were the most satisfactory. Use of $CaCO_3$ as a buffer resulted in decreased glycol production, whereas addition of NaOH to maintain an optimal pH of 6.0 to 6.2 exhibited no adverse effects. A possible explanation was suggested for dissimilation of 2,3-butylene glycol upon attaining low sugar levels in the fermentation beer.

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Physical Tracers for Bacterial Aerosols

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Investigators in the field of aerobiology who are concerned with determining bacterial survival under various environmental stresses, find it necessary to distinguish between the loss of viable bacteria from aerosols due to biological decay (death) as opposed to physical decay due to such factors as unrestricted diffusion, impaction on available surfaces, and fall-out. The distinction is important, for example, in assessing the effects of air sterilization mechanisms or in simple evaluations of microbial response to controlled conditions of temperature and relative humidity.

The usual viable cell assay of samples taken at intervals during the life of an aerosol are used to estimate a decay rate which represents the total of the physical and biological components. To derive the biological decay rate from this parameter it is necessary to determine the physical component. This may be accomplished by total cell assessment from the aerosol samples or by some indirect measure provided that the latter is associated with the same particle size distribution as the test organisms.

Attempts to determine the total cell numbers in an aerosol sample directly must rely upon laborious microscopic examination which is hindered by the presence of debris and which is still more difficult when the organism under test is of small size.

The use of cells tagged intracellularly with P^{32} has been described by Harper, Hood, and Morton, 1958. This technique offers the most realistic approach to the estimate of physical decay of the microorganisms but has the disadvantage that the preparation of intracellularly tagged cells is tedious and time consuming.

Two additional methods for determining the physical decay rate of aerosols will be reported and their advantages discussed in this paper. The methods include the use of bacterial spores and radioactive S^{35}. In both cases the tracer material is added directly to the bacterial suspension.

MATERIALS AND METHODS

Cultures. Bacillus subtilis var. *niger* cells were grown in casein acid digest medium and then autolyzed to eliminate vegetative forms. The suspensions were centrifuged to a heavy mud and stored at −40 C until time of use. After resuspension in 0.2 per cent gelatin and 0.4 per cent anhydrous sodium phosphate solution the spores were heat shocked (65 C for 30 min). *Serratia marcescens* cells were grown in a tryptose, phosphate, and cerelose medium; centrifuged, then resuspended 1:1 in supernatant, and pellet frozen. The cells were then stored at −40 C until use when they were resuspended in gelatin and phosphate solution. *Pasteurella tularensis* cells were grown in a casein acid digest medium and stored at 4 C until used.

Aerosolization. Pure cultures of bacteria or mixtures of cultures and tracer were disseminated into aerosol chambers ranging in volume from 5×10^4 to 2×10^5 L with structural and operational characteristics similar to those of the chamber described by Ray (1959). Spray nozzles were employed to produce aerosols in these units.

Sampling. Aerosol samples were collected by means of all glass impingers (AGI), described previously by Tyler and Shipe (1959). To these were attached pre-impingers which in general collect particles greater than 5 μ and allow particles less than 5 μ to pass into the AGI (May and Druett, 1953). Collecting fluids were gelatin and phosphate solution except in *P. tularensis* trials when gelatin and saline solution was employed.

Assay. After appropriate dilution with distilled water, mixed samples of *B. subtilis* and *S. marcescens* were streaked on a medium containing 2 per cent Bacto-tryptose,[1] 1 per cent glucose, 0.5 per cent NaCl, and 2 per cent Bacto-agar[1] with 0.005 per cent potassium tellurite added for the inhibition of *S. marcescens.* The samples were also streaked on a medium containing 2 per cent Wilson's peptone, 0.5 per cent glucose, 0.5 per cent NaCl, and 3 per cent Bacto-agar with brilliant green at a concentration of 1:2 million, added for the inhibition of *B. subtilis.*

