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A method for measuring the retained dose in experiments on airborne infection

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INTRODUCTION

In experiments on airborne infection one usually wishes to know the dose which each animal has been given. The dose depends on the cloud concentration, the volume inhaled, and the percentage retention of inhaled particles; the cloud concentration alone is readily measurable during the experiment. Techniques have been described, for use in work on respiratory retention, that measure the volume of cloud inhaled and its concentration before and after inhalation, but there are difficulties in applying these to infection experiments and we know of no published example of such use. A common practice is to calculate the dose from measurements of respiratory minute volume and percentage retention made at some other time.

An alternative procedure, established by pioneers of quantitative work in this field (Rosebury, 1947; Henderson, 1952), is to measure the cloud concentration and then calculate the intake from a knowledge of the respiratory minute volume of the species exposed (e.g. Guyton, 1947). This takes no account of the proportion of particles exhaled, but this is unimportant in comparative studies so long as the ratio of calculated intake to retained dose remains the same. The conditions governing this ratio for a particular experimental animal are the size of the inhaled particles and the actual respiratory minute volume (as compared with that assumed). For experiments done in standardized conditions with clouds of single cell particles, the ratio may apparently be assumed constant, and this is substantiated by the excellent reproducibility of dose/response results.

We can make a better estimate of the total retention or lung retention from measurements made with radioactive spores (Harper & Morton, 1953) but this procedure also depends on the constancy of experimental conditions and animals. There is an obvious need for a direct method which does not depend on measurements of respiration or retention made at another time. Furthermore, we know that individual animals give widely varied retention (Harper & Morton, 1953; and see below) and this leads to the interesting question whether response in individual animals within a group is determined by the varying retention as well as by varying biological susceptibility.

We propose here a new technique, which is at present applicable only to alveolar retention in the guinea-pig but may find wider usefulness: a single experiment with mice gave similar results. The idea was based on our observation that the rate of

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removal of spores from the lung was quite slow: see also Henderson, Peacock & Belton (1956). If this removal followed a regular course, so that the remaining spores after any time lapse showed a constant relation to the initial retention, radioactive spores could be used to measure the retained dose of a pathogen. The animals would be exposed to a mixed cloud of radioactive spores and pathogen; after the appropriate holding time, the residual spores would be assessed; from this the original retention of spores would be calculated; and hence, knowing the ratio of spores to pathogen in the cloud, the original retained dose of pathogen would be determined.

The first experiments reported here showed a promising constancy in rate of loss of retained spores: they were followed by experiments in which guinea-pigs were exposed to mixed clouds of *Brucella suis* and spores, which provided further evidence on the rate of loss and also enabled us to compare measured doses and observed infection.

MATERIALS AND METHODS

Brucella suis strain PSIIIk was grown in a casein partial hydrolysate medium with aeration.

Radioactive *Bacillus subtilis* var. *niger* ('Bacillus globigii') was prepared by the method of Harper & Morton (1952), modified in that the spores were treated with 10% formalin (about 4% formaldehyde) for 18 hr. at 37° C. before washing: this considerably reduces the rate of loss of ^{32}P as compared with resting viable spores.

Radioactive *Br. suis* was grown on fortified tryptose agar (McCullough, Mills, Herbst, Roessler & Brewer, 1947) containing 200 mC./l. of ^{32}P, treated with 10% formalin for 2 hr. at 37° C., and washed six times.

Clouds were generated in the Henderson apparatus (Henderson, 1952), slightly modified for exposure of four, instead of two, guinea-pigs at a time. Cloud samples were taken in the Porton 'raised' impinger (May & Harper