AIRBORNE MICRO-ORGANISMS: A TECHNIQUE FOR STUDYING THEIR SURVIVAL

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INTRODUCTION

This paper describes an improved technique for studying the influence of natural or artificial conditions on the survival of airborne micro-organisms.

If an aerosol is contained within an unventilated chamber, there is a fall of concentration compounded of physical loss and decay of viability. The physical loss is due to deposition on the surfaces, to which the particles may be brought by settling, by convective flow, by diffusion, or by electrostatic attraction. In a ventilated space the concentration is reduced also by dilution.

For some purposes it is sufficient to measure the total loss: if, for example, a technique of air hygiene is being studied, it may be thought sufficient to know the reduction in viable airborne particles without finding whether they have died or been physically removed. On the other hand, it is necessary to be able to separate the physical and biological effects, if a proper study is to be made of the influence of various factors. If, for example, experiments are done in which one environmental factor is varied, there is no assurance that observed variations in total decay rate are entirely or even partly due to changes in the viable decay rate: the effect may be purely physical.

There are several ways of discriminating between physical and viable decay. The total number of airborne particles may be measured by total cell counts under the microscope, or by light-scattering measurements on the aerosol. Both methods are open to objection: for example, direct counts of micro-organisms require large numbers of cells in the sample and absence of foreign particles, and are inapplicable to viruses; light scattering is influenced by particle-size changes which result from heterogeneity of the cloud or from changes of humidity, and can only be used over a limited range of concentration. Another method is to measure a 'decay constant' for the system: either the physical loss of some inert simulant, or the total loss of viable particles in the absence of the factor under test. The assumption is then made, without experimental verification, that this 'decay constant' is truly constant and independent of the factor under test. It is unnecessary to criticize this method in detail: to us it seems that it can only be considered when the decay rate under test conditions is much greater than the decay constant, and even then is to be accepted with reluctance.

There is a method which is free from these objections, and which provides each experiment with its own internal yard-stick. That is the use of a 'tracer', of such nature that it may be assumed to be unaffected by those influences which alter the viability of the test organism, while it is equally subject to physical loss. We

were using bacterial spores for this purpose when some shortcomings were found and we developed the use of radioactively-labelled organisms. This paper describes the investigation.

MATERIALS AND METHODS

Bacillus globigii (*B. subtilis* var. *niger*) was grown on Tarr's medium containing 0·25 % glucose. Spores were harvested in water, heated at 60° C. for 90 min., washed three times and resuspended in water. Radioactive *B. globigii* spores were prepared by the method of Harper & Morton (1952) on medium containing 100 mC./l. of ^{32}P as phosphate, and treated with formaldehyde as described later.

Brucella suis (strain PS III k) was grown in a casein partial hydrolysate medium. Radioactive *Br. suis* was grown on a tryptose agar medium containing 200 mC./l. of ^{32}P as phosphate, reaped in 10 % formalin, and washed six times in water. (This level of activity is the most that can be used if development of long filamentous forms is to be avoided.) For use as suspending fluid in some experiments, the (inactive) casein hydrolysate culture was killed by autoclaving: the cells were then removed by centrifugation if the experiment was to be done with the Collison atomizer.

Spray

(*a*) *Homogeneous*. Suspensions were sprayed from a Collison atomizer in the apparatus described by Henderson (1952) in such conditions that only the larger wet particles contained cells and even then not more than one in most cases. These clouds evaporated to give an aerosol in which nearly all particles consisted of a single cell.

(*b*) *Heterogeneous*. Suspensions were sprayed from a simple atomizer with concentric fluid and air jets in such conditions that the cloud after evaporation consisted of a range of cell aggregates with mass median diameter of about 4 μ.

Cloud holding

The clouds were held in a variety of apparatus

Type	Half-life for physical decay
3 l. jar	½ hr.
66 l. jar	3 hr.
Test chamber, $1{\cdot}6 \times 10^5$ l.	12 hr.

