crystals do eventually appear.

Silver: I suppose they could appear if you assume that you get a suitable nucleus formed with the right sort of properties. Dr. Record, who is concerned with freeze-drying studies, found that one apparently can hold droplets of sucrose in virtually zero RH conditions for weeks and not see a crystal form. Sodium glutamate, magnesium glutamate, and other sugars do the same. Some substances will crystallize easily -- dulcitol will start coming out at about 30% RH, and you never see it come out until you do get down to that level of dryness. Dr. Record has some samples of glucose that he had dried right down to so-called freeze-dry states and they were just super-saturated, glassy masses. They just don't crystallize.

VFTF: Well, do they hold some water in spite of the hydrogen bonding?

Silver: This is an interesting question. You see, what happens when the substance crystallizes is that there is little to show that water is being lost from the system. Nevertheless, of course, the fact that you have got a thick viscous fluid surrounding an organism, to my mind, permits further evaporation to take place. You may now have a solid, and what sort of matrix exists in this situation? But in theory, anyway, evaporation could go on. It may be that with the super-saturated droplet you have a rather artificial condition; the true humidity you have in your suspended droplet is not that which the ambient RH will necessarily tell you.

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\* See Page 186.

VFTF: Can you actually see the substance as it crystallizes?

Silver: Oh, yes. I don't know what you would want to do with this other than to prove that if the substance does crystallize, it is going to be harmful. Surely this doesn't surprise anybody, but it is nice to be able to see it.

Zimmerman: I think that there are two different conditions that involve the solution and the crystallization of sugars. One is the behavior of pure solutions with sugars; the other is the behavior of contaminated sugars. From what I have read, a thing that causes sugar refiners grief is the fact that a small amount of contamination in a sugar solution will change its behavior completely; the solution will refuse to crystallize when they know there is another 40% of sucrose present. And the explanation which has been suggested to me by reading is that sugars in general form these lactal ranges; 1,5; 2,5; and 2,6. So you have a variety of molecules with different structures, and the property of crystallization is related to the symmetrical arrangement of uniform molecular configurations in a regular pattern. Since there is a multiplicity of patterns of sugar molecules; that is, sugar molecules of different structures, they obstinately refuse to crystallize except after extended periods of time. If you deliberately contaminate them, as we did some time ago, you find that sugar solutions, which when they are pure all have different patterns of crystallization, will then assume pretty much the same general characteristic behavior.

Silver: I should add, when I talk about crystallization, I am talking about crystallization in the presence of bacterial cells. I am not just limiting myself to what happens to pure solutions.

VFTF: Do you suppose that the bacterial cell, a microorganism, an infectious agent that has to be transmitted from one host to another via the air route, in a sense would have some genetic advantage if it released some material into its environment?

Silver: Of course there'll be lots of materials in the environment of the cell. For instance, in a tubercle bacillus, the droplet is peculiarly protected by the nature of the material of mucus, and so forth. If this would protect it, then it could be some advantage which would be reflected in some metabolic characteristic of these cells.

VFTF: Well, in any case, of course, we have been thinking about transmission of disease as a problem involving rehydration rate. Again, just how important is that, in what manner and at what speed do cells

rehydrate as they go through the respiratory tract and deposit themselves on the lung, and what form of collection is possible which reproduces the process? I don't know whether we want to discuss this, but criticism of any type of ordinary impinger is just that it fails to achieve this. How much closer, if at all, is taking a glass fibre out of a box and dropping it into fluid? Is this a poor representative or not of the source of rehydration when compared to a particle dropped on the lung?

WITE: It seems to me that one of the unfortunate things about emphasis upon lab situations is that a lot of money has been spent on the investigation of microorganisms in experimental droplets, and very little money is being spent on the kinds of environment that microorganisms find themselves in under natural conditions of transmission of infectious agents by the air. Has anyone studied either at Porton, NBL, or Detrick what happens to microorganisms in mucus, or various types of mucus medium?

Kethley: We have done a great deal of work using beef extract solids, or saliva solids, to simulate this as nearly as possible, and we find the same problems we found with all of the other suspending materials. It depends upon the organism you are using and a number of other factors; in other words, results are responsive to temperature, source, humidity, strain of the organism, time of the year -- all of these variables intervene. But cells do survive better than in some of the generally used simple preparations. Certainly not as well as in the excellent admixture studies Dr. Zimmerman has described. So there is a similarity of problems, even though there seems to be a great difference in the composition of these substances. However, crystallization is never a problem because of the contamination of such materials. It is impossible to demonstrate the crystallization of such materials. It is impossible to demonstrate the crystallization of potassium chloride, for example, which is your commonest crystalloid, in beef extract or saliva. You can dry the stuff out and make smears and send them to the X-ray lab and never find any crystals; so that crystallization is not a true property

Goldberg: In studying aerosols, by and large, one observes quite good survival at a very low RH, poor recovery in the intermediate RH, and good viability at higher RH. Do you have any clues by this droplet technique of a transition zone from intermediate to low RH which

would tend to give a clue as to the physical parameters we might manipulate in that way?

Silver: This is a very interesting point. I think it is agreed that this is a common observation. Of course, if you really set to it to see whether this is merely the way nature organized it or whether it is a laboratory-induced variable, you can alter the shapes of these curves in a large number of ways. But I don't know that we should go too far in trying to explain the normal observations. I am impressed by the variation that results from the more casual changes in the formulation of, let's say, the growth medium or even the temperature or growth rate. Let's take an example like Escherichia coli again. If you formulate the medium for growing E. coli in a certain way, and give the product to George Harper, he may find that in his drum at high humidity he has to wait until the next day before he can notice much of a fall-off in viability. Is this a "natural" state of affairs? Conversely, by putting in a pinch of something else to get a complete transformation in viable recovery you may swamp the sort of transition that you usually get when going from one RH to another. Admittedly, this remains a problem, but to my mind I don't want to be side-tracked by this effect of the different RH. I am trying to look at the problem in a more general way.

Dimmick: I think that we have another problem that should be mentioned. While we have instances where we have almost no death at the time we aerosolize, we have other instances where we get a rather rapid loss of viability followed by a few cells that survive almost indefinitely. These come from the same population. If you recultivate survivors, they usually produce the same sort of death pattern as before, and yet somehow these cells are different. Perhaps investigation of these few survivors, the mechanism that causes them to live, is one of the factors that we should pay some attention to.

Silver: Well, we have just begun now. Some more people in Porton have become interested in this problem and started thinking about working on a microscale in which you look at individual droplets, quite small ones now, containing small numbers of organisms looking at the whole of the field. In other words, you examine the total contents and see how they respond to a wide variety of tests, how quickly they grow and how they respond to different metabolic feeding conditions. In fact, on a slightly bigger scale, we have been doing

that now for the last year or so, and I can't take time off to talk about results, I'm afraid, but they confirm what you suggested; that you get practically a whole spectrum for the recovered organism from those which grow after the lag phase in a fairly normal way, much the same as if you inoculate a broth, down to those which show no sign of life at all. In between you can get a whole spectrum of behavior, growing oblong sausages not dividing, dividing but dividing slowly, dividing a few times and then stopping.

Dimmick: Have you, yourself, actually made observations of this sort? This seems to me to be very important.

Silver: Oh, yes. It is particularly true that if you spray from water you tend to exaggerate these conditions. The cells clearly have a reduced capacity to manufacture everything that they need and this manifests itself in a number of different ways. Optically, the thing grows, say, to 50, 60, 80  $\mu$  long, but just cannot divide. It is clearly capable of growth. Others grow to three or four times the normal length, net, in the log phase and then divide.

VFTF: Well, if initial division is hindered you still get only one colony. Anything you get would be a lag in terms of your measurement.

Silver: There is no doubt it is certainly growing all right; you can show this in an exaggerated way if you transfer it to other media. Apparently there is something in the culture medium which gives the cell some sort of start and some facility which keeps it going at least for a time until it can manufacture what is necessary for normal growth and division. Dr. Cox, incidentally, will say something more on the lines of the way we have been looking into this field, using a slightly novel technique.

Morton: Has anyone tried to use synchronous cultures?

Silver: Yes, but I can't speak authoritatively on this. Synchrony has lost favor in Porton. I don't know why. They looked at it for a time (Dr. Herbert and his colleagues), and suddenly dropped it.

Dimmick: Well, it seems to me it might be critical for the understanding of the heterogeneity of the population concerned. You may not know the population is heterogeneous unless you do this sort of thing and even then there is the possibility of some heterogeneity being present. You recall, a couple of years ago I demonstrated distorted survivor curves from cultures I attempted to synchronize.

Silver: I can't remember why, but they started to work with the same sort of ideas in mind and suddenly dropped it and decided they were no longer interested in synchrony.

Sawyer: I think very remarkable progress currently is being made in the area of analysis of biochemical reactions of single cells, like single hepatic cells, epithelial cells, and so forth. Can anyone comment about work of this sort? It seems to me Dr. Silver has a means here of using these small drops which are behaving, in a way at least as they would in the aerosol, to obtain single cells and to employ microminiature biochemical techniques to determine what is going on, metabolically, within the individual bacterial cell, trying to find factors in this way that correlate with their behavior.

Silver: We have done nothing yet but I can say, since we have a new member joining us on the staff soon, that this will be the sort of field he will be working in.

## SOME OBSERVATIONS ON THE INFLUENCE OF SUSPENDING FLUIDS ON THE SURVIVAL OF AIRBORNE VIRUSES

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Until quite recently, studies on the survival of airborne virus particles have been carried out with material sprayed from chemically complex fluids, usually containing protein, in which the viruses have been harvested. Whole chick embryo suspension, allantoic fluid, vesicle fluid in serum buffer, and tissue culture supernatant are some that have been used in Microbiological Research Establishment (MRE). This raises the question of what influence the suspending fluid has on the survival of airborne viruses at different levels of relative humidity (RH). Hemmes, Winkler, and Kool (1962) have shown that although the composition of the suspending fluid influences the level of visible decay of airborne T<sub>5</sub> coliphage, it does not interfere with the influence of RH, as variations in decay rate with RH follow the same trend in all the media used. They have also shown that the opposite influence of RH on the survival of polio and influenza viruses occurs when these two viruses are sprayed from a mixed suspension, or separately from different fluids, and conclude that the influence of RH is more or less independent of the suspension media. However, a recent paper by Webb, Bather, and Hodges (1963) has shown that the influence of RH on the survival of airborne Rous Sarcoma virus can be strikingly altered by the use of different suspending fluids, and almost eliminated if sprayed from 6% inositol.

I have also been examining the influence of suspending fluid on the survival of airborne viruses. This paper reports the results obtained when purified polio and vaccinia viruses are sprayed from a number of suspending media, and compares them with those obtained earlier (Harper, 1961) when the same viruses were sprayed from their harvesting fluids.

#### MATERIALS AND METHODS

Suspensions. Poliovirus (Brunhilde) was grown in ERK-D cells and purified by ultracentrifugation followed by the use of anion exchange columns prepared according to the method of Sober et al. (1956). Suspensions contained between  $1.5-2.0 \times 10^{11}$  plaque forming units/mgm of protein.

Vaccinia virus, obtained from infected rabbit skin pulp, was purified by differential centrifugation followed by sucrose density gradient separation (Zwartouw, Westwood, and Appleyard, 1962). The final suspension contained about  $1 \times 10^{10}$  pock-forming units/mg of protein and the hemagglutination titer was less than 1/8.

Electron microscopy showed the suspensions to be free of extraneous material. The purified suspensions distributed in small amounts were stored in dry ice, and one bottle removed for each experiment. I am indebted to the Virology Section, MRE, for the preparation of these suspensions.

Aerosol generation and storage. Purified virus was suspended in between 13 and 200 vol of the test fluids, depending on the virus content of the suspensions, 10  $\mu$ c carrier-free  $P^{32}$  added as a physical tracer, and the pH adjusted to 7.0-7.2 before spraying with dilute HCl or NaOH. Clouds generated by a Collision atomizer were sampled 1 sec after spraying, and then stored in a 75.1 rotating stainless steel drum. The stored clouds were sampled at intervals during the 23-hr holding period. Methods of cloud collection, virus assay and determinations of viability were the same as those used previously (Harper, 1961).

Test conditions. Clouds were held in darkness at a temperature of 20-21 C at 20%, 50% or 80% RH.

#### RESULTS

I have used the term "some observations" in the title of this

paper because there has not been a great deal of replication of tests so far.

First let us look at the level of viability found in young clouds sampled 1 sec after spraying. Table 1 shows the viability of purified poliovirus after spraying from a number of fluids. Earlier results obtained with a crude suspension sprayed from tissue culture supernatant fluid are shown at the top of the table. At high RH, viability is high, irrespective of the suspending fluid used, whereas at intermediate and low RH there is considerable variation in viability. However, with the possible exception of gelatin, viability is highest at high RH and lowest at low RH with all the suspending fluids used.

TABLE 1. Poliovirus (Brunhilde strain).  
% viable 1 sec after spraying.  
20-21 C

Suspending Fluid	% Relative Humidity		
	20	50	80
Crude	19*	66*	120*
Water	1*	85*	97*
Phosphate Buffer 0.2 M	0.6	102	110
Cysteine 0.1%	0.8	60	118
Gelatin 0.5%	2.4	116	69
Calf serum 1%	0.2		
Tissue culture S.N.F.	14		
NaCl 0.5%	11*	(5.2)	110*
KCl 0.65%	7.1	18*	91
K <sub>2</sub> SO <sub>4</sub> 0.78%	4.7	11	123
Na <sub>2</sub> SO <sub>4</sub> 0.63%	8.9	33	106
CaCl <sub>2</sub> 0.47%	0		95

\* Arithmetic means of between two and five tests. Other values are the results of single tests.

Table 2 shows what happens to purified vaccinia in similar conditions, using a smaller number of suspending fluids. Viability is high in all the test conditions and appears to be relatively independent of the suspending fluid used. These results with young clouds show that with a virus sensitive to RH (polio), and despite evidence that level of viability can be considerably influenced by the composition of the suspending fluid, the influence of RH is unchanged.

Vaccinia virus, previously found to be insensitive to RH in young clouds, retains this characteristic when sprayed from a number of fluids.

TABLE 2. Vaccinia virus. % viable 1 sec after spraying. 20-21 C

Suspending Fluid	% Relative Humidity		
	20	50	80
Crude	97*	93*	112*
Water	100	102	52
McIlvaine buffer (0.002 M)			111
Water + 1% serum			110
McIlvaine buffer + 1% D.H.S.			89*
NaCl 0.5%	96	102	114*
K.Cl. 0.65%	135		82

\* Arithmetic means of between two and five tests. Other values are the results of single tests.

Tables 3 and 4 show levels of viability found after storing clouds for 24 hr in the dark. Viability of poliovirus kept at high RH after spraying from water, phosphate buffer, cysteine or gelatin is much superior to that found at intermediate or low RH where, at the most, only a trace amount of viable virus is recovered. Failure to recover viable virus at low RH, where it had previously been found to survive in significant amounts, was a bit disturbing. To see whether the ability of this virus to survive at low RH had been lost during the purification process, a test was made in which the suspending fluid was the supernatant fluid from a heat-killed suspension of polio grown in ERK-D cells, the original growth medium of the purified material. In this test, viability at low RH is satisfactory, indicating that failure to recover viable virus is a function of the suspending fluid and not due to any change in the virus. At this stage of the investigation it appeared that the survival of poliovirus stored in clouds at intermediate and high RH was little influenced by the composition of the suspending fluid. A number of salts were then tested for their effect on survival and these were found to have a marked influence on viability. At low RH, clouds sprayed from solutions of sodium chloride, potassium chloride and potassium sulphate show

viabilities of the same order as those found previously with crude suspensions. At intermediate RH, viabilities are higher than those from crude suspensions. However, at high RH, previously found to be the best conditions for survival, viabilities are low when sprayed from these salts. The considerable influence of RH found with a number of the suspending fluids is greatly reduced when spraying from the two potassium salts. The substitution of potassium in potassium chloride by calcium results in failure to recover viable virus at low RH without markedly affecting survival at high RH. When the potassium is replaced by sodium, the influence of RH is reversed. Substitution of potassium in potassium sulphate by sodium results in failure to recover viable virus at low and intermediate RH but does not make much difference to survival at high RH.

TABLE 3. Poliovirus (Brunhilde). % viable 23 hr after spraying. 20-21 C.