Mixed samples of *P. tularensis* and *B. subtilis* were diluted in gelatin and saline solution and streaked on two media: tryptose agar for the assessment of *B. subtilis,* and glucose cysteine agar (BBL)[2] medium with 2 per cent packed blood cells and 10 to 20 units per ml of penicillin for the assessment of *P. tularensis.*

Samples containing radioactive S^{35} were diluted in distilled water. Aliquots (0.1 ml) were pipetted into metal planchets, evaporated to dryness overnight, and assayed the following day in windowless gas flow counters (Tracer Lab model SC 50).[3]

Parameters. Aerosol data are expressed in these tests in terms of per cent recovery and decay rate. The former is the proportion $\times$ 100 of the number of units recovered as aerosol by a particular sampler to the number of units disseminated. Decay rate is approximately the linear decrease of the natural logarithm of concentration with time, expressed as per cent per min.

Replication and Analysis. Treatments were tested in five to eight replicate aerosol studies. All numerical aerosol data were transformed to logarithms and analyzed statistically to detect significant differences among treatments. A probability of less than 5 per cent, that an observed difference between treatments was due to chance alone was defined as statistically significant.

[1] Difco Laboratories, Inc., Detroit, Michigan.

[2] Baltimore Biological Laboratories, Inc., Baltimore, Maryland.

[3] Tracerlab, Inc., Boston, Massachusetts.

Results

Culture compatability. Preliminary experiments to test compatability of organisms with tracers were conducted using 24-hr contact periods at a temperature of 4 C. The mixtures tested included *S. marcescens,* or *P. tularensis* cells with *B. subtilis* spores as a tracer, and *P. tularensis* cells with radioactive S^{35} as a tracer. The results, summarized in table 1, indicated no adverse effect of tracers on viable counts of the test organisms over the 24-hr holding period.

Aerosol compatability. Cultures of *S. marcescens* at a concentration of 20×10^9 cells per ml were mixed with *B. subtilis* spores at three concentrations and disseminated into a 200,000-L test vessel. Table 2 presents the aerosol per cent recoveries at 2- and 30-min cloud age, and the decay rates of *S. marcescens* for the particle size range less than 5 μ diameter and greater than 5 μ diameter. Levels of recovery in the smaller size range decreased significantly as tracer concentration increased. This same effect was also evident for the re-

TABLE 1

Effect of several tracers on viable counts of test cultures with 24-hr contact periods

Tracer	*Serratia marcescens* Counts $\times 10^9$/ml	
	Without tracer (0 hr)	With tracer (24 hr)
Bacillus subtilis (2×10^9/ml)	216	200
	Pasteurella tularensis Counts $\times 10^9$/ml	
B. subtilis (2×10^9/ml)	115	113
S^{35} (0.014 mc/ml)	122	115

TABLE 2

*Aerosol per cent recoveries and decay rates of Serratia marcescens and Bacillus subtilis disseminated in intimate mixture**

Treatment	*S. marcescens* (20×10^9/ml)					
	<5 μ Aerosol particles			>5 μ Aerosol particles		
	2 min	30 min	Decay rate	2 min	30 min	Decay rate
	%	%	%/min	%	%	%/min
Without tracer	5.90	0.42	9.01	31.2	1.29	10.7
B. subtilis at 2×10^9	5.89	0.34	9.64	32.7	1.41	10.7
B. subtilis at 20×10^9	4.42	0.18	10.9	30.1	0.98	12.3
B. subtilis at 40×10^9	2.98	0.12	10.9	29.1	0.50	13.5
Tracer concentration	*B. subtilis*					
2×10^9/ml	11.9	9.38	0.92	40.4	4.41	7.53
20×10^9/ml	9.55	7.50	0.92	43.3	3.33	8.80
40×10^9/ml	7.13	5.36	0.92	43.4	2.88	9.22

* Trial conditions: 85 per cent relative humidity; 75 F.

sistant spores of *B. subtilis* suggesting that the effect was due to physical causes rather than viability losses. It could be postulated that a shift in particle size distribution (toward a larger mass medium diameter) occurred as cell numbers increased. Such a hypothesis would result in a higher rate of fall-out and this was verified by significantly higher decay rates in larger size fractions as spore concentrations were increased. Certainly, however, there was no effect on *S. marcescens* caused by using *B. subtilis* as a tracer at the lowest concentration of 2×10^9 spores per ml.