The physical half-lives given are for aerosols of single cells of radioactive-killed *Br. suis*: the decay follows the exponential pattern.

Sampling

Samples were taken in 'raised impingers' (May & Harper, 1957) working at critical flow: the sampling fluid was 0·1 % gelatin saline with 0·25 % 'Manucol' and Dow Corning Antifoam A. Viable counts were done by surface drop plates:

in the following discussion, viability means production of visible colonies when assessed this way. Radioactive counts were made in M.6 liquid Geiger–Muller counters.

EXPERIMENTAL

Bacterial spores in the resting state remain viable indefinitely over a wide range of temperature and humidity. They have therefore been used by ourselves and others as tracers against which the viable decay of vegetative cells can be compared. A possible objection to their use is that in the appropriate conditions they pass very rapidly into the 'heat-sensitive' state and are then comparable to vegetative cells in sensitivity to desiccation. In anticipation of their failure to behave as stable tracers for this reason, spores of *B. globigii* (BG) were incubated for 1 hr. at 37° C. in two different whole cultures of *Past. tularensis*: there was no change in viable spore count, and the heat sensitivity did not exceed 10 % as determined by heating to 60° C. for 30 min. The same result was given by a suspension of BG spores in water. It was then found, however, that BG spores became 100 % heat sensitive when tested, in the same conditions, in five different whole cultures of *Br. suis*. It was also found that stability in the mixed suspension did not assure absence of sensitization in the aerosol phase: resting BG spores remained unchanged when suspended in an autoclaved whole culture of BG, but an aerosol formed from this showed 98 % heat sensitive within 5 min.

Viable spores are therefore not generally suitable as tracers. We have previously described the preparation of spores labelled with radio-phosphorus (Harper & Morton, 1952): these should make a satisfactory tracer, for the phosphorus is assimilated within the spores and is, of course, unaffected by physical influences. An improvement on the original method of preparation is to kill the spores with formaldehyde: after reaping they are washed three times, suspended in 10 % formaldehyde, held overnight at 37° C., and washed six times to remove formaldehyde and free ^{32}P. The advantages of using killed spores are that they retain the phosphorus more tenaciously, and that it is preferable not to have viable spores (of uncertain viability because of the radio-phosphorus) in the system. Formaldehyde treatment is preferable to autoclaving which reduces the phosphorus content to about 60 % of that in viable or formaldehyde-killed spores.

These killed radioactive spores became the standard tracer in our laboratory work. In a typical experiment (Table 1), the washed killed radioactive spores were mixed with the test suspension, a *Br. suis* whole culture. A suitable amount was then sprayed and the cloud held in closed containers at controlled humidity and temperature. Samples were taken at intervals in impingers: part of the collecting fluid was diluted and plated for viable count, and the rest was digested with nitric acid and assessed in a liquid Geiger–Muller counter. (We consider the acid digestion desirable to stop phosphorus or organisms being adsorbed on the GM counter or other glassware: it also sterilizes pathogenic samples.)

It is important to remember that in such experiments the physical efficiency of sampling does not generally matter, provided it is the same for test organism and

tracer. We are concerned with the change in ratio of test organism to tracer: the tracer should take care of all physical loss.

The general applicability of radioactive BG spores as tracers depends on the closeness with which they simulate the cells under test. Although they are larger and denser than vegetative cells such as we were then investigating, no significant

Table 1. *Viable decay of* Br. suis *estimated by radioactive BG technique.* Br. suis *in culture fluid, radioactive BG tracer added. Collison atomizer in Henderson apparatus. Cloud collected in* 3 *l. jars.* 34° C., *relative humidity* 50 %

	Total airborne content of one jar		Viable count / Radioactive count	
Time (min)	10^3 cells *Br. suis*	GM (counts/min.)		% viable
0	734	1540	476	(100)
1	466	1396	334	70
2	307	1252	245	51
4	156	690	226	48
8	107	916	117	25
16	31	544	57	12
32	6	479	13	3