Suspending Fluid	% Relative Humidity		
	20	50	80
Crude	1.1*	Trace*	85*
Water	0*	0*	50*
Phosphate buffer 0.02 M	0	0	23
Cysteine 0.1%	0	0	32
Gelatin 0.5%	0	Trace	89
Calif serum 1%	0		
Tissue culture SNF	3.7		
NaCl 0.5%	1.2*	(0.9)	0.2*
KCl 0.65%	4.3	2.2*	9.1
K <sub>2</sub> SO <sub>4</sub> 0.78%	3.1	2.9	9.6
Na <sub>2</sub> SO <sub>4</sub> 0.63%	0	0	3.9
CaCl <sub>2</sub> 0.47%	0		15

\* Arithmetic means of between two and five tests. Other values are the results of single tests.

Vaccinia virus sprayed from water (Table 4) shows the same response to RH as that previously found when a crude suspension was sprayed from McIlvaine's buffer plus 1% dialyzed horse serum. When the purified virus is sprayed from buffer plus serum, viability at high RH is much higher than that found previously with the crude

suspension. The protective effect of serum is also evident when the buffer is replaced with water. Tests with virus sprayed from sodium chloride show similar viability at the three levels of RH, and from potassium chloride there is an indication that the influence of RH is less than when sprayed from water. McIlvaine's buffer also gives some protection at high RH.

TABLE 4. Vaccinia virus. % viable 20 hr after spraying. 20-21 C

Suspending Fluid	% Relative Humidity		
	20	50	80
Crude	15*	12*	0.02*
Water	38	4.5	0.6
McIlvaine buffer			5
Water + 1% serum			20
McIlvaine buffer + 1% serum			18*
NaCl 0.5%	25	31	32*
KCl 0.65%	30		10

\* Arithmetic means of between two and five tests. Other values are the results of single tests.

The limited number of tests carried out so far have not yielded enough information for useful discussion of the possible processes leading to loss of viability in airborne virus particles. They do show, however, that the survival of polio and vaccinia viruses, like that of some bacteria, is in part dependent on the chemical composition of the fluid from which they are sprayed. The demonstration that the influence of RH can be largely eliminated and in one case reversed by the choice of suspending fluid, goes a long way toward explaining conflicting reports of the influence of RH on the survival of airborne viruses.

The finding that the survival of two unrelated viruses, one a DNA, and the other a RNA virus, can be considerably improved in what had previously been found to be the conditions showing most rapid decay by spraying from sodium chloride or potassium chloride, is of considerable interest.

Although I have not been able to demonstrate such striking

differences in survival as Webb et al. (1963) found with Rouse Sarcoma virus, my results with poliovirus support his findings more closely than those of Hemmes et al. (1962). This may in part be due to the age of clouds sampled; Hemmes et al. (1962) measured viability for up to 1-1/2 hr after spraying, Webb et al. (1963) up to 5 hr after spraying. My results with clouds aged for 23 hr before sampling show a very different picture. An example of the effect the duration of an experiment can have on assessing the influence of a particular suspending fluid is given in Table 5. This shows the viability of poliovirus at different cloud ages after spraying from sodium chloride. The large initial loss of viability at low RH overshadows the much slower decay rate in this condition with the result that 1 hr after spraying, viability is 7-fold greater at the high than low RH, after 4 hr viability is similar in both conditions, and after 24 hr highest viability is found at low RH.

TABLE 5. Poliovirus (Brunhilde). % virus viable at given times after spraying from 0.5% NaCl. 20-21 C

Hours After Spraying	% Relative Humidity	
	20	80
0	11	105
1	3.9	27
4	2.5	2.4
23	1.2	0.2

MEANS OF TWO TESTS

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#### DISCUSSION

VFTF\*: Maybe you mentioned it and I missed it, what did you use as a tracer?

Harper: p<sup>32</sup>.

VFTF: Having the tracer and thus knowing how much material, physically at least, was still suspended, was there evidence that there was loss of infectivity or biological activity on the part of the virus?

Harper: What you are comparing with Eularensis is growth on an artificial medium as opposed to infection of a susceptible host. As by definition, a virus has to infect a cell(s) to demonstrate its presence, every virus titration is in fact an infectivity titration; therefore you would not expect any effect to be as great as when comparing growth on artificial media with animal infectivity. A colleague of mine (Hood) has just published the results of an investigation with influenza virus in which he compared infectivity in mice with viability measured by two different techniques. He used the PR8 and Asian strains and assessed impinger samples in whole eggs and by the chorioallantoic membrane piece technique. There was no difference between viability measured with the two assay methods or in either ID<sub>50</sub> or LD<sub>50</sub> for mice exposed by the respiratory route with young and aged clouds. Does this answer your question?

VFTF: Yes, it does. This can also be demonstrated with Coxiella.

Morton: With hemagglutinating viruses, did anyone compare infectivity with hemagglutination activity after spraying from various fluids and after varying period of aging?

Harper: Not to my knowledge.

VFTF: If a virus is aerosolized, is there any relationship between the materials we know will stabilize the virus in solution, such as servatol, or certain metals such as magnesium, in the case of poliovirus; does it have a similar effect in the case of the aerosol? My second question is -- how were these viruses purified?

Harper: The only substances I have tested are the ones I have mentioned. When we first started this work I asked some of my virological colleagues to suggest suitable suspending fluids. The suggestions I received indicated that some of the stabilizing mixtures were

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\* VFTF = Voice from the floor.

rather chemically complex. As I received a lot of conflicting advice, I took up a suggestion that I start with water and found that the purified viruses survived very well when sprayed from water.

The methods used to prepare the suspensions were: poliovirus, Brunhilde strain, was grown in ERK-D cells and purified by ultracentrifugation followed by the use of anion exchange columns prepared according to the method of Sober and others. Suspensions contained between  $1.5$  and  $2.0 \times 10^{11}$  plaque forming units/mg of protein. Vaccinia virus, obtained from infected rabbit skin pulp, was purified by differential centrifugation followed by sucrose density gradient separation. This is a method published by Zwartouw and others. The suspension contained about  $1 \times 10^{10}$  pock-forming units/mg of protein and the hemagglutination titer was less than  $1/8$ . Electron microscopy showed that the suspensions were free of extraneous matter. The suspensions used in this work were prepared by my colleagues in the virus section of MRE.

Jensen: In the 1 sec reading, these were compared to what percentages?

Harper: We put a physical tracer into our suspensions and measured the ratio of plaque or pock count to tracer count in the suspensions and in all the samples collected during the course of the experiment. What we finished up with is a series of ratios. We regard the suspension sample ratio as equivalent to 100% viability and express the cloud sample ratios in terms of percentages of that ratio: that is, per cent viabilities.

Jensen: Yesterday you seemed to imply that you took your samples before 1 sec.

Harper: You must not regard 1 sec as a short period of time in which the RH could have very little effect. In some circumstances, particularly with poliovirus at low RH, all the virus is killed within 1 sec of aerosolization.

## PROTECTING AGENTS AND THEIR MODE OF ACTION

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This subject, in an oversimplified way, will be treated from a physical aspect by considering what happens to a system consisting of a protecting agent, comparing this model with that of a bacterium contained within a droplet of pure water, and examining in what aspects these two models may differ as the droplet:

- (1) Loses water owing to evaporation,
- (2) Comes to equilibrium with air of controlled humidity, temperature and pressure, and is maintained under these conditions for a given interval of time,
- (3) Gains water upon reconstitution during collection in an aqueous medium.

Such possibilities as:

- (4) Effects of metabolites and metabolic inhibitors,
- (5) Differences in viability which result from the choice of growth conditions and bacterial species,
- (6) Damage to the bacterium caused by the mechanism of aerosol generation and of collection,

will be excluded for the purpose of this paper.

As a result of processes (1), (2) and (3) -- i.e., the processes which occur as a direct consequence of aerosol experiments -- other factors such as (7) - (10) will be considered, viz,

- (7) The rate of loss of water,

- (8) The effect of relative humidity (RH) upon this and upon the equilibrium concentration attained by the protecting agent,
- (9) The rate of regain of water upon collection,
- (10) The distribution of the protecting agent and its effect in terms of solvent modification, i.e. a solvent system in which many water molecules are replaced by some other species.

For the model of a bacterium contained in a droplet, consider Fig. 1, which may be split into three zones:

- Zone I - defined as that zone between the droplet/air interface and the cell wall,
- Zone II - defined as that zone between the cell wall and the cytoplasmic membrane,
- Zone III - defined as that zone contained within the cytoplasmic membrane.

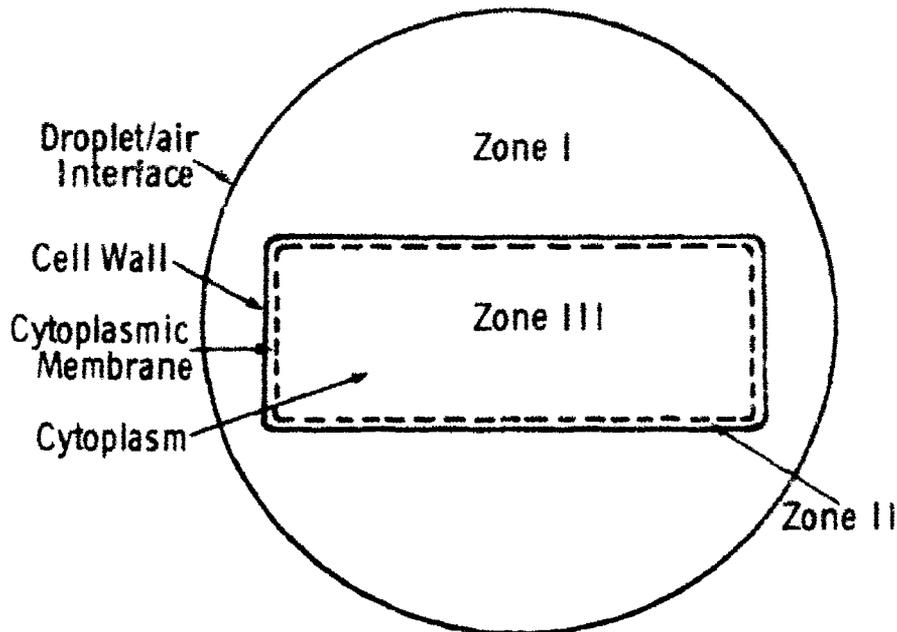


Fig. 1. Model of a rod shaped bacterium contained in a droplet

Furthermore, let the system be defined also in terms of its equilibrium state (S), with  $S^1$  representing the initial state which corresponds to that of an unevaporated droplet (also that state in the bacterial spray suspension). As a consequence of water evaporation, the system comes to equilibrium with air of controlled humidity, pressure

and temperature -- this equilibrium state will be  $S^i$  and will be dependent upon the humidity. At equilibrium, the water activity ( $a_1$ ) in zones I, II, III must be equal and the same as the water activity of the humidified air surrounding the droplet. If the water activity is not the same throughout, then mass transfer will occur in order to establish equilibrium. The equilibrium state  $S$ , therefore, may be related to water activity as is shown in Fig. 2, where

$S^i$  = initial equilibrium state,

$a_1^i$  = corresponding water activity, close to 1,

$S^j$  = test equilibrium state,

$a_1^j$  = corresponding water activity, of value between 1 and 0,

$S^o$  = boundary equilibrium state,

$a_1^o$  = corresponding water activity, equal to zero,

$W^-$  = rate of loss of water,

$W^+$  = rate of gain of water,

$S^c$  = equilibrium state achieved upon collection,

$a_1^c$  = corresponding water activity, controlled by the composition of the collecting fluid.

As a simplification  $S^i$  and  $S^c$  are shown to be the same, but this need not be so since  $a_1^i >$  or  $< a_1^c$ .

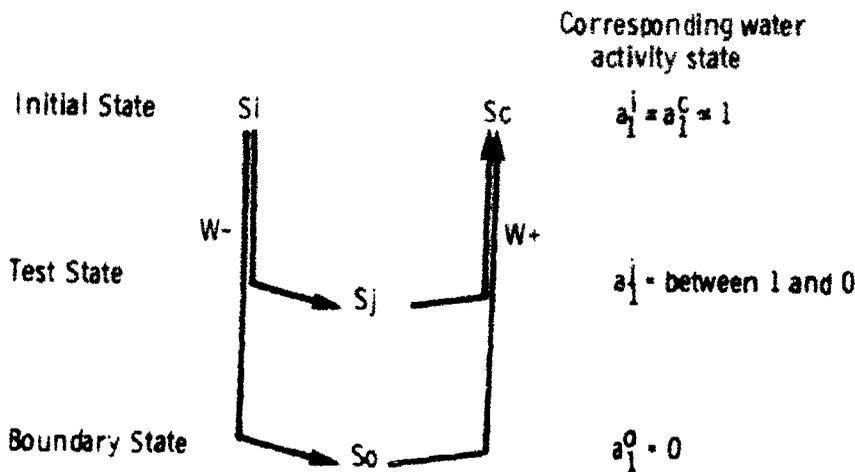


Fig. 2. Relationship between equilibrium state ( $S$ ), water activity ( $a_2$ ), dehydration ( $W^-$ ) and rehydration ( $W^+$ )

## THEORETICAL

### A. The rate of loss of water

Simple theory (Green and Lane, 1957) gives,

$$W = \left( \frac{dm}{dt} \right)_0 = 2 \pi d \cdot D \cdot (p_1 - p_1^j) \quad (1)$$

where  $(dm/dt)_0$  = rate of loss of water mass from the droplet in still air,

$d$  = diameter of droplet,

$D$  = diffusion coefficient of water vapour,

$p_1$  = water vapour pressure of water at the droplet surface,

$p_1^j$  = water vapour pressure of water in the surrounding air.

$$\text{and } W = \frac{dm}{dt} = 2 \pi d \cdot D \cdot (p_1 - p_1^j) (1 + b \cdot R_e^{-1/2}) \quad (2)$$

where  $dm/dt$  = rate of loss of water mass from the droplet in moving air,

$b$  = constant characteristic of the evaporating substance,

$R_e$  = Reynolds number (characterizes the airflow around the droplet).

$$\text{Hence } W = \frac{dm}{dt} = 2 \pi d \cdot D \cdot \bar{p}_1 \cdot F \cdot (a_1 - a_1^j) \quad (3)$$

where  $F = (1 + b \cdot R_e^{-1/2})$  = ventilation factor,

$a_1 = p_1 / \bar{p}_1$  = water activity in the droplet surface,

$a_1^j = p_1^j / \bar{p}_1$  = water activity of the surrounding air,

$\bar{p}_1$  = saturated vapour pressure of water.

From equation 3 it may be seen that the rate of water loss is related to the droplet size and to the ventilation factor (also dependent upon droplet size). For given values of  $d$  and  $F$ , the evaporation rate is related to  $a_1^j$  (the RH chosen as the test condition), such that as the RH decreases, the evaporation rate increases. For given values of  $d$ ,  $F$ , and  $a_1^j$ , equation 3 also shows that the evaporation rate depends upon the water activity at the droplet surface. Therefore, if bacteria are sprayed from pure water (i.e.  $S^i = a_1^i = 1$ ), the rate of evaporation will be maximal, since the value of  $a_1$  does not change until most of the water has evaporated from the droplet. However, if bacteria are sprayed from a solution of a protecting agent (i.e.,  $S^i = a_1^i < 1$ ), then as evaporation of water proceeds, the concentration of the protecting agent, or more strictly its activity, changes.

Since,  $a_2 = m_2 \gamma_2$  (4)

and in  $a_1 = \frac{-m_2 \phi_2}{55.51}$  (5)

- where
- $a_2$  = activity of the protecting agent,
  - $m_2$  = molality of the protecting agent,
  - $\gamma_2$  = molal activity coefficient of the protecting agent,
  - $\phi_2$  = osmotic coefficient of the protecting agent

The activity of water,  $a_1$ , in the droplet decreases as will the evaporation rate, which approaches zero as the droplet approaches equilibrium. A more detailed analysis is complex, but it would seem that the nature of the protecting agent is of importance with respect to evaporation rate, since this will depend upon the strength of the protecting agent-water interactions, and the solubility, volatility, viscosity, etc. of the protecting agent.