Similar studies were conducted employing spores of *B. subtilis* mixed with *P. tularensis*. In this case, aerosol assessment was confined to particles collected by the all glass impinger following the pre-impinger sampler, that is, to particles less than 5 μ in diameter. Significantly lower initial recoveries and significantly higher decay rates of *P. tularensis* were obtained when it was disseminated at a concentration of 150×10^9 per ml with added spores at a concentration of 50×10^9 per ml. A biological effect as well as a physical effect was probably operating here. However, these effects were not evident when the concentration of spores in the mixture was decreased to 1×10^9 per ml. In this case there were no significant differences detected between *P. tularensis* aerosols with tracer and without tracer on the basis of either initial recovery level or decay rate (table 3). It can be concluded that since these spores (at low concentration) are a suitable tracer for an organism as fastidious as *P. tularensis* they would also be suitable for many other vegetative bacteria.

A further series of tests was conducted with S^{35}, a mass tracer characterized by the emission of weak β-rays. No safety problems were encountered during testing as was evidenced by the lack of increase in normal radiological background levels in working areas. *P. tularensis* was again chosen for test because it was considered to be highly susceptible to possible radiation damage.

The results obtained with the test cells and with S^{35} are given in table 4. There were no significant effects of S^{35} on the aerosol recoveries or decay rate of *P. tularensis*.

Validity of spore and S^{35} decay rates. In addition to indicating compatability of test organisms and tracers it was necessary to obtain some assurance that the tracer decay rates reflected the physical decay of the test organisms. Accordingly, *P. tularensis* cells were tagged intracellularly with radioactive P^{32} by the method described by Harper *et al.* (1958). These dead, fixed cells were then added to a *P. tularensis* culture similar to those described earlier. The mixture was aerosolized and decay rates were determined from the P^{32} recoveries in particles less than 5 μ diameter. In this instance there was no doubt that the isotope was distributed throughout the aerosol in the same manner as the test organisms. The results are presented in table 5 along with the decay rates obtained with *S. marcescens* with 2×10^9 per ml spore tracer, *P. tularensis* with 1×10^9 per ml spore tracer, and *P. tularensis* with 0.014 mc per ml S^{35} tracer.

The primary point of interest was the similarity of the physical decay rates of spores and S^{35} to that of tagged cells. Normal experimental errors can account for the differences among these values.

It was not expected that total decay rates or biological decay rates would be similar among tests.

TABLE 3

*Aerosol per cent recoveries and decay rates of Pasteurella tularensis and Bacillus subtilis disseminated in intimate mixture**

	P. tularensis (150×10^9/ml)		
	4 min	32 min	Decay rate
	%	%	%/*min*
Without tracer	2.00	0.18	8.29
With tracer	1.24	0.14	7.42
	B. subtilis (1×10^9/ml)		
	8.72	5.70	1.50

* Trial conditions: 85 per cent relative humidity; 75 F.

TABLE 4

*Aerosol per cent recoveries and decay rates of Pasteurella tularensis and S^{35} disseminated in intimate mixture**

	P. tularensis (150×10^9/ml)				S^{35} (0.014 mc/ml)			
	4 min	18 min	32 min	Decay rate	4 min	18 min	32 min	Decay rate
	%	%	%	%/*min*	%	%	%	%/*min*
Without tracer	0.292	0.178	0.0922	4.04				
With tracer	0.207	0.105	0.0594	4.37	3.28	2.81	2.42	1.07

* Trial conditions: 85 per cent relative humidity; 75 F.