Table 2. *Apparent viable decay of* Br. suis. Br. suis *sprayed from water containing radioactive BG tracer. Collison atomizer in Henderson apparatus. Cloud collected in* 3 *l. and* 60 *l. jars (average of four results each).* 22° C., *relative humidity* 80 %. *Sampled after* 60 *min.*

	% of cloud remaining airborne	Apparent viability (%)
3 l. jars	32	47
60 l. jars	70	34

difference was expected. The calculated Stokes settling velocity is two or three times that of a *Br. suis* cell, but as it is only about 0·2 cm./min. the difference would not show in a short-term experiment: furthermore, the cloud-holding conditions were such that some convective mixing would be expected. Subsequent experience has shown that it is valid to use the spores in experiments which involve little physical decay, but if the holding time is prolonged until a substantial part of the cloud has settled out there will be a significant difference between the amounts of tracer and test organism remaining airborne. This was shown by an apparent difference in viable decay rates between identical clouds held in small and large containers (Table 2). The most probable explanation of the difference was that the spore tracer was settling out more rapidly than the *Br. suis*: this would have the effect of decreasing the apparent viable decay rate, to a greater extent in the smaller containers because of the more rapid physical decay in them. This was confirmed by experiments in which radioactive *Br. suis* and viable BG spores were sprayed together (Table 3). In these experiments sensitization of the spores was avoided by (*a*) using washed *Br. suis* cells, or (*b*) adding 0·1 % D-alanine (recommended by Mrs J. F. Powell) to the whole culture.

These experiments show clearly the more rapid loss of BG spores. It will be noted that the culture fluid reduced the difference, presumably by adding the same weight of solid residue to each type of cell.

Table 3. *Physical decay of* Br. suis *and BG spores compared. Radioactive* Br. suis *with viable BG spores. Collision atomizer in Henderson apparatus. Cloud collected in 3 l. jars*

	Time (min.)	% of cloud remaining airborne		Ratio of *Br. suis* to BG
		Br. suis	BG	
In water (2 expts.)				
19° C., relative humidity 55 %	30	67	47	1·4
	60	45	25	1·8
	120	30	11	2·7
In culture fluid (3 expts.)				
21° C., relative humidity 58 %	10	40	40	1·0
	30	20	17	1·2
	60	9·3	6·1	1·5
	120	3·6	2·1	1·7

The conclusion we reached was that the best tracer for any particular test organism would be a radioactive killed preparation of the same organism. It would not be acceptable to have the radioactive tracer within the test cells, for these might be damaged by radiation during growth or afterwards. We have, however, verified that radiation from the tracer cells does not harm the test cells: mixed suspensions have been held up to 4 days without effect, and also it is estimated that the radiation dosage in a typical experiment does not exceed 0·001 MLD, far below the level of possible effect.

There seems to be no difficulty in preparing these suspensions by simple modification of our basic method. We have, for example, prepared radioactive *Past. tularensis* on blood–glucose–cysteine agar containing 150 mC./l. of radioactive phosphate. It is a convenience to reap highly infective organisms in 10 % formaldehyde solution *before* washing. It is evidently possible to select one of these tracers for test cells of similar size; we have in *P. tularensis*, *Br. suis* and *B. globigii* spores a well-spaced range of useful sizes.

Details of some typical tracer suspensions are given in Table 4.

On storage, phosphorus is slowly lost from the cells into the suspending fluid. Table 5 shows the increase in supernatant activity when suspensions are stored at 4° C., determined by centrifuging and resuspending in the same fluid at various times.

Loss of ^{32}P from the cells could introduce error into aerosol experiments if the conditions of generation of the cloud were such that particles were formed containing no tracer cells: these particles would probably be much smaller than the tracer cells, but being radioactive they would give a GM count as if tracer cells were present. It is therefore our practice to wash tracer suspensions just before use (although it can be seen from Table 5 that they could be left several days without risk of significant error).