An additional consequence of the loss of water by evaporation will be that cooling of the droplet will occur. The extent of this will depend upon similar factors as for the rate of loss of water, since from equation 3

$$W = \frac{dm}{dt} = 2 \pi d D F \bar{p}_1 (a_1 - a_1^j)$$

and  $(T^j - T) = \frac{LD}{K} F' \bar{p}_1 (a_1 - a_1^j)$  (reference 1) (6)

- where
- $T^j$  = temperature of the surrounding air,
  - $T$  = temperature of the droplet surface,
  - $L$  = latent heat of evaporation,
  - $K$  = thermal conductivity of air,
  - $F'$  = heat transfer factor.

Hence, any modification attributed to an altered rate of loss of water caused by the presence of a protecting agent may, in fact, be owing to an altered droplet temperature. Also, those properties which are functions of  $a_2^j$  (i.e. the activity of the protecting agent at the  $S^j$  equilibrium) will be affected in accordance with droplet temperature, as will those processes which occur as a consequence of changing rapidly from the  $S^i$  to the  $S^j$  equilibrium (see Section B).

#### B. The equilibrium concentration

At the  $S^j$  equilibrium, the water activity in zones I, II and III will be equal to that in the surrounding air, i.e. all have a value

of  $a_1^i$ . Hence, from equations 4 and 5 the corresponding activity of the protecting agent,  $a_2^j$ , is controlled by  $a_1^i$ , since

$$\mathcal{E}_2 = 1 + sm_2 + tm_2^2 + um_2^3 + vm_2^4 \quad (7)$$

$$\ln \mathcal{E}_2 = wm_2 + xm_2^2 + ym_2^3 + zm_2^4 \quad (8)$$

where s, t, u, v, w, x, y, z are coefficients

This means that in the absence of other solutes, the activity achieved by the protecting agent depends only upon the value of the test pH and is independent of  $a_2^i$ , the initial activity. However, this will be important with regard to the size of the droplet at equilibrium, since the greater the initial concentration, the greater will be the equilibrium diameter. This may be of great influence, because in a solution the concentration of the solute at the surface differs from that in the bulk of the solution and depends upon whether the solute is positively or negatively adsorbed at the surface, i.e., lowers or raises the surface tension of water. McBain and Humphrey (1932) found this layer to extend for 50-100  $\mu$  beneath the surface of a solution, and therefore aerosol droplets may achieve surface, rather than bulk, solution activities and properties.

If other solutes are present at the  $S^i$  equilibrium in comparable activities to the activity of the protecting agent, then the composition (in terms of activities) of the droplet at the  $S^j$  equilibrium will depend upon  $a_1^i$  and upon the composition at the  $S^i$  equilibrium, together with the nature of solutes involved. Therefore, should protecting agents or other substances be toxic at high concentration, the value of  $a_1^i$  will govern mainly if such a concentration is exceeded, reached, or not attained.

A further consequence of the presence of a protecting agent is that the solvent system has been changed. For cells sprayed from pure water, then at the  $S^j$  equilibrium, the situation is one where the cell components are in direct equilibrium with water vapour, i.e. a solid-vapour equilibrium. In the presence of a protecting agent the situation is different, because solid liquid/vapour equilibria can occur. Depending upon the distribution of the protecting agent, solid/liquid solid vapour, and liquid vapour equilibria may exist. Of these, the solid liquid constitutes the modified solvent system, and in regions where it exists, there is opportunity for the protecting agent to operate through functions related to its activity,  $a_2^j$ , e.g., a modifier

of dielectric constant, ionic strength, activity of other substances with which it is in association, but not as a water activity modifier for a system at equilibrium. Such functions might be expressed in terms of the greater or lesser susceptibility of protein, lipid and nucleic acid to an altered biological function, for example, to denaturation or to a physically different environment where a dielectric constant might be so changed that a reaction is prevented or accelerated.

The actual value of  $a_1^j$  (and perhaps its rate of achievement within the bacterium) may also be important for changes in structure that arise solely as a result of altered water activity. Examples are changes in structure of the nucleic acids and other polymeric compounds, such as lipoproteins and proteins. At higher values of  $a_1^j$ , phenomena associated with swelling can occur, whereas at lower values of  $a_1^j$ , helical configurational transitions can take place to give, eventually, non-helical disordered states for the nucleic acids. Such transitions usually show hysteresis, i.e. transitions which are not instantly, and perhaps completely, reversible (Falk, Hartman, and Humphrey, 1963). These changes, like those arising through solvent modification, will depend upon the distribution of the protecting agent.

If the distribution is known, some indication may be obtained of possible mechanisms involved, as shown in Table 1.

TABLE 1. Relationship between distribution of protecting agent and its mechanism of action

Case	Zone occupied by agent	Zone effected	Mechanism
1	I	I	$W - a_1^j f(a_2^j)$
		II	$W - a_1^j$
		III	$W - a_1^j$
2	I, II	I	$W - a_1^j f(a_2^j)$
		II	$W - a_1^j f(a_2^j)$
		III	$W - a_1^j$
3	I, II, III	I	$W - a_1^j f(a_2^j)$
		II	$W - a_1^j f(a_2^j)$
		III	$W - a_1^j f(a_2^j)$

The zone occupied by the protecting agent is, in the first instance, dependent upon its permeability through the cell wall and cytoplasmic membrane, but the zone can be modified by experimental procedures. For example, if an agent such as raffinose is chosen, which from plasmolysis data is thought to penetrate the cell wall of Escherichia coli 163 (Jepp) extremely slowly (Record, Taylor, and Miller, 1962), then by mixing bacteria and a solution of this agent immediately prior to droplet formation, and given a short equilibration time for the achievement of  $S^j$ , raffinose will be maintained external to the cell wall, i.e. case 1. During a short life at the  $S^j$  equilibrium, it would seem unlikely that much raffinose would cross the cell wall owing to the fantastic viscosity achieved. However, if bacteria and raffinose solution are equilibrated at  $S^i$ , then the protecting agent occupies zones I and II, i.e. case 2. At the  $S^j$  equilibrium it would again seem unlikely that raffinose would transfer across the cell wall because of the viscosity control of its diffusion and also because of the small solute activity gradient across the cell wall, viz.  $(a_2^j)_I - (a_2^j)_{II}$ . By this example of the technique, for given values of  $d$ ,  $F$  and  $a_1^j$ , the importance of the occupation of zone I and II may be estimated.

### C. The rate of gain of water

After attainment of the  $S^j$  equilibrium state, the cycle is completed by collection of the droplet to give the equilibrium state  $S^c$ , which will depend upon the composition of the collecting fluid.

Protecting agents that do not crystallize or evaporate can modify the rehydration process and the response of the bacterium upon reconstitution by a variety of mechanisms, for example,

- (1) a changed interface for the  $S^j$  to  $S^c$  conversion, i.e. solid/liquid instead of solid vapour,
- (2) the formation of phase separation, e.g. polyethylene glycol phosphate water, and dextran polyethylene glycol water systems, which can exist as one or two phases, depending upon compositions (Abbottson, 1960),
- (3) the rate of diffusion of the protecting agent away from the droplet surface collecting fluid interface, and therefore the rate of diffusion across zone I (and therefore into the bacterium) since this may be diffusion controlled and will also be a net rate.
- (4) the distribution of the protecting agent, since its presence in zone II (e.g. a plasmolyzed cell) will result in the cytoplasmic

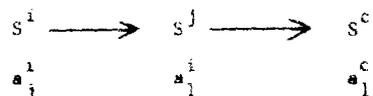
membrane not being able to return to the cell wall until the protecting agent has diffused out of zone II. The degree to which the presence of the agent transiently prevents the recovery of the cytoplasmic membrane (and therefore the swelling rate and activity of the components of zone III) will depend upon, for example, the mass of the agent in zone II, the volume contraction of the agent solution on loss of water during equilibration with  $a_1^j$ , the degree of cell-wall shrinkage and its recovery rate, and the diffusion coefficient of the protecting agent through the cell wall.

(5) similarly as for (3), the rate of diffusion of the protecting agent out of zones II and III (if present), since the rate of diffusion of water into the bacterium will be a net rate.

These mechanisms may be further modified by the temperature of the collecting fluid, and by those properties which are functions of its activity,  $a_2^c$ , e.g. water activity  $a_1^c$ , viscosity, ionic strength, dielectric constant, etc., and hence also the permeability of the solutes present in the collecting fluid.

The rate of rehydration could be of importance, by the same reasoning, applied to the dehydration process, together with at least one additional reason - specifically, the possibility of spheroplast formation. If the rate of rehydration is too rapid, then osmotic pressure developed by the components of the cytoplasm, together with a contribution of any protecting agent in zones II and III, may be sufficient to cause rupture of the cell wall, especially if the wall shrinks as a result of experiencing the  $S^j$  equilibrium and has an appreciable relaxation time (in comparison with the time for the  $S^j$  to  $S^c$  transition) required for its conversion back to a more normal state.

In summary, the situation is envisaged as the following change of equilibrium states caused by the movement of water:



$S^i$  is the initial equilibrium state defined by  $a_1^i$ ,

$S^j$  is the test equilibrium state defined by  $a_1^j$ ,

$S^c$  is the collected equilibrium state defined by  $a_1^c - a_1^i$ .

(1) rates and times of conversion are related to  $W^-$  and  $W^+$ , and are influenced by the presence or absence of a protecting agent and

the values of  $a_1^i$ ,  $a_1^j$  and  $a_1^c$  chosen.

(2)  $S^i \longrightarrow S^j \longrightarrow S^c$  conversion for maintenance of biological activity may be dependant upon time and  $a_1^j$ , since conversions that arise as a direct result of aerosol experiments may

(a) involve movement of small molecules and rearrangement of the structure of nucleic acids, proteins, lipoproteins, etc., such that biologically inactive species can occur.

(b) be subject to the presence of a substance whose activity at any time will be dependant upon  $W$ -,  $W+$ , and  $a_1^j$ , and upon competitive action of a protecting agent with which the substance is in association.

(c) be related to a solvent-modifying property of the protecting agent which is a function of its activity and also, therefore, of  $W$ -,  $W+$ ,  $a_1^j$  and its distribution.

#### EXPERIMENTAL AND DISCUSSION

The rate of loss of water ( $W$ -) depends upon the droplet diameter and also upon the ventilation factor for given values of  $a_2^i$ ,  $a_1^j$ , etc. The use of a fibre technique at different ventilation rates and with droplets of diameter of  $140 \mu$  (wet) enables a study to be made of evaporation rate over a range that included evaporation rates greater than those achieved in the aerosol. Data in Table 2 suggest that if viability is a direct function of the rate of loss of water, then for the gram negative organism E. coli 163 (Jepp) the critical rate is likely to be much greater than, much smaller than, or so critical as to lie between those rates studied. This is similarly so if the critical function is the equilibrium time which fell between 10 and 120 sec. Although one culture of E. coli 163 became sterile at equilibration times of 15 and 20 sec, other cultures of E. coli 163 have not demonstrated this effect.

Figure 3 shows, for an aerosol age ( $t$ ) of 15 min, the per cent viability (traced by Bacillus subtilis spores) of E. coli 163 with sucrose in zones I and II. The experiments were performed during the same day and the suspensions were prepared from the same cultures of bacteria. Collecting fluids of phosphate buffer (PBMA) and sucrose (1M/l) were used. Enhanced viability as the sucrose concentration increased may have resulted from an increased competitive action (i.e. solvent modification), although droplet size and equilibration time probably changed with sucrose concentration.

TABLE 2. Per cent viability of *Escherichia coli* 163 traced by *Bacillus subtilis* studied by the fibre technique at 23 C, as a function of equilibration time (T) and exposure time (t)

t (min)	Collecting fluid	% viability																
		23	0	50	65	40	100	48	79	59	81	25	36	67	44	82	45	28
15	PBMA																	
15	IMSu	0	0	78	114	101	98	79	103	74	95	66	82 <sup>*</sup>	67	51	69	77	75
30	PBMA	0	0	45	69	58	89	57	80	34	21	11	20	77	40	37	52	47
30	IMSu	0	0	68	108	88	94	58	73	83	90	39	78 <sup>*</sup>	90	40	44	63	65
30	0.4MSu	6	84		77	66	53	58			61	34		34	16	41	61	
30	PBMA <sup>*</sup>	49	97	71	48	49	58	64	80	50	51	42	51	81	78	69	25	27
T (sec)		15	20	50	20	30	35	120	30	72	20	45	35	17	13	25	10	25
Protecting agent		0.2 M Sucrose	0.1 M Sucrose	0.1 M Sucrose	0.1 M Sucrose	1 M Glycerol	0.2 M Sucrose											
BF %		50	50	50	50	50	50	50	50	50	50	50	50	50	20	20	20	
Culture number		27663	15763	21663	19763	20263	19763	25763	25763	25763	25763	25763	25763	25763	25763			

PBMA = phosphate buffer + manucol + antifoam.

0.4MSu = 0.4 M/l sucrose in PBMA.

IMSu = 1.0 M/l sucrose in PBMA.

\* droplets exposed to saturated air for 4 min prior to collection.

⊗ collected in 2 M/l sucrose in PBMA.

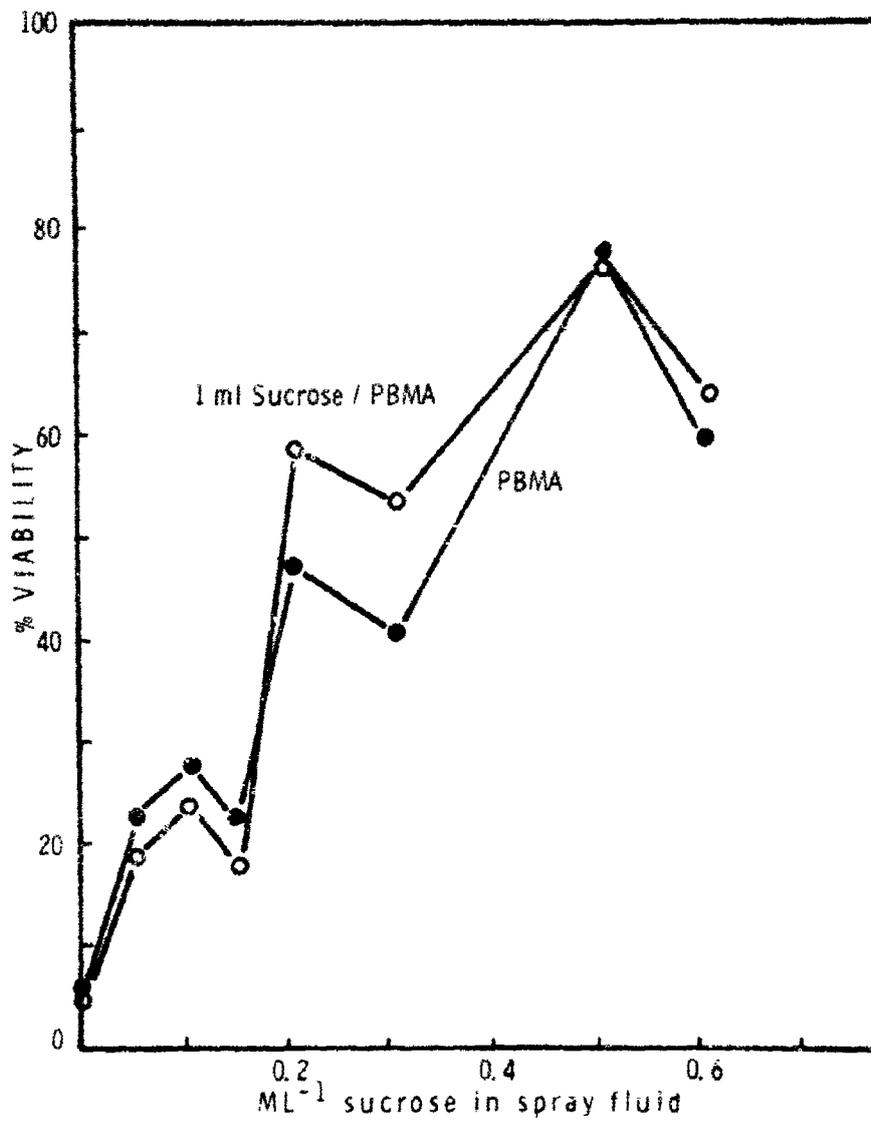


Fig. 3. Aerosol survival of *Escherichia coli* 163 at 50% RH 23 C as a function of initial sucrose concentration for an exposure time of 15 min.

Collection by impinger containing PBMA and 1 M sucrose/PBMA.