TABLE 5

*Total, physical, and biological decay rates of four bacterial aerosols**

Test Organism	Tracer	Total Decay Rate†	Physical Decay Rate‡	Biological Decay Rate§
		%/*min*	%/*min*	%/*min*
Seratia marcescens	*Bacillus subtilis*	9.64	0.92	8.72
Pasteurella tularensis	*B. subtilis*	7.42	1.50	5.92
P. tularensis	S^{35}	4.37	1.07	3.30
P. tularensis	*P. tularensis* tagged with P^{32}	7.26	0.94	6.32

* The tabulated decay rates apply only to the fraction of the aerosol contained in particles less than 5 μ in diameter.

† Derived from viable assay of test organism.

‡ Derived from assay of tracer.

§ Difference between total and physical decay rates.

Factors affecting physical decay were controlled precisely but factors affecting viability varied among these tests.

Discussion

The results of these studies indicated that *B. subtilis* spores and radioactive S^{35} constituted excellent tracers for *S. marcescens* and *P. tularensis* aerosols and thus presumably for a wide range of vegetative forms.

One might question the use of *B. subtilis* spores as a purely physical tracer under the conditions described. However, we have not been able to demonstrate in any of our tests that the spores germinate and become susceptible to environmental conditions during aerosolization or during the life of the cloud. Samples of the aerosol at ages up to 30 min have been assayed, heat shocked, and again assayed with no discernible decrease in viable numbers. The similarity of decay rates as estimated by spores and by isotopes is further evidence that the *B. subtilis* spores are a suitable physical tracer.

Extreme caution should be followed in altering tracer concentrations. Although it may be desirable from the standpoint of greater sensitivity to increase concentrations of either *B. subtilis* spores or S^{35}, one may alter the characteristics of the test suspension to such an extent that the results obtained may be artificial or, in the case of isotopes, the safety hazards may increase to the point where operation is no longer feasible.

With respect to S^{35}, concentrations were restricted so that the aerosol did not contain amounts in excess of the maximal permissible limits for workers in controlled areas. In other words, a worker could have entered the test unit during the conduct of trials without serious exposure. Although the aerosol concentration could be increased without undue health hazards, there would be a concomitant increase in safety problems associated with handling the culture before dissemination, with assaying the samples where the activity has been concentrated in a small liquid volume, and with the disposal of the aerosol.

In choosing between S^{35} and spores one must consider not only the factors discussed previously but also the relative sensitivities which can be obtained. In these studies the spores were employed at a concentration of 2×10^9 per ml. The quantity of S^{35} employed (1.014 mc per ml) yielded counts in the mixtures of about 1.55×10^7 per min per ml. Since the minimal count in our studies, after adjustment for normal background radiation, was chosen to be 50 cpm per ml, whereas the minimal bacterial count is 150 organisms per ml (plating 0.2 ml), the relative sensitivity difference was about 48:1 in favor of the spore method. However, different safety concepts could alter this relationship to a considerable degree in either direction. Also, of course, it is entirely feasible to concentrate the S^{35} by evaporation of the entire sampler contents (20 ml). This would, in our tests, have resulted in a 20-fold increase in S^{35} count.

It should be mentioned that experimental errors as determined by trial to trial differences were found to be similar for both S^{35} and spores. It was also found that the time lapse between sample collection and completion of assay was about the same for each type of tracer.

Summary

Procedures for estimating physical decay rates in aerosols were developed employing *Bacillus subtilis* var. *niger* spores and radioactive S^{35} as physical tracers in intimate mix with the test organism. It was determined that viability of *Serratia marcescens* and *Pasteurella tularensis* was not affected by intimate contact with the tracers at 4 C for 24 hr. Similarly, the aerosol characteristics of these organisms were not affected by the presence of appropriate concentrations of tracer materials. The spores do in fact undergo physical decay only, as evidenced by the lack of vegetative forms in aerosol samples. Both *B. subtilis* spores and S^{35} resulted in decay rate estimates similar to the physical decay estimated from P^{32} intracellular tracer.

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