Table 4. *Radioactive-killed cell suspensions grown on media containing initially* 167 *mC./l. of* ^{32}P

	10^6 GM counts/min./ml.	GM counts/ min./10^4 cells	% ^{32}P utilized	% activity in supernatant
Br. suis	15·2	4·1	28	0·19
	13·2	3·4	25	0·16
	19·2	5·7	36	0·17
	16·1	3·9	32	0·17
	21·0	6·0	40	0·19
Past. tularensis	6·8	4·1	13	1·10
	9·0	5·5	18	0·84
	9·7	4·9	18	0·46
	9·7	4·8	18	0·86
	7·9	4·1	15	1·10

Note. Column 1 gives the activity of the suspension: when freshly prepared, nearly all the ^{32}P is within the cells, as shown by the low supernatant activity in Column 4. Column 2 was calculated using total cell estimates from optical density. Column 3 shows the percentage of the ^{32}P, added to the medium, which is found in the washed suspension.

Table 5. *Loss of* ^{32}P *from cells in aqueous suspension at* 4° C. *as shown by increased supernatant activity. Activity in aqueous solution shown as percentage of total suspension activity*

	Days storage at 4° C.									
	0	1	2	4	5	6	7	9	10	12
Br. suis	0·2	1·3	1·8	1·9	3·1	—	—	—	—	—
	0·2	1·3	1·3	2·3	2·5	—	—	—	—	—
	0·3	1·2	1·8	3·0	4·1	—	—	—	—	—
	0·2	—	4·8	—	—	—	7·0	10·0	—	—
	0·2	3·5	—	5·3	5·4	—	6·0	6·1	—	—
Past. tularensis	0·3	1·5	—	2·3	—	3·6	—	—	6·5	9·4
	0·6	1·5	—	2·3	—	4·2	—	—	6·1	8·0
	0·5	2·0	—	4·8	—	3·4	—	—	5·0	8·6
	0·5	2·8	—	3·9	—	4·5	—	—	7·1	7·9

We have used these tracers in aerosol-survival experiments involving holding periods of many hours and have confidence in the results obtained, although we cannot give direct proof of the identical physical behaviour of test cell and tracer. They are visually identical under the microscope; but perhaps the best evidence is the absence, in a large number of experiments, of the anomalous results which would be expected if the tracer cells differed significantly from the viable test cells in physical behaviour.

The problem of tracers for viruses has been considered: the difficulty of preparing radioactive virus free from contamination by radioactive debris is such that another approach is desirable. If the viruses are dispersed in such conditions that a substantial part of the cloud consists of single virus particles, the larger bacterial cell tracers may show unacceptable differences in behaviour from the virus: in these circumstances, use of a soluble radioactive tracer might give the better physical approximation. On the other hand, if the virus particles are larger, because of

aggregation or the presence of other material in the suspension from which the cloud has been formed, a bacterial tracer would have the advantage. On present evidence, however, soluble radioactive phosphate seems likely to prove an acceptable tracer.

SUMMARY

The concentration of viable cells in an aerosol of micro-organisms is reduced by physical loss and biological decay. To study biological decay it is necessary to distinguish between the two processes. The preferred way of doing this is to mix the test aerosol with a tracer which is subject only to physical loss: the tracer and test cells should be mixed before dissemination.

The recommended tracers for bacterial clouds are bacteria made radioactive by growing on a medium containing ^{32}P, then killed with formaldehyde and washed. To ensure closest identity of physical behaviour it is necessary that the tracer should be of the same species as the cells under test.

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REFERENCES

HARPER, G. J. & MORTON, J. D. (1952). *J. gen. Microbiol.* **7**, 98.
HENDERSON, D. W. (1952). *J. Hyg., Camb.*, **50**, 53.
MAY, K. R. & HARPER, G. J. (1957). *Brit. J. industr. Med.* **14**, 287.

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