Culture number 17563

Tracer, *Bacillus subtilis*

PBMA = phosphate buffer + manucol + antifoam

Data in Table 3, which indicates the per cent viability obtained with different distributions of raffinose, suggest that the presence of raffinose in zone II (i.e. between the cell wall and the cytoplasmic membrane) resulted in a collection phenomenon, while protecting action could be expressed with raffinose outside the cell wall. Since raffinose is thought to be barely permeable through the cell wall, and since aerosol formation very rapidly followed the addition of raffinose to the bacteria in experiments with raffinose mainly external to the cell wall, it would seem unlikely that solute transfer across the cell wall occurred in these experiments during the  $S^1 \longrightarrow S^2$  conversion and during life in the aerosol.

The results shown in Table 4, obtained by Dr. Silver using E. coli 163 in the presence of glycerol, erythritol, and glucose, with E. coli 162 and glucose, and with Serratia marcescens in the presence of glycerol and sucrose, were similar in that a protecting agent external to the cell wall resulted in most, if not all, of the observed response. Glycerol and erythritol may be exceptions to this, but as these substances are permeable enough to penetrate the cytoplasmic membrane, their distribution was not as certain as for the larger molecules.

Therefore, because raffinose external to the cell wall, i.e. case 1, can result in a substantial enhancement of viability, loss of viability is prevented by the mechanisms shown below, viz.

<u>Zone</u>	<u>Mechanism</u>
I	$W- W+ a_1^j f(a_2^j)$
II	$W- W+ a_1^j$
III	$W- W+ a_1^j$

provided that the system is at equilibrium with  $a_1^j$ .

Hence, if enhanced viability arises from a direct solvent modifying property of raffinose, the cell wall apparently is a labile structure of E. coli 163 (provided that solute transfer across the cell wall did not occur). If too rapid a rate of removal of water is responsible for loss of viability, then the sensitive structure/s could be in zones I, II and/or III, the the presence of raffinose at an initial concentration of 0.3 M/l external to the cell wall is sufficient to prevent the lethal rate from being achieved. Similarly, if the rate of water is too rapid, this layer is sufficient to prevent

TABLE 3. Per cent viability of *Escherichia coli* In3 traced by *Bacillus subtilis* studied by the aerosol technique at 23 C, as a function of distribution of raffinose and of exposure time. Collection by impinger.

ZONE I M/l	0.05										0.30										0.08										0.30										0.08										0.30										0.30										0.30																																																																																																													
	0.05										0.05										0.30										0.05										0.30										0.05										0.30										0.05										0.30										0.05										0.30																																																																															
ZONE II M/l	0.05										0.30										0.05										0.30										0.05										0.30										0.05										0.30										0.05										0.30																																																																																									
t Collecting (min)	fluid										fluid										fluid										fluid										fluid										fluid										fluid										fluid										fluid																																																																																																			
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15	22										23										75										41										73										11										35										55										12										50										46										61										24										58										25										38										32																			
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RH %	50										50										50										50										50										50										50										50										50										50										50										50										50										50										50																																							

t = exposure time PBM = phosphate buffer + mucicol + antifoaam Su = sucrose/PBM  
 ZONE concentrations are those immediately prior to aerosol generation.

TABLE 4. Per cent viability of bacteria traced by *Bacillus subtilis* studied by the aerosol technique at 21 C, as a function of distribution and exposure time. Collection by impingers (I) and bubble\* (B)

ZONE I M/1 ZONE II M/1 t Collecting fluid (min)	% Viability												
	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	0.5
5 A	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5
5 B	0	1.0	0	1.0	0	1.0	0	1.0	0	1.0	0	1.0	0.5
5 C	10I	28I											
15 A	0B	0.2B	2I	1I	10B	1B	30I	46I	10I	48I	50I	53I	19I
15 B	0.2B	0.8B	3I	1I	11B	3B	68B	10I	31I	38I	37I	46I	21I
15 C	29B	47B	5I	36I	11B	11B	45B	11B	119I	97I	33I	22I	
Organism													
Culture number													
PH %													
Protecting agent													
	E. coli 163 20162 55	E. coli 163 16261 20	E. coli 163 13161 50	E. coli 162 10162 58	S. marcescens 24262 70	S. marcescens 2361 15	S. marcescens 2361 15	S. marcescens 2361 15					
	glycerol	erythritol	glucose	glycerol	glycerol	glycerol	sucrose						
Collecting fluids													
A	phosphate buffer + phosphate buffer + manuocol + antifoam manuocol + antifoam												
B	15% raffinose 15% raffinose + 1M erythritol												
C	1M glycerol 5M glycerol												
	distilled water + manuocol + antifoam												
	1M glycerol												
	2M glycerol												
	5M glycerol												
	1M sucrose												
	2M sucrose												

occurrence of the lethal rate. Ingress rates less rapid than this can cause loss of viability owing to osmotic shock, provided that the system is sensitive to this order of ingress; i.e. osmotic shock need not be a primary death mechanism.

Since 100% viability was not obtained with raffinose when in zone I or in zones I and II, and since the per cent viability as a function of time also decreased, raffinose only lowers the decay rate rather than prevents loss of viability, and therefore the protecting action of raffinose may arise through the prevention of some death mechanisms, while others continue at the same, or reduced, rates as those in the absence of raffinose. These possibilities are supported by experiments using *E. coli* (type B) and phage T7 (Cox and Baldwin, 1964), which suggest that some systems of this bacterium and this phage are capable of maintaining biological function during the course of the processes which occur as a consequence of aerosol experiments for cloud ages of the order of half an hour. By 2 hr some further impairment of biological function apparently occurs, since under these conditions not all the bacteria supported phage growth and became lysed.

Because raffinose was able to confer a protecting action, then perhaps larger molecules than raffinose, such as polyethylene glycol (PEG) and dextran of high molecular weight, might be expected to affect the viability when these are used as protecting agents. Figure 4 shows the per cent viability obtained using PEG/sucrose mixtures at 50% RH, and that PEG alone caused a great loss of viability. As the sucrose concentration increased so did the viability and the competitive action. Figure 5 suggests that the viability response is complex in the presence of PEG if bacteria are incubated in vitro with PEG at room temperature for 30 min. Since the breakpoint in the curve is ca 25 w/v% for a range of molecular weight fractions of from 200-15,000 for *E. coli* 163, the effect may concern adsorption and interfacial tension at a solid/solution interface. This idea is supported by the clumping behavior of *E. coli* 160 and 163 in the presence of PEG, and because a stable dispersion was obtained at PEG levels that gave low viabilities. The phenomenon was also complicated by the choice of plating media and suggests that the overall effect may have been caused by changed wettability, permeability, enzyme activity, etc.

Dextran at 50% RH for *E. coli* 160 and *E. coli* 163 was found to be a poor protecting agent; results were similar to those of bacteria

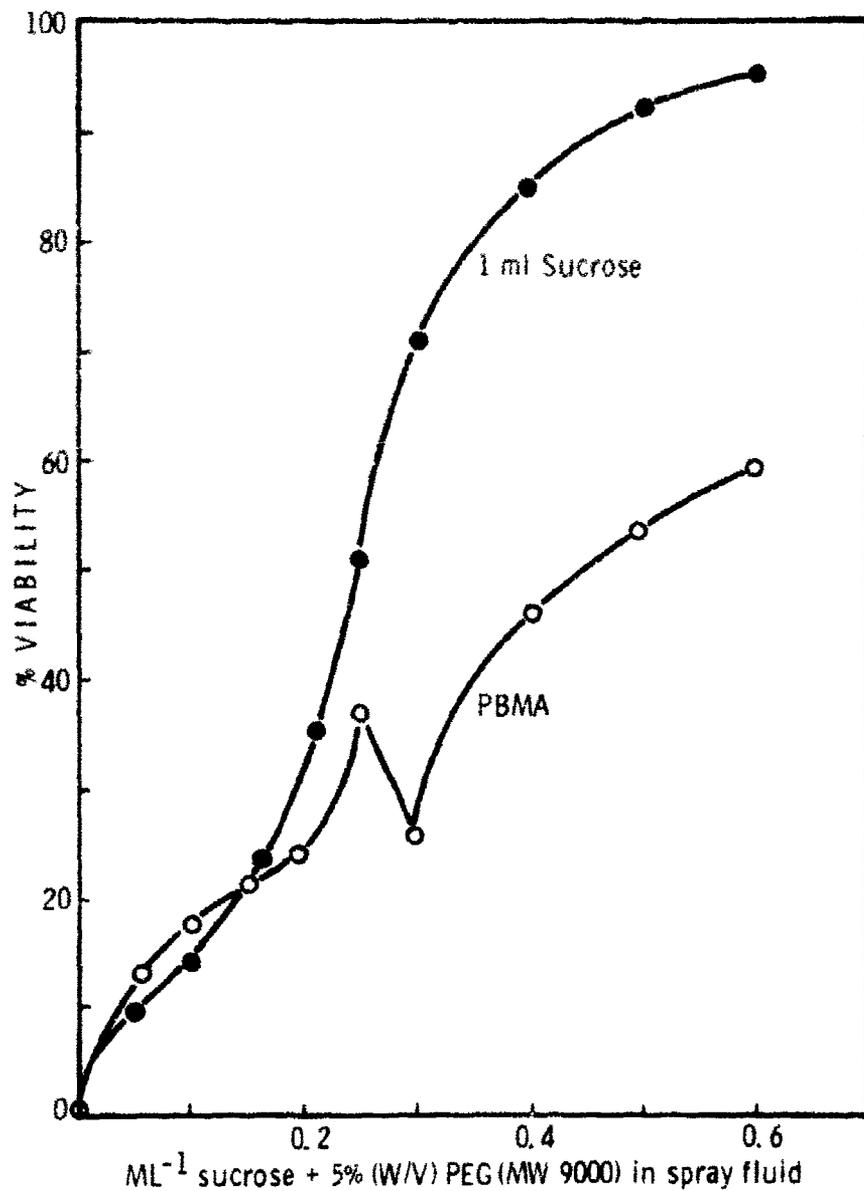


Fig. 4. Aerosol survival of *Escherichia coli* 1b3 at 50% RH 23 C, as a function of sucrose + PEG in spray fluid for an exposure time of 15 min.

Collection by impinger containing PBMA and 1 M 1 sucrose.

Tracer, *Bacillus subtilis*

Culture number 10563

PBMA = phosphate buffer + manucol + antifoam.

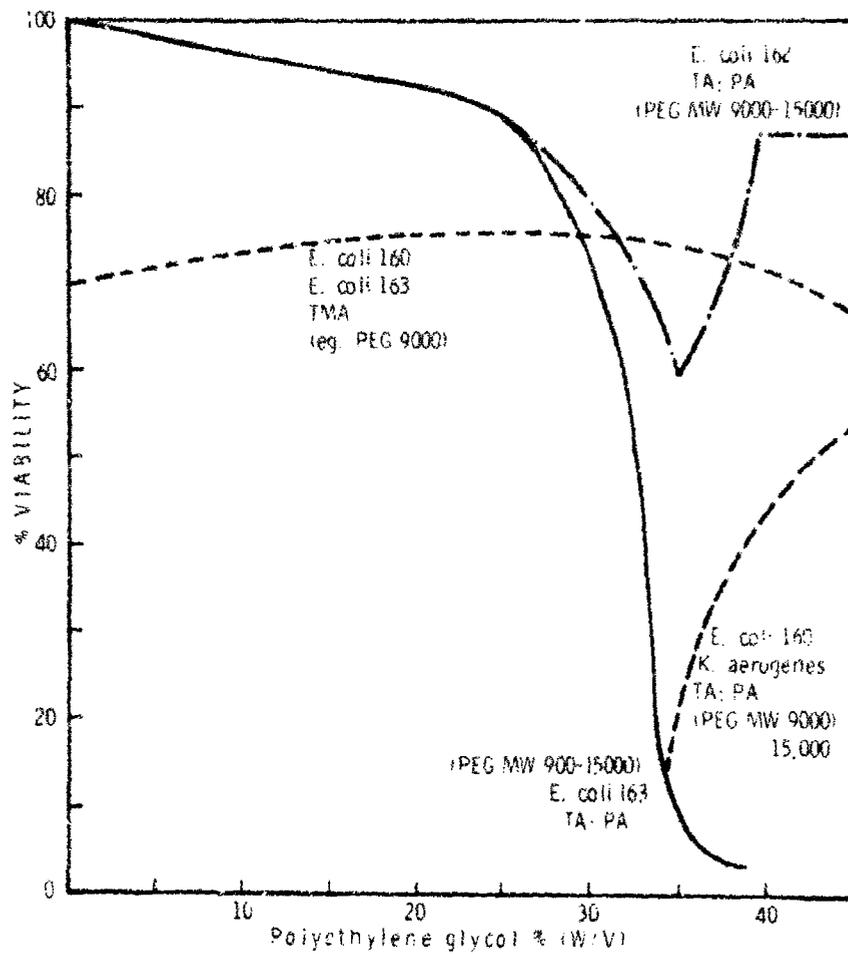


Fig. 5. Response patterns for bacteria in contact with PEG for 1/2 hr at room temperature as influenced by the plating out media.

TA = tryptone agar  
 PA = peptone agar  
 TMA = tryptic meat agar

sprayed from water into air. Dextran and especially PEG lower the surface tension of water and can cause phase separation. Hence, the possibility of markedly different surface free energy changes exists for these two classes of compounds, i.e. those which raise or those which lower the surface tension, when present in high concentration. The possibility of a large surface free energy change also occurs when cells are sprayed from water, since interfaces are likely to change from solid/liquid to solid/vapour, and back to solid/liquid upon collection of the aerosol. Therefore, to elucidate the possible mode of action of protecting agents for the gram negative bacteria, compounds unable to permeate the cell wall readily, but having a range of physical properties, need to be studied in conjunction with investigations using phage. In this way some of the mechanisms causing loss of viability after a change of state of water can be elucidated.

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#### DISCUSSION

Silver: I want to comment a little on this paper. What we are trying to do here at this stage of the work is to pin down, if we can, the physical and physico-chemical phenomena associated with the life and death of organisms. Of course these phenomena are not just restricted to the bacterial cell - it is the whole of the system, which includes the techniques as well. We want to look at the behavior of collecting fluids and plating media; if organisms don't grow on petri dishes, has this anything to do with the organism itself, or is it a purely physical phenomenon which is induced by the physical properties of the so-called protective substance? You might as well say polyethylene glycol does nothing else, but will stop the organism from

growing, where in fact the organism is still alive. Consider dextran (M.W. 115,000) for instance: it can't possibly kill the organism, because it is never in a position to. Presumably it can only operate from outside the cell surface. By way of elaboration, this is what we are trying to do and we shan't move on, unless we have further details to consider the interference of metabolic activities generally -- until we feel that we are moving on fairly certain ground.

Cabelli: I would appreciate it a great deal if you would go over the slide where you just used sucrose alone, and if I understood correctly, the difference in survival rate of percent survival numbers is due to the shift in the equilibrium time.

Cox: That is a possibility.

Cabelli: If this is the case, how did the numbers compare; that is, equating change in concentration of sucrose to a change in the composition of the systems and to equilibration time? In other words, what was the difference in time that would be involved in the difference in equilibrium? You didn't make any conjectures as to the range of times -- were they in micro-seconds?

Cox: If one sprays from water, using for example a Collison spray, most of the droplets equilibrate in times of the order of a fraction of a second. I think it is true to say that if one adds sucrose, for example, then this does extend the equilibration time, especially under conditions of high ventilation rate.

Goldberg: On that particular slide, what range of time were you talking about?

Cox: That was for aerosols, as opposed to droplets supported on fibres, and so I do not have experimentally derived equilibration times.

Goldberg: Well, what about your computations?

Cox: The equation given earlier is too approximate and was included only to show some of the factors involved in the rate of evaporation of droplets.

Morton: I wonder if I can ask Dr. Cox to tell us a little bit more about (this is speculation, of course) the possible competitive adsorption of substances like sucrose and polyethylene glycol (PEG), particularly in respect to the work that he did in suspensions. I can't anticipate, of course, what he has to say, but I think he could show some relationship between the two, and also in regard to the addition of sucrose and what he thought about the role of these high

molecular: how they might act on the surface of the cell.

Cox: This was the sort of response we obtained -- per cent viability as a function of percent polyethylene glycol (Fig. 5 of paper; further details are to be published elsewhere). What we tried to do was to see whether or not sucrose could compete with polyethylene glycol, and thereby prevent the observed response. In fact, it appears that the per cent viability as a function of moles of sucrose per liter in the presence of different amounts of polyethylene glycol, using E. coli 163, caused a competitive action of sucrose in the presence of 30% PEG (M.W. 1540). Whereas if polyethylene glycol concentrations were increased to 60%, then no matter how much sucrose we had, up to 0.4 M/l, we couldn't get a reversal of the polyethylene glycol effect in a test tube. In the aerosol the situation is different, because when one sprays from polyethylene glycol alone, then for equilibrium at 50% RH the concentration of polyethylene glycol is pretty high and probably attains about 80% PEG. This is a rough estimate, because I don't have any water activity data for PEG solutions. In the aerosol we found this "competitive action" of sucrose (Fig. 4 of paper). Now this could have arisen, for example, from two reasons: either we were getting competitive action at binding sites between sucrose and polyethylene glycol, or the effect of sucrose was one of a thermodynamic competition in that the concentrations achieved in the aerosol droplet by PEG were less than the critical region of Fig. 4; i.e. the sucrose was acting as a PEG diluent. Other data on the effects of polyethylene glycol, and in this case in glucose mixtures, have been obtained by Record, Taylor, and Miller (1962). At different combinations of PEG and glucose they found that the two molecules together gave a more enhanced survival for freeze-dried material than was obtained with either glucose or polyethylene glycol alone. This was not only true of polyethylene glycol, it was also true of other high molecular weight compounds such as dextran and bovine serum albumin. We have found the same sort of phenomenon with the fibre technique using mixtures of dextran and sucrose -- this is a high-molecular weight dextran 115,000 (See Fig. D-1.)

The technique here was to take different compositions in terms of moles of sucrose per liter, plus 5% dextran, to form tiny droplets, expose them to air at 50% RH then collect them in phosphate buffer or sucrose, or after a slow rehydration process collect them in phosphate buffer.

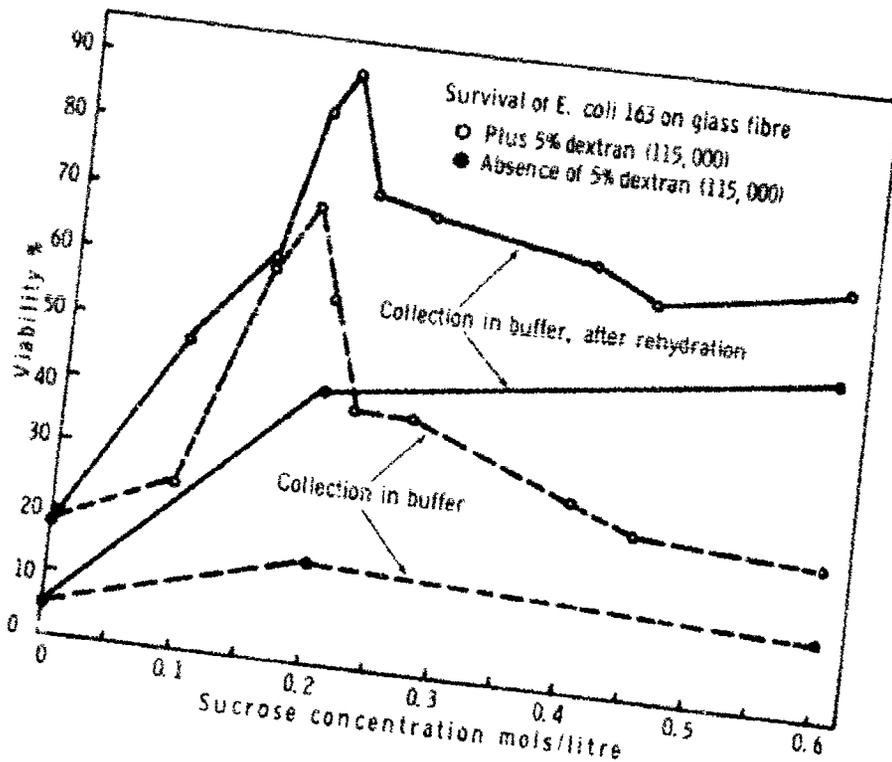


FIG. D-1

Since dextran is of such a high molecular weight (115,000), I think we can be more certain than we are in the case of raffinose, that dextran is probably acting outside (or within the cell wall, but not inside) the cell, in which case this is very interesting, especially in the light of responses obtained with polyethylene glycol. As I mentioned before, in the presence of polyethylene glycol one observes a change in clumping behavior of bacteria. *E. coli* 160 is a very good demonstration of this, because it has a large tendency to clump and does so at PEG concentration other than those corresponding to the minimum of Fig. 4. One wonders, therefore, if the action of some of these very high molecular weight compounds is not at the cell wall and is in some way modifying the response of the bacterium. It leaves one to consider such things as surface-free energies.

For a liquid against air it has a given surface tension against air, and a mixture of two liquids that are not miscible develop an interfacial tension. For a solid-liquid interface, as in a bacterial suspension, such problems may occur as the spreading of liquids over solids and whether or not the cohesive forces of solid to solid are

greater than solid to liquid. If the adhesive forces of solid to solid are greater, clumping occurs, whereas if the solid-liquid cohesive forces are greater, then a dispersion results. So one of the things that we have tried to do is to see if the viability response to polyethylene glycol can be explained in terms of either changes in watability or something of that nature. It is extremely difficult to make any quantitative measurements of interfacial tensions and surface tensions, especially of solids, but some experiments have been performed on solid polymers and it is true to say that the interfacial tension of a solid against air is much greater than it is against the liquid if that liquid spreads over that surface. Some of the work of this type is done from the consideration of the way in which glues operate; it is interesting that substances such as dextran, sucrose, sorbitol, glycerol, etc. are used in the manufacture of adhesives.

One of the consequences arising from aerosolizing from water, as I mentioned this morning, is that the interface changes from solid-liquid to solid-air; whereas if one used something like sucrose or some other substance which does not crystallize or evaporate, a solid-liquid interface exists and the actual change that the surface experiences is much less than if one uses a substance that does crystallize or evaporate. It is just about conceivable that the actual stability of the cell wall and/or cytoplasmic membrane may depend upon interfacial tensions and surface tensions, because if one looks upon these structures in terms of an emulsion, oil in water, or water in oil type, then stability problems are likely to occur, and so I think we should pay attention to the stability of the cell wall and the cytoplasmic membrane region, and perhaps in this way we might find one of the reasons why bacteria die in the aerosol, especially over the initial equilibration stage.

VFTF\*: During the course of the Symposium, nobody has mentioned anything about experiments involving the study of the non-ionic surface active agents. You mentioned surface tension interfaces between the solid and liquid and solid-air phases. I wonder if the surface active agents of the non-ionic type have any effects or relationships with the other types of stabilizing agents that have been discussed. Something on that must have been studied.

Cox: Polyethylene glycol can be looked upon as a non-ionic

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\* VFTF = Voice from the Floor.

surface active agents, as can ethane diol, glycerol, dextran, etc. Of the hydroxy compounds that we have tested for protecting action in the aerosol, those raising the surface tension of water, such as sucrose and raffinose, seem to confer a stabilizing action, whereas those which lower the surface tension tend to be poor stabilizing agents. However, if this difference in surface adsorption properties is the reason why the polyhydroxy compounds are or are not good protecting agents, then processes which occur in the aerosol and in droplets supported on glass fibres would seem to be different, since as mentioned by Dr. Silver in his presentation, glycerol does not behave as a protecting agent for E. coli 163 in the aerosol, but does so for the fibre technique.

VFTF: We have done some investigations with a few of these surface active agents without noticing any appreciable modification in the aerosol behavior; they neither provided stability nor induced instability.

## RADIATION, RELATIVE HUMIDITY AND THE MECHANISM OF MICROBIAL DEATH IN AEROSOLS

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I would like to start by saying that it is very pleasing to see somebody else thinking in terms of water movement because, as most of you know, we in our laboratories have been working along these lines since 1956. Before I describe these experiments, I want to state that, with regard to the question of cell survival in aerosols and damage due to the impinger collection, in all our work we have found no significant effect of the impinger fluid, either with aerosols that have been generated from a cell suspension with a protective agent, or from distilled water; and we have used organisms where we can find very little effect of the actual spraying mechanism itself. It is true that one does find a little effect of the impinger; by and large this is never any greater than a factor of two, and I am sure that most people using aerosols will agree that it is very, very difficult to tell the difference between a cell count of, say,  $1 \times 10^6$  and  $2 \times 10^6$  in an aerosol. This kind of argument we felt is insignificant.

In aerosols a susceptible organism will drop some 3-4 logs in count at low relative humidities (RH) in quite a short period of time. We have used organisms that will do just this because it is not worthwhile trying to find out why a cell dies in an aerosol if, in fact, it does not die.

In an effort to find out why a cell is sensitive, we have studied many types of cell with or without various protective agents as, I am sure, most of you know. I am not sure whether to consider it an honor that no one so far has mentioned inositol. We have been using this compound to protect cells after extensive studies on the action of polysaccharides in one form or another, straight-chain compounds, ring compounds, and so on. These studies led us to conclude that the activities of these compounds relied primarily on the presence of hydroxyl groups and possibly amino groups. I was quite amused by the mention of sodium arsenite this morning; it appears one takes a compound such as raffinose which desiccates the cell because it will not penetrate and then adds sodium arsenite to prevent this extraction of water -- a sort of cyclic research.

The work I am going to describe was done to test an hypothesis, namely that compounds such as inositol replace removed water. We have found inositol to be an extremely good protector of airborne organisms provided one does not add compounds such as sodium chloride, thiourea, or any other hydrogen bond breaking compound; if you do, then you will find less or perhaps absolutely no effect of the inositol, or for that matter, any like protector molecule.

The underlying idea was to test this hypothesis by using radiation. We all know that ultraviolet (UV) light will kill cells. You heard a paper yesterday on that score and, of course, it has been known for a long time indeed that UV light will kill off microbes very, very quickly. I have stated that, by and large, we are happy with the impinger sampler and are not worried about what may happen in the impinger -- because we know any effect is comparatively insignificant. We are concerned, then, with what happens to viability in the aerosol. Of course, there is always a little man in back who will say: "Ah, yes -- but I looked at the survivors under the microscope and they changed shape, grew longer or fatter." Well, as I look around the audience I see an awful lot of different shapes, but put you in the middle of the desert and you will behave the same way. I don't think such comments have any real bearing on the present argument. It is true that some cells, having gone through an aerosol procedure, will perhaps divide once, maybe not at all, may get long, but will not form colonies. Obviously something is wrong with them, otherwise they would carry on normally. So we have preferred to use viability, which

means that any cell incapable of dividing, or of forming a colony, or of any kind of reproduction, is dead. We feel, you see, that anything that is incapable of reproducing might just as well be dead.

In the first series of experiments, we utilized UV light to determine what effect this would have on the survival of cells in an aerosol with respect to RH. We were looking to see whether RH could modify the effects of UV. Now in our particular case we did not use the mercury germicidal line. Anybody dealing with radiation biology knows very well that there is a threshold level beyond which increasing the intensity will not increase the death rate and we did not wish to work with wavelengths where it is difficult not to blast the cells with so much radiation that we would not see an effect of RH. This is like somebody trying to find out why a glass holds together by hitting the thing with a sledge hammer.

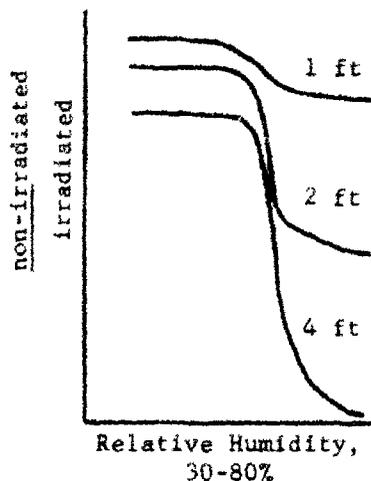


FIG. 1. Effect of white light intensity in terms of distance from source on survival of airborne Serratia marcescens as a function of relative humidity.

Figure 1 shows the ratio between survivors in the dark and the survivors under irradiation from a General Electric ES sunlamp, plotted against the RH at which the aerosols were held during irradiation. In this figure, and those following, I am showing only trends, because it is the similarity of points of change that is important. Definitive

data have been published previously<sup>(1,2)</sup>. The irradiation time in this particular case was 15 min. You can see quite clearly that, with the lamp at a distance of 4 ft from a drum (which has been equipped with plastic to transmit light of about  $2,800 \text{ \AA}$ ), the irradiation had little effect at 80% RH. As the RH is decreased below about 65%, there is a very rapid increase in the sensitivity of cells in aerosols to the radiation. If the intensity of the radiation is increased by just moving the lamp forward (there is really no need to go into units and how many watts/sq cm, at this particular stage of the game anyway), the same increase in death rate at around 60-65% occurs, although the magnitude of the decay rate at 80% increases quite considerably. If you give high intensity irradiation with the lamp very close to the drum, then the effect of the RH is by and large lost, but there is still a slight tendency for the change to occur at 65% RH. Now, the point at this moment was, "is this new information?" -- and the answer is "No". This particular kind of behavior was noted in the early '30s and is shown in the book by Wells on Air Hygiene<sup>(3)</sup>. We have, therefore, only confirmed what was already known and perhaps removed some of the controversy.

The next question was: "Is there any effect of different wavelengths?" We examined this by means of filters tested in the Spectrophotometer. As you can see (Fig. 2), using the same ratios and the same RH, the sudden increase at about 65% RH is apparent, using 28-3,00  $\text{\AA}$  band and with the 34-4,500  $\text{\AA}$  band, although longer exposures were required. We could still detect the effect of RH even up as far as the yellow band and the pattern was the same. At the higher humidities there was no effect of irradiation with the lamp 4 ft from the drum and as the humidity was lowered there was a sudden change in sensitivity around 65%.

The only difference between the high and low death rates is the absence of humidity. Because we used washed organisms aerosolized from distilled water, there is no question of osmotic pressure damage coming into the picture at all. This effect here appears to be a difference in the water content of the cell. We then argued that if this is true, then water molecules in this cell can prevent UV damage under these conditions and a compound such as inositol, if it is behaving as we have said, should protect cells against these wavelengths of light at low RH, because it should replace water molecules.

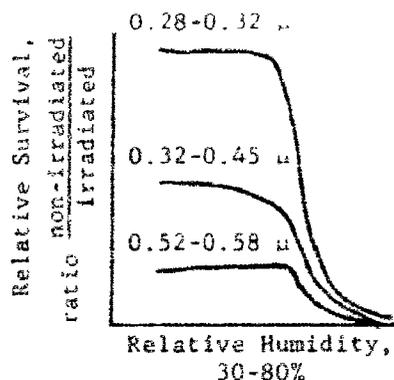


FIG. 2. Effect of wavelength on survival of airborne *Serratia marcescens* as a function of relative humidity.

In the presence of inositol there was no effect whatsoever of the radiations. Now there is absolutely no way you can explain an effect of such magnitude by assuming that the cell wall is being clogged up, or that the substance is not getting into the cell. We already know that it gets into the cell. We accomplished this long ago by using dry weight data, interference microscopy, and so on.

Table 1 is an illustration of the effect of light intensity on the protection afforded by inositol against the light. You can see that as you increase the intensity of radiation into the drum, the effectiveness of inositol disappears. Not shown in the table is the fact that, if you increase the intensity of the radiation, the effectiveness of your RH disappears. So the inositol phenomenon is, by and large, very much the same as water.

TABLE 1. Effect of irradiation, 2,800-3,200 Å, on survival of airborne *Serratia marcescens* with and without inositol

Survival after 30 min aerosol time				
Irradiation distance	9"	18"	24"	No irradiation
<u>Inositol</u>				
+	10 <sup>3</sup>	10 <sup>4</sup>	9 x 10 <sup>5</sup>	1.2 x 10 <sup>6</sup>
-	--	--	2 x 10 <sup>3</sup>	---

In the next figure (Fig. 3) I have summarized all the work we have done over many years and some work done by other people. The abscissa is the relative decay rate of the organisms in the dark or under irradiation, and the trends shown here are not all on the same scale since I only want to show the changes in values. I am quite prepared to accept the fact that in dark aerosols these  $K$  values, over a full 5-hr period, are thermodynamically meaningless, because the decay vs. time is not a straight line -- but it is a very convenient way of expressing the results. Under irradiation the plot is a straight line and the decay rate can be expressed in this way satisfactorily.

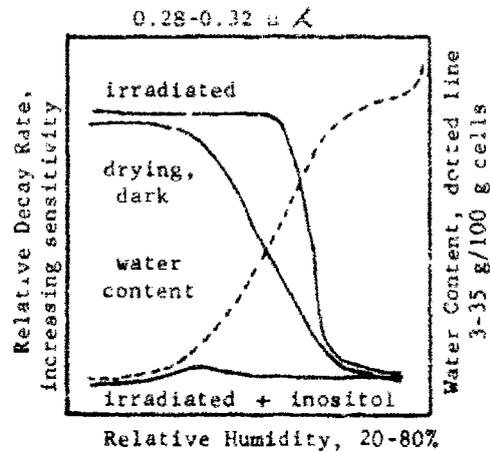


FIG. 3. Combined relationship between water content of air-dried cells of *Serratia marcescens* and sensitivity to drying and UV light as a function of relative humidity.

The plot,  $K$  for radiation, vs. RH, is the one I have just shown you with regard to the UV light and the other is that of cells in dark aerosols when they are aerosolized from distilled water. The latter becomes severely modified by any kind of added solid and you may get the best survival at 60% or at some other humidity. However, this distilled water pattern has been confirmed by a number of other people. As the humidity decreases, there is a sudden increase in decay rate at 65% RH but it does not increase quite so rapidly in the dark aerosol as it does in the irradiated ones. The bottom line represents our experience, and the experience of some other workers, with inositol as protecting agent. There does seem to be a small region around 40% RH

where its protective ability is not quite so good, but, by and large, very good protection is afforded cells at all RH levels and against UV irradiation.

The dotted curve is the quantity of water in the cell at these RH's. Some of these data are the result of my own investigations, and some of the investigation of other workers on the amount of water present in various proteins and nucleoproteins. Those workers who have studied the water content of various RH generally agree that even drying at 80% RH leaves only something in the order of 30 g of water/100 g of cellular solids. This particular amount of water is agreed to be the quantity of water actually bound to the proteins. It is just enough to give a monolayer of water and it is this water only that remains bound to the components of airborne cells. As to RH, you can now see what happens. Life or death appears to rely on the bound water of the cell, so what we are doing in aerosols is not just taking water away from the cell but removing a structural entity from a macromolecule. Apparently these water molecules are vital to the biological integrity of macromolecules.

The other thing I would like to point out is that this water is not available as a solvent for anything. It is water bound to cellular solids and is part and participle of a macromolecule; it does not act as a carrier of ions, or anything else; it behaves much as the water in organic crystals.

Now if we view these plots, we can see that the decrease in bound water at 70% RH coincides with the time at which an increase in death occurs. The curves are almost a mirror image of one another and, in fact, the point at which the water starts to drop very rapidly, at about 70% RH, is the point at which the decay rates, both in the dark and light, increase most rapidly. The other point here is that bound water will prevent damage due to UV light under these conditions and we know that inositol will prevent this damage also; in other words, it must be replacing the water. To test the hypothesis further, we used X-rays because we all know that X-rays are supposed to break water molecules.

Figure 4 shows the result of preliminary experiments on the action of X-radiation from a 250 KV X-ray machine on the death rate of airborne cells. In this figure the decay rate is expressed in

biophysical manner ( $1/D^{37}$ ). As you can see, X-rays produce the opposite effect to that of UV with respect to RH. There is the same sudden change at about 65% RH, but instead of getting protection at the higher RH ranges, very high decay rates are obtained, and as the humidity is lowered decay rates decrease. Many people have stated that dry cells are more resistant to ionizing radiation than are wet cells, but to my knowledge no one have ever correlated this with RH. The interesting point from these preliminary studies is that inositol will protect even against X-rays up to a certain point; and maximum protection by inositol is afforded cells at around 65% RH.

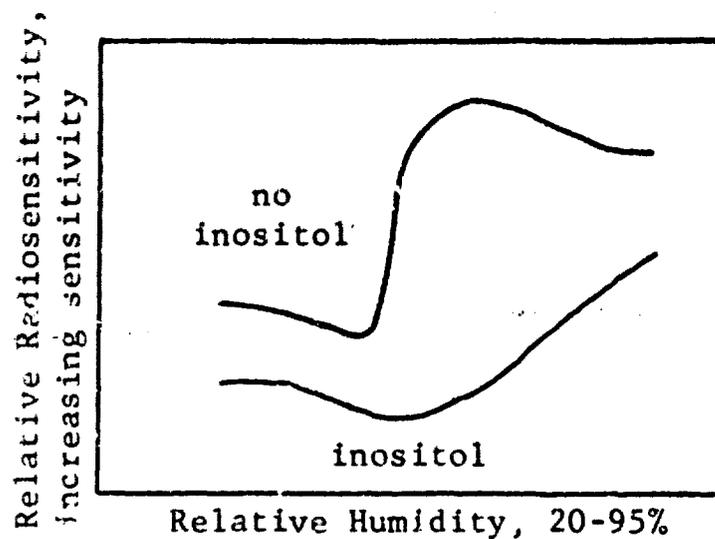


FIG. 4. Effect of X-rays on sensitivity of *Serratia marcescens*, with and without inositol, as a function of relative humidity.

At the moment, the role of DNA in the death of cells is uncertain, but we are fairly confident that inositol by virtue of its protective action with X-ray, UV light and straight desiccation, is able to replace water molecules when they are removed from macromolecules by means of varying the RH. Again there is no way in which you can explain the X-ray picture through osmotic or impinger effects or by assuming that inositol affixes itself to the outside of the cell wall. We all know where the damage is done as far as UV light is concerned, and we also have some rough ideas as to what X-ray does to water molecules.

To summarize, then, we must look at the whole picture of this

aerosolization business. We know lots of things; we know first of all that certain compounds protect through hydroxy or amino groups. If these groups are blocked by methylation, then protection is lost. We know that the action of UV light can be prevented by water, and we also know that under conditions where an RH effect is apparent, we can prevent the action of the UV light by protective compounds such as inositol. We also know that certain cells are very stable to aerosolization and that inositol and odd sugars are natural ingredients of those cells. We know too that during aerosolization one of the main things to happen to a cell is the prevention of its ability to synthesize protein, and hence long lag phases are observed. We also know that the organism loses its ability to synthesize adaptive enzymes, and a cell appears to die because of its inability to synthesize new enzymes. We also know that the cell wall opens up and certain things leak out. We know also that this leakage cannot be correlated with death or with survival. Now that we have all this knowledge behind us, we are pressing ahead and extending our studies to viruses (some of which we have already published) and also to animal cells.

This is the picture we have in mind right now. Within the cell there are macromolecules of this kind (this is a nucleoprotein, but it could be something else); you have UV light of up to  $2,800 \text{ \AA}$  absorbed by the nucleic acid fraction and anything about  $2,800 \text{ \AA}$  will be absorbed by the protein moiety. Energy transfer resulting in damage to a large conjugate molecule is considered to be more easily achieved in the absence of water whereas with X-ray direct action on these water linkages will disrupt the molecule. The concept is fairly simple, and all we are suggesting is that inositol and some of these other compounds, even some of the carbohydrates, can replace structural water and preserve the cell under certain conditions of dryness.

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## DISCUSSION

Dimmick: I am glad to hear what you said about the biological decay being thermodynamically meaningless because that is what I have felt for a long time myself, and I came prepared to argue vociferously here since I care a lot about that point. I think that what you are doing is very important. We need in aerobiology, I believe, more of a fundamental and theoretical approach, one based upon a well-formed hypothesis and well-conducted experiments. I think you have done that, but I still wonder if inositol, or inositol-like compounds, are the whole story here. I am still puzzled about the freeze-dried bacteria surviving so long without water, and I am puzzled about the possible influence of inositol, or other substances, on the whole physiological state of the cell -- whether or not we are getting additional reactions aside from that of a purely molecular action of inositol on a particular site.

Webb: Well, I don't see how we can explain the action of inositol with the three factors -- desiccation, X-rays or UV light -- any other way than to assume a molecular action. If you have an alternative, I should be glad to hear it. As far as freeze-drying is concerned, energy is always needed to distort or change a molecule -- the mere removal of water will not necessarily alter the molecule, especially if the process is carried out at very low temperatures, as every enzymologist knows. We also know that cells do not die in aerosols held at low temperature, even if the RH is also low. We have to consider all these facts in a workable hypothesis as well as our radiation data.

Dimmick: No, I can't argue the reaction due to your irradiation studies.

Webb: Well, that is why we did it. The whole idea of doing these studies was to test whether inositol would in fact do this. It did.

Dimmick: I have no doubt that it does, but I do doubt the extension to the idea that taking the water molecules away from any particular molecule will thus destroy the cell.

Webb: As I have stated, it may not. Temperature has to be considered and quantity of water left. In any case, it doesn't destroy all of them. A certain low percentage of them will survive without water, probably indefinitely.

Dimmick: Why?

Webb: Because when the water is removed from the protein or nucleic acids, or whatever the macromolecule happens to be, its new configuration or bonds is formed by chance, and some allow it to return to

normal on rehydration. This is a chance affair which may vary with different species but there will be a few that will survive, and will survive indefinitely until you start putting water back in. Then you have a different phenomenon altogether, because the first thing that happens on rehydration, particularly if conducted very slowly, is the absorption of the water and tremendous heat production. This heat alone can kill unless you rehydrate with large amounts of water. There is also lots of competition for water, and water itself may act on a molecule and distort it. I know the people at Detrick have studied this phenomenon with dried cells.

Neckly: I think that has a strong bearing on the point of why the bacteria die when you rehydrate, but you also indicated that death was due to water removal. If you rehydrated at room temperature, we found that you have greater kill than if you do the rehydration in the ice-box at 2<sup>0</sup>, for instance. So, perhaps it is simply the heat production at the sites on proteins that kills. Perhaps these two ideas are still compatible then, as I first thought that your whole idea was that it is the taking away of water that produces killing, and we had observed killing when you put the water back in.

Webb: You get killing both ways. If you take cells right down to dryness and follow water loss, then put the water back in slowly, you get a good hysteresis. It seems that the water does not go back in the same place. If you rehydrate with lots of water you cut down two things -- localization of heat and competition between the groups for molecules of the water. I feel the latter is equally as important as far as rehydration kill is concerned and, of course, dehydration and UV death.

VFIF\*: I wonder how you explain the difference that apparently arises in your findings and interpretations with the fact that other people feel that the formation of thymine dimers is the cause of the inactivation as the result of the UV on DNA.

Webb: I am not trying to say anything about thymine dimers. All I am saying is that inositol and water prevent damage. You see, the UV has to be absorbed by the bases to cause death and this kind of absorbed energy has to dissipate somewhere along the line. All I am saying is that the water and inositol obviously act as dampers of energy transfer and allow energy dissipation without molecular damage. In doing so, perhaps they prevent dimer formation.

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\* VFIF = Voice from the floor.

BEHAVIOR OF COLORADO TICK FEVER,  
VESICULAR STOMATITIS, NEUROVACCINIA AND  
ENCEPHALOMYOCARDITIS VIRUSES  
IN THE AIRBORNE STATE

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In recent years, many reports have appeared describing the influence of temperature and relative humidity (RH) on airborne bacteria; however, with the exception of some work prior to 1950 with influenza virus, the only reports on viruses have been those of Hennessy in 1960 on influenza and poliovirus, and of Harper in 1961 on influenza, poliovirus, vaccinia and Venezuelan equine encephalomyelitis viruses. No reports are known to us comparing the aerosol behavior of different viruses that have been produced and assayed in a single host species. This paper primarily presents some results of such a study utilizing four viruses, namely, vesicular stomatitis, neurovaccinia, Columbia-SK and Colorado tick fever viruses, each aerosolized in the NBL rotating drums.

In 1958 Goldberg introduced the use of large, slowly rotating stainless steel drums of the type depicted in Fig. 1 as instruments suitable for containing aerosols of pathogenic organisms during prolonged studies on the effects of temperature and RH on the survival of airborne microorganisms.

Physical loss in such drums is less than 50% in 24 hr. At NBL such loss is monitored photometrically by means of a device which determines the light scatter of airborne particulates. Biological loss is based upon growth or infectivity titrations of organisms in air samples collected at suitable intervals. In the studies to be described,

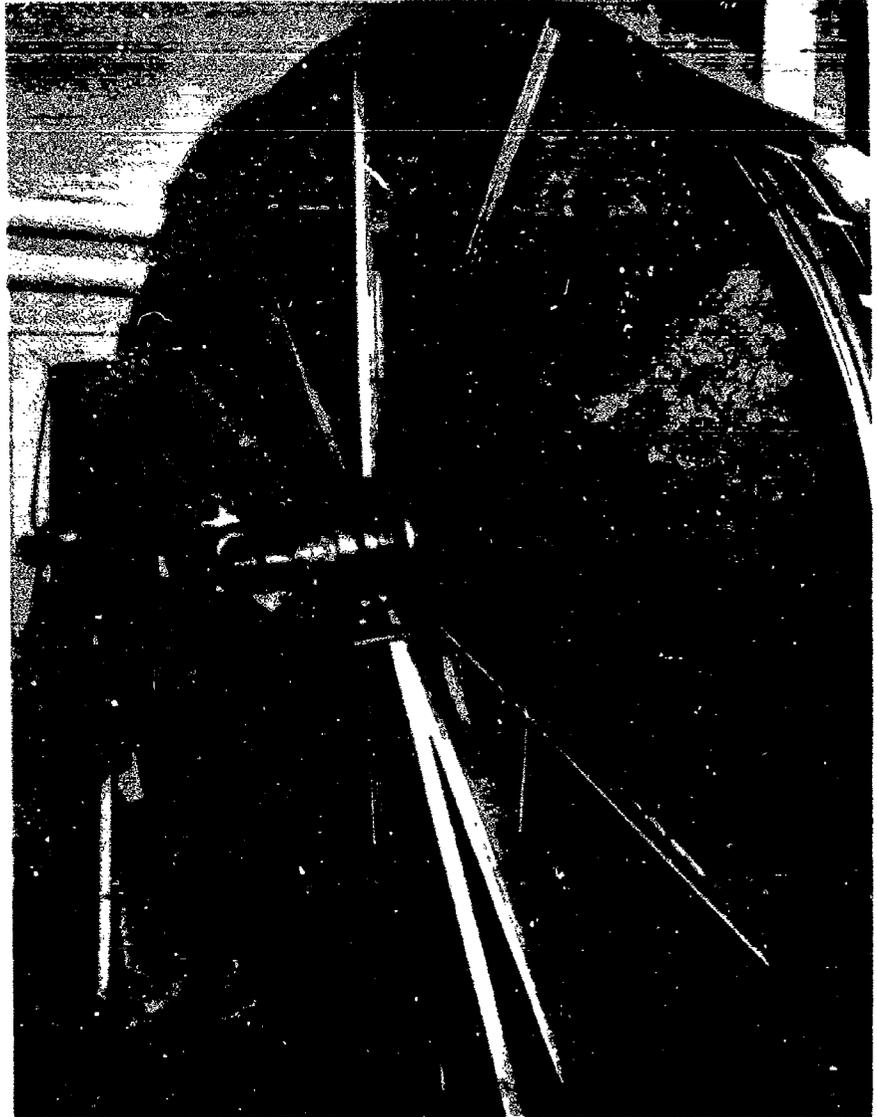


FIG. 1. NBL Rotating Drum

each virus was harvested from the infected brains of moribund suckling mice. The brain tissue was usually homogenized in 0.5% lactalbumin enzymatic hydrolysate in Earle's balanced salt solution, residual tissue fragments being removed by centrifugation. In some instances, 0.1% bovine albumin fraction V was added to the above fluid. Colorado tick fever virus was usually harvested in 100% rabbit serum, although a brief series of comparative aerosol trials at 70 F indicated no preference for this suspending fluid over the fortified lactalbumin hydrolysate.

Virus was aerosolized into the rotating drums at controlled air temperature with RH established at nominally 10% intervals over a range, for the most part, extending between 20-80% RH. Virus was entrained from a Wells-type refluxing atomizer in the air stream, entering the drums during a 10-min period. This was followed by a 5-min equilibration period prior to collection of a 5-min aerosol sample in an all-glass impinger containing lactalbumin hydrolysate and antifoam. Samples were collected from the air stream and, at suitable intervals up to 6 hr of aerosol age, from the drums. At the lower temperatures tested, samples were also collected at 28, 48 and 72 hr of aerosol age. Virus survival was assayed on the basis of infectivity of the samples titered by intraperitoneal inoculation in suckling mice; in many experiments with vesicular stomatitis and neurovaccinia viruses, plaque assay in chick fibroblast monolayers was also used.

Data typical of our observations are plotted in Fig. 2, which illustrates the decay of airborne vesicular stomatitis virus at 90 F and a nominal 35, 40, 45 and 50% RH. Typically, such data indicates a rapid early decline in surviving virus, followed by a second, substantially more resistant stage. Such two-stage decay curves are essentially similar to those that have been reported for many bacteria.

Rotating drums, as employed in this study, are not particularly useful for detailed examination of the first rapid stage of viral decline; accordingly, further comment in this paper should be understood to apply to the second-stage portions of aerosol decay observed during the period from 15 min after the start of aerosolization to 6 hr of aerosol age thereafter.

One may inquire what reproducibility of observations could be attained in aerosol studies as we have conducted them. Two examples

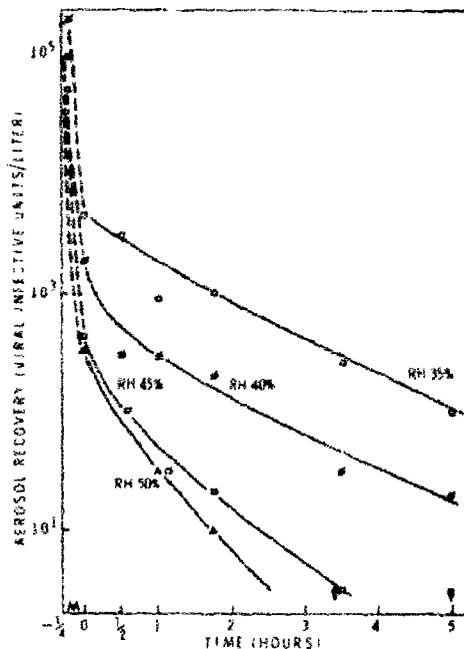


FIG. 2. Effect of relative humidity (RH) on vesicular stomatitis virus aerosol decay at 90 F

are shown in Fig. 3. The two curves illustrate the marked variation occasionally encountered between two batches of mouse passage virus seemingly prepared in the same manner and possessing similar initial titers, but showing substantially different aerosol stability. This variation may be contrasted with the results obtained from aerosolization of either one of these batches in one drum in comparable trials on several successive days. Under such conditions, using a single batch, comparable observations fluctuated over a range of titers somewhat less than 1 log. Assays in tissue culture, as might be expected, gave better replication than did assays in mice; otherwise, results obtained by either method were comparable. Reproduction of operating conditions and of observations became more difficult in aerosols maintained for several days, the apparatus being incapable of maintaining predicted temperature more precisely than approximately  $\pm 2$  F over such a time period. Temperature and RH variation in trials on successive days are also illustrated by examples in Fig. 3, wherein the temperature range for four successive runs was 90-92 F and the RH range 24-40%.

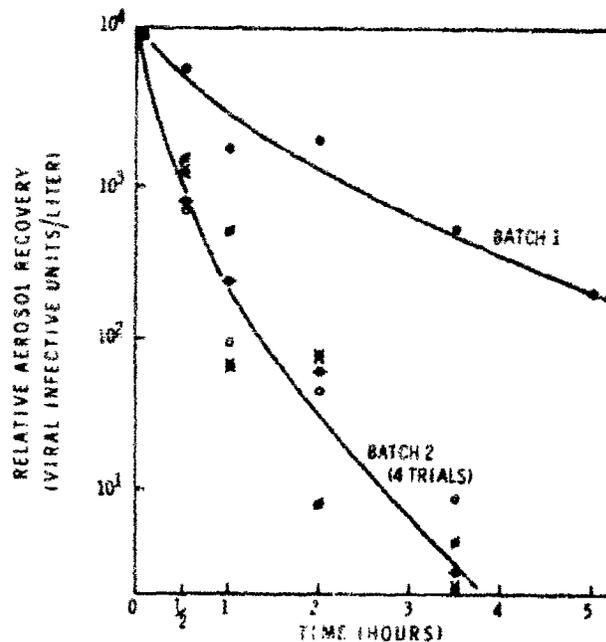


FIG. 3. Variability in similar vesicular stomatitis virus aerosols.

Accordingly, although reproduction of operation conditions and of observations was difficult to obtain and maintain, with the result that data cited in this paper cannot be considered strictly quantitative with respect to the designated temperatures and RH, replication of observations within the ranges indicated above is considered quite adequate to establish the trends and contrasts with which the remainder of this paper is concerned.

Within such limitations, the response-patterns of vesicular stomatitis virus aerosols have been examined at four temperatures over a wide range of RH. The results are illustrated in Fig. 4, where the relative viral loss after 5 hr aerosol age is plotted for 50, 70, 80 and 90 F at RH ranging between 20-80%. It is apparent that the response to changes in RH is complex, with maximal stability occurring in the regions of 20 and 80%, whereas minimal stability was observed near 50%. With successive increases in temperature, the rate of aerosol decay was enhanced, but the general pattern of response to RH remained essentially the same. Again, the loss of viral activity was most marked near 50% RH; in this region at temperatures of 80 and 90 F, no virus was recovered after 4 and 3 hr aerosol age, respectively.

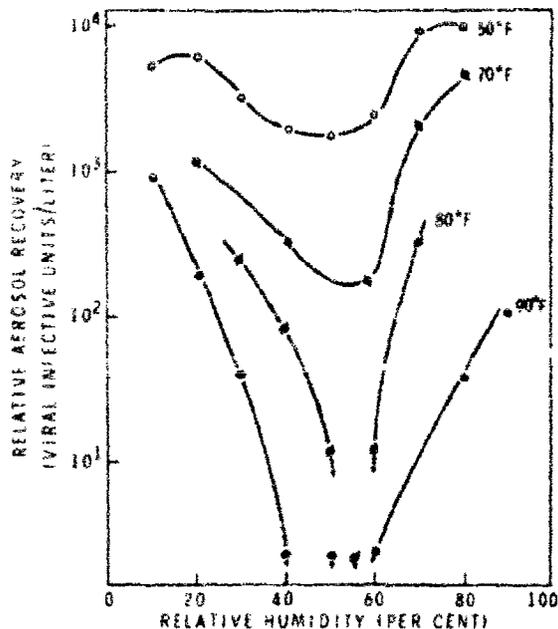


FIG. 4. Effect of temperature and relative humidity on vesicular stomatitis virus aerosols aged 5-6 hr

Similar, but more limited, studies have been made of three other viruses selected for their capacity to produce high titer virus in infected suckling mouse brain following intraperitoneal inoculation. These were single strains of neurovaccinia and of Columbia-SK viruses, and several strains of Colorado tick fever virus. With minor exceptions, all were produced, suspended, aerosolized and assayed from aerosol samples in a similar manner. Yet, as shown in Fig. 5, each of these viruses differed markedly in its aerosol stability at RH ranging between 20 and 80%. The response pattern of the neurovaccinia strain was similar to that of the vesicular stomatitis virus; however, the neurovaccinia virus was substantially more temperature-resistant in that its aerosol stability at 80 F was comparable to that of vesicular stomatitis virus at 70 F. Both of these viruses showed midrange RH sensitivity, but high stability at both extremes of low and high RH. This may be contrasted with the response-pattern of the other two viruses, namely, Columbia-SK and Colorado tick fever, both of which showed good stability at high RH, but high sensitivity at low RH. Further, these

two viruses were different, both with respect to RH region of maximal decay rate and the degree of aerosol sensitivity at low RH. Accordingly, the response-pattern of each virus characteristically differed from that of the other three.

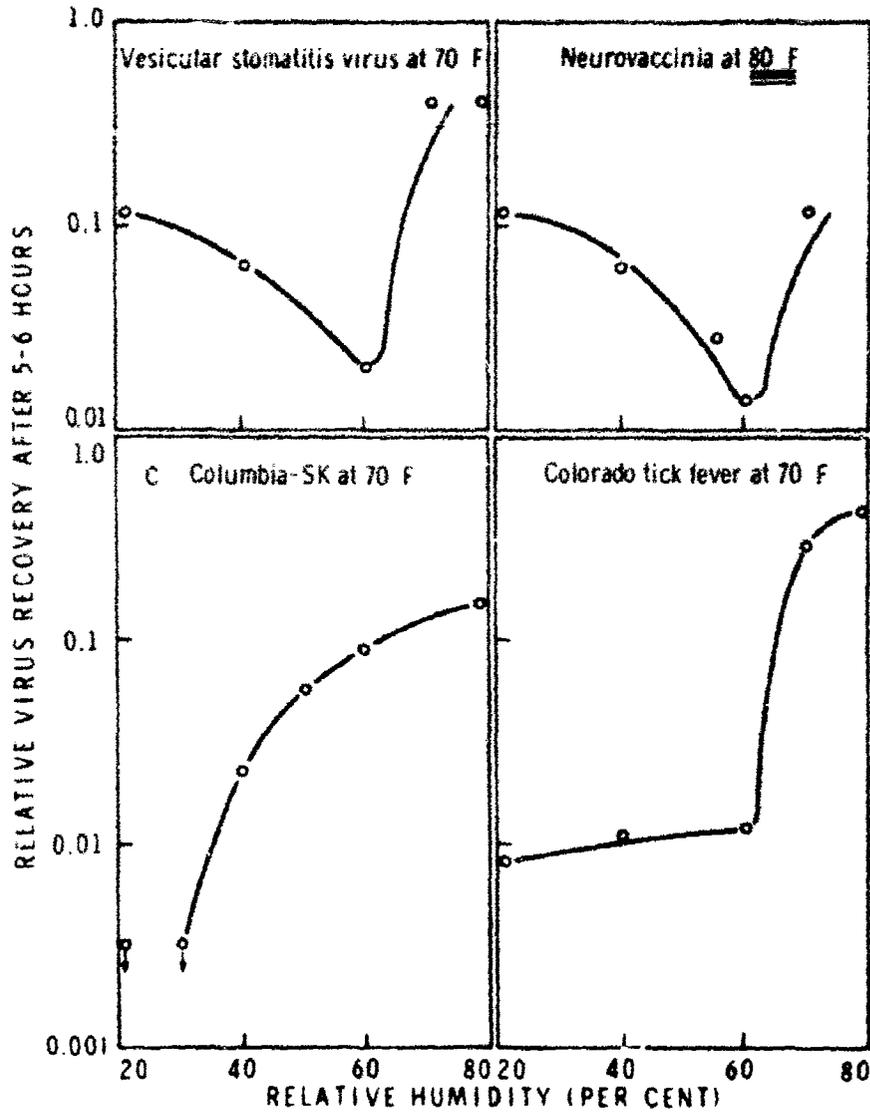


FIG. 5. Effect of relative humidity on four viruses in air.

In the introduction to this paper, we implied that the variation in the response-pattern of various viruses might in some degree be attributable to their production in different hosts. We have recently attempted to demonstrate this by producing vesicular stomatitis virus both by mouse passage and by passage in chick fibroblasts. These studies are still incomplete, but in general they indicate that a single virus produced in two different host systems may indeed vary markedly in aerosol characteristics. Secondly, different viruses produced in a single host system may manifest an aerosol-response pattern that, to a large extent independent of treatment, is characteristic of the particular virus employed.

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#### DISCUSSION

L. Miller: You had two kinds of curves -- did you have a DNA and RNA in each set of curves?

Watkins: Not each set. We have two other viruses in there -- I don't know which they are.

L. Miller: You have no idea why they should be so different -- no chemical or physical explanation?

Watkins: At this stage, no. We have worked some with vesicular stomatitis produced in chick fibroblast and find that it is markedly more resistant than when it is produced in mice, and on a very preliminary glance at it over a range of RH, it follows the same pattern but at a more resistant level. We haven't gone beyond that.

THE INFLUENCE OF STORAGE, AEROSOLIZATION  
AND REHYDRATION ON THE PERMEABILITY  
OF PASTEURELLA TULARENSIS TO PHOSPHATE IONS

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During the development of a solution for collecting aerosolized Pasteurella tularensis, Cabelli (1962) observed that the viable recoveries of this organism were greatly enhanced by the presence of phosphate ion in the collecting fluid. He also observed that a number of carbohydrates, particularly melezitose, trehalose and sucrose, and the polyhydric alcohol, inositol, improved recoveries. Cabelli suggested that the effectiveness of these compounds was related to their ability to obviate the effects of changes in cell permeability due to the stress of collection.

In the work reported here, the behavior of the phosphate ion was examined in greater detail in order to elucidate processes which occur within the bacterial cell and at the cell membrane during collection. Since properties of aged cells are different from those of fresh cells, some of the changes occurring during aging were also to be examined in both unaerosolized and aerosolized cells.

Phosphates and phosphorus compounds are important in a number of synthetic cellular processes and in intermediary metabolism. Phosphates also constitute one of the buffer systems of the cell. It is logical to expect that a disturbance in phosphate transport could affect culturability of the cell. If reasons for the phosphate requirement were elucidated, a sound basis would be provided for prevention of the deleterious effects of collection and holding.

A number of workers have observed that phosphate metabolism is altered during aging and stress. During the aging process phosphatase activity increases in animal cells and there is uncoupling of oxidation from phosphorylation (Bourne, 1962). Stresses, such as carbon dioxide intoxication, alter body fluid phosphate levels in both invertebrates and vertebrates (Brown and Prasad, 1957; Hayes, 1961). Cellular phosphate metabolism in P. tularensis may be affected in one or more of the following ways:

1. The ability of the cell to take up phosphate ions may be decreased due to decreases in activity of enzymes which are related to the transport of phosphate or due to injury to a specific phosphate transport site in the plasma membrane.

2. Injury to the suspending fluid interface may result in an increase in the size of "pores" in this interface. Ions and small organic metabolites could then diffuse in or out depending on the direction in which the concentration gradient existed. In an unfavorable collecting fluid such as gelatin-saline (0.1% gelatin, 0.85% NaCl), a cell could lose many low molecular weight cell constituents.

3. Injury may increase the intracellular space available to phosphate ions.

4. Stresses may activate esterases which hydrolyze organic phosphates, thus increasing the intracellular inorganic phosphate which can then diffuse outward or exchange with extracellular phosphate ions.

In the experiments described here, the organism studied was the Schu 5 strain of P. tularensis. To study ion movement, cell suspensions were washed in a sucrose (5%)-inositol (2.7%) solution, and then resuspended in the test solution. Aerosols were produced using a University of Chicago Technical Laboratory Atomizer in the Reynier chamber. Clouds were dynamic and collections were made for 10 min, using the all-glass impinger with a fill of 18.5 ml and an air flow rate of 6 liters/min. Trichloroacetic acid-soluble phosphorus was determined by the method of Fiske and Subbarow (1925) and total phosphorus by the same method on sulfuric acid digests of collecting fluids and washed cells.

Preliminary experiments were performed to determine a suitable wash fluid for the bacterial cells. The composition of the wash

solution was based on an observation made by Cabelli (1962) which indicated that melezitose-inositol solutions were extremely effective in enhancing culturable cell recoveries and that sucrose was nearly as effective as melezitose. Melezitose is expensive and difficult to obtain in the kilogram quantities required for preparation of collecting solutions. Sucrose was therefore utilized as a substitute. Washed cells were suspended in solutions containing 2.7% inositol and varying concentrations of sucrose. The cells were held at 4 C, room temperature and 37 C for 24 hr. The solution containing 5% sucrose permitted recovery of 95% of the original cell population. In 22% sucrose, only 56% of the original population was culturable. Intracellular inorganic phosphorus in the cells held at the high sucrose concentration was 88% of that observed in cells held in the 5% solution.

At a holding temperature of 37 C, intracellular inorganic phosphorus of cells held in concentrated sucrose solution was only 38% of that observed in cells held in the dilute solution. No sucrose-inositol solution was favorable for holding 24 hr at 37 C. However, in the solution of lower sucrose concentration, the recovery of 0.4% of the original population was greater than twice the recovery in the solution containing 22% sucrose. In solutions containing sucrose in concentrations intermediate between 5 and 22%, values for cellular inorganic phosphate lying between these extremes were obtained. At room temperature, values for the recoverable proportion of the original population and the phosphate content of the cells lay between those obtained at higher and lower temperatures.

In the cell preparations held at room temperature, it was apparent that more inorganic phosphate was lost than could be accounted for by inorganic phosphate found in the cell held at 4 C. This suggested that, as the temperature increased, inorganic phosphate was produced by enzymatic activity or by decomposition of organic phosphate. In Table 1, data obtained with the 5% sucrose-2.7% inositol solution are presented. In one experiment, total cellular phosphate was also determined. The last two columns in the table indicate that almost all of the phosphate lost is inorganic phosphate, since the levels of the inorganic and total phosphate are nearly the same, within the limits of experimental error.

TABLE 1. Effect of temperature on cellular phosphate in Pasteurella tularensis (Strain 5)<sup>a</sup>

Temp C	Trial	ug Inorganic P per cc Cell Suspension	ug Total P per cc Cell Suspension	ug Inorganic P per cc Supernant	ug Total P per cc Supernant
4	I	57	-	7	-
	II	60	415	11.5	12.3
27	I	51	-	17	-
	II	55	387	17	17.1
37	I	37	-	31	-
	II	25	205	31	32

\* Washed cells suspended for 24 hr in 5% sucrose-2.77 inositol solution. Initial concentration of cells per cc suspension: Trial I  $10 \times 10^{10}$ ; Trial II  $8.1 \times 10^{10}$ .

Figure 1 indicates the method used to prepare cells for phosphate analysis. The 10-min collection period results in approximately the same number of cells initially in all three collecting fluids, as indicated by plate counts. Table 2 indicates the composition of SYN fluid. Of particular interest is the presence of phosphate, inositol, and sucrose. After holding 18-24 hr, recoveries of culturable cells in SYN are demonstrably superior to those in gelatin-saline. Table 3 contains the results of studies conducted at 50% relative humidity (RH). These data indicate that there is a decrease in the inorganic phosphate in gelatin-saline after 18 hr of holding. Differences at 1 hr among cellular inorganic phosphate concentrations in these collecting fluids probably have less meaning than those differences observed at 18 hr. Differences after holding are observed between SYN and the other fluids at 34 days and 48 days. Differences between sucrose-inositol and gel saline are significant at 33 days, but not at 47 days. This suggests that sucrose-inositol is no longer able to protect aged cells against the effects of aerosolization at 50% RH. In cells collected at 80% RH, higher concentrations of cellular inorganic phosphate are maintained in SYN after 18 hr of holding. At the higher RH, differences between sucrose-inositol and gelatin-saline after 18 hr of holding are significant at 20 days, but not at 47 days.

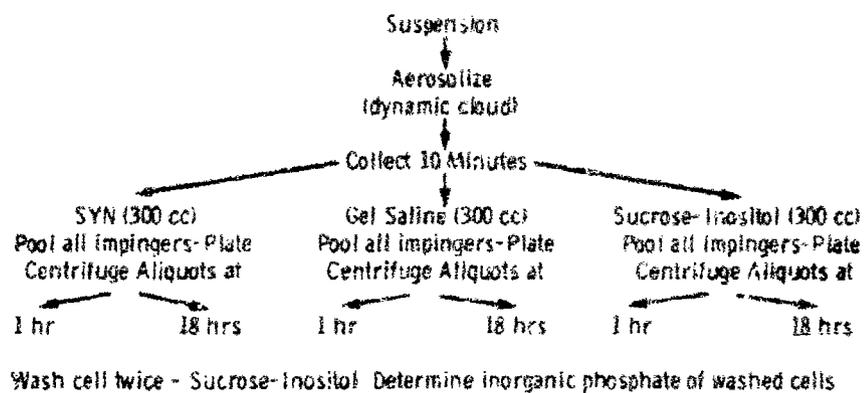


FIG. 1. Preparation of cells for inorganic phosphate analysis.

TABLE 2. Composition of SYN Fluid.

	<u>Per cent</u>
Sucrose	2.6
i-Inositol	2.7
Thiourea	0.1
l-Cysteine.HCl	0.1
l-Histidine.HCl	0.05
Spermidine.PO <sub>4</sub>	0.002
K <sub>2</sub> HPO <sub>4</sub>	0.38
KH <sub>2</sub> PO <sub>4</sub>	0.11
pH adjusted to 7.0	

TABLE 3. Inorganic phosphate content of aerosolized\*  
*Pasteurella tularensis* (Schu 5)

Cell Age Days	Holding Time Hours	ug Inorganic Phosphorus Content (± standard error) of Cells $\diamond$ Collected in		
		SYN <sup>‡</sup>	Gel Saline	Sucrose-inositol
19	1	3.70 ± 0.43	1.87 ± 0.28	3.77 ± 0.04
	18	2.55 ± 0.25	1.04 ± 0.0	-
32	1	3.10 ± 0.69	2.53 ± 0.48	2.23 ± 0.17
	18	3.25 ± 0.63	0.71 ± 0.17	1.77 ± 0.07
47	1	1.84 ± 0.64	1.63 ± 0.18	1.53 ± 0.20
	18	3.04 ± 0.39	1.79 ± 0.09	1.93 ± 0.27

\* Relative humidity, 50%; Temperature, 50 F; Dynamic Cloud, Reynier Chamber, 18.5 ml impinger fill, all-glass impinger, 6 l/min flow rate, 10 min collection.

$\diamond$  Cells harvested from 35 cc impinger fluid.

<sup>‡</sup> Culturable cells collected per cc SYN - 19 days -  $4.78 \times 10^9$   
 32 days -  $3.10 \times 10^9$   
 47 days -  $1.31 \times 10^9$

These observations are consistent with the hypothesis that organic phosphate is lost from the cell as it is held in a phosphate-free fluid for an extended period of time. Up to a culture age of 34 days, a sucrose-inositol solution is more effective than a gel-saline solution in preventing outward movement of phosphate from the cell.

Inorganic phosphorus levels, although significantly different in the cells collected in these three fluids, did not drop to undetectable levels. This suggested that there was a source of phosphate ions within the cell. Figure 2 shows a schematic diagram of the theoretical phosphate equilibria existing in the cell in the steady state. Under stress, and certainly as the temperature is changed, the position of the equilibrium could be shifted. This could be a mechanism for maintaining the cellular inorganic phosphate within defined limits. The process of transport at the cell-external solution interface could be the critical one in maintaining the internal intracellular phosphate within limits consistent with culturability.

Table 4 contains a comparison between inorganic and total

phosphate content of aerosolized cells before and after holding. In gel-saline, an undesirable collecting fluid, inorganic phosphate drops approximately 30%, while in three trials, total phosphate drops 60%. This indicates that under stress a breakdown of total phosphate occurs and that inorganic phosphate is maintained at the lowest limit the cell membrane can retain.

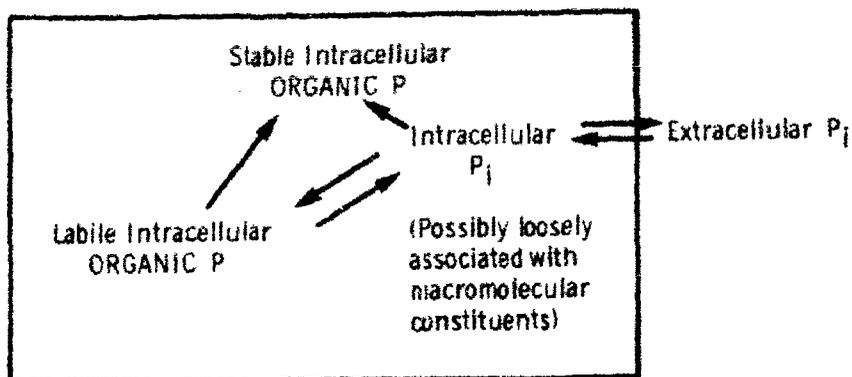


FIG. 2. Theoretical steady state, phosphate equilibria in a cell

TABLE 4. Cellular inorganic and total phosphate in aerosolized *Pasteurella tularensis* (Schu 5)

Collecting Fluid	% change after holding 18 hr			
	Inorganic phosphate	Total phosphate		
		Trial II	Trial I	Trial II
SYN	-16.7	-10.1	+ 4.3	+23
Gel-Saline	-29	-63	-64	-55
Sucrose	-15	-30	-	+ 2.5

Trials I and II - 50% RH, 50 C. Chicago atomizer, Reynier Chamber, Dynamic cloud, 10 min collections, all-glass impinger, 6 l/min flow rate 18.5 ml fill.

Trial III - 80% RH, other conditions similar.

In summary, there appears to be a factor which is age dependent in the ability of the aerosolized cell to maintain phosphate against a concentration gradient. Organic phosphates appear to be the source of a portion of the inorganic phosphate lost by the cell.

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#### DISCUSSION

Middlebrook: You didn't mention anything about the study of poly-phosphates and I wondered if you had done any studies of poly-phosphate in morphological terms of granules or measurement of poly-phosphate in the cell at different ages, aerosolized and so forth.

Hayes: I think this would be a very worthwhile thing to do but I haven't had time to do it.

LeJeune: We have had problems with aspiration of BC 1; I think Dr. Cabelli has also experienced trouble with collecting fluid in which you could aspirate BC. We found that by tripling the concentration of phosphates we eliminated this aspiration loss -- this may have some bearing on the same problem. Why it should be manifested in P. tularensis in an aerosol and yet not show up in a spore until you get it back into the collection fluid and start aspiration -- whether the same process or same mechanism is involved -- I don't know. But certainly this could help to explain why the mere addition of more phosphate to the collecting fluid solved our aspiration loss.

VFTF\*: In this respect you might like to know that Dr. Frank Harold has experimented with different types of phosphate and poly-phosphate metabolism and his findings might be extremely useful in this sort of study.

VFTF: We, too, have found that the viability of the cell is highly dependent on collection method and on the phosphate concentration, similar to the results presented here.

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\* VFTF = Voice from the floor.

SUMMARY OF DISCUSSION OF PROBLEMS  
RELATING TO THE SAMPLING  
OF MICROBIAL AEROSOLS\*

T. W. Kethley, Moderator

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A committee composed of Messrs. Dimmick, Ehrlich, Kethley, Madin, Silver, and Wolfe chose the topic "Sampling Microbial Aerosols" for Saturday morning's discussion. For the purpose of conciseness, only the most pertinent ideas have been included herein. Names of individual speakers have, for the most part, been omitted. References to practical problems and to theoretical questions not directly associated with sampling were retained since they point to needed areas of research.

Professor Kethley moderated the discussion, pointing out that sampling is still an art. The sampling method employed must be selected according to the specific answers desired since no single sampler, or sampling technique, can possibly satisfy all requirements.

It was suggested that if each investigator reported the results of at least a few samples from a reference sampler, we might be better able to interpret the data presented in our own frame of reference. It was pointed out that whenever a mechanical sampler was considered for use by everyone, all of the sampler's peculiarities and all specifics regarding sampling procedures must be spelled out; otherwise incorrect sampling techniques could yield erroneous results.

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\* Mr. William Miller, Fort Detrick, Frederick, Maryland, provided editorial assistance (Editor).

There was a suggestion that a "predictable" percentage of organisms would be killed by a mechanical sampler. Many consider this unacceptable; no sampler should affect viability of the organism. Others argued that as long as the percentage is known and consistent, there is no problem. It seemed reasonable to suppose that a standard sampling technique implied establishing standards for particular groups of problems, but not for the entire sampling problem existing throughout aerobiology. A few argued that an important part of the problem concerns those factors influencing the survival capacity of organisms. The influence of "rehumidification", as reported at this conference, was listed as an example. Although death mechanisms are important, we simply do not have enough information at this time to make meaningful statements.

We need to be able to predict what will happen to animals exposed to the number of viable cells determined by our sampling methods. There is a decided interest as to the relative importance of the various acts leading to infection; i.e. the conditions prevailing when the organism is airborne, when the organism is traveling toward the host, and when the organism alights in the alveoli, as well as the degree of infectivity at any given point as influenced by temperature of the organism, relative humidity, etc. Of course, there is a vast difference and no necessary connection between the animal as a sampler and the animal as a host responding to infection.

Mr. Wolfe pointed out that we should look at sampling as a reference point and not try to simulate what will happen if an animal inhaled a given number of organisms; this can be determined by simultaneous experiments with animals. Is it not possible, someone asked, to have an infective organism that does not form colonies in laboratory situations? The question was answered affirmatively, and it was further pointed out that there is indeed some danger in confining one's experiments to non-living samplers. If behavior of the organism is well understood, good correlation between the mechanical and the host sampler ought to be obtained. However, if one had a mechanical sampler simulating the physical capability of the lung to collect particles, it would still not simulate the capability of the lung to collect viable particles, or the capability of the lung to eliminate pathogens.

Other related problems were whether or not viruses can be collected satisfactorily in samplers currently in use. In addition, both in the laboratory and in the field, the concentration of viable cells may vary from more than  $10^6$ /liter to as low as 1/liter. Is a single sampler adequate?

Professor Kathley then outlined the problem as developed to this point; namely that we need three types of samplers: (a) one which simulates the respiratory system closely -- for specialized work; (b) one for field work when organism concentrations are usually low and long sampling times are undesirable -- a sampler that would enable sampling of large quantities of air in a short time; and (c) a highly flexible, complicated if need be, sampler for laboratory work -- one that would enable the worker to control parameters like aspiration rate, temperature, sampling rate, fluid quantity, and any other desirable parameter. Obviously, all of these problems could not be settled without further knowledge, and the question was discussed whether it would be desirable to have a standard reference sampler. Several samplers were suggested as possible reference samplers, but it was evident that only the impinger and the Andersen (stacked sieve)\* sampler might be applicable for this purpose. It was emphasized, for example, that the impinger is (a) simple, (b) rugged, (c) inexpensive, (d) easily obtained, (e) easy to learn to use, and (f) the parameters could be specified in considerable detail. During this discussion it was agreed that the Andersen sampler should also be a standard reference sampler when concentrations of organisms were too low for the impinger. After some discussion of the most desirable flow rate for the impinger, Mr. Goldberg pointed out that the impinger known as the AGI-30 was available from commercial sources<sup>†</sup>, a point that had bothered many persons, that its flow rate was 12.5 liters/min which was a compromise between suggested rates, and that this, at least for the present moment, was the most suitable instrument. General agreement was then reached that the AGI-30 should be the standard reference sampler, providing that the particular techniques and materials used should be clearly spelled out by each investigator when his

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\* Andersen Samplers and Consulting Service, 1074 Ash Ave., Provo, Utah.

† Ace Glass Inc., P.O. Box 192, Vineland, New Jersey, and  
Chas Glass Co., 570 Broadlawn Terrace, Vineland, New Jersey.

work was reported<sup>(1)</sup>.

Discussion of the Andersen sampler then continued. It was recommended for use in sampling air of hospitals and there was an indication that it was well suited to the method of transfer to a tissue system, hence might be suitable for collection of virus. Again it was pointed out that if the Andersen sampler was to be a reference sampler, the techniques and materials, that is the kind of media, the level to which the plates were poured, the particular flow rate, etc., should be specified. The modified Andersen sampler was discussed, but it was pointed out that this particular model will probably not be available in a manufactured form for some time.

There followed a vigorous discussion of many of the parameters of the Andersen sampler. Its primary usefulness is that it is a high volume sampler and it does perform some measure of particle sizing. Considerable interest was generated in the relationship of particle sizes found in the natural environment to those which might enter the alveoli, especially in regard to a suggestion that an additional stage having smaller holes might be added to the Andersen sampler, but there was a question as to whether natural forces tend to create particles, even with viruses, smaller than ones capable of being collected in usual samplers. It was evident that there is a considerable interest, coupled with a lack of information, regarding the host-parasite relationship. Both are biological systems, and both are changing, and the problem of sampling is closely associated with the problem of "infectivity" and "viability". Having admitted that the problem is serious, and having admitted that there are no adequate answers at the present, the audience generally agreed that the Andersen sampler was the best field sampler, as a reference standard, that we currently have available, and that its use should be recommended<sup>(1)</sup>.

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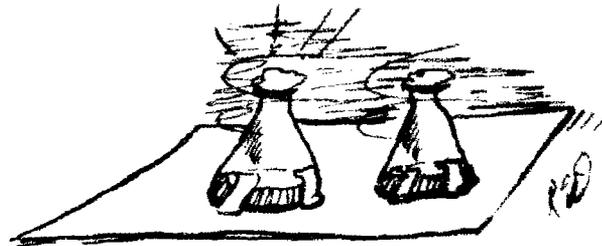
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"Last one to reach  $10^8$  is  
a dirty ol' clostridium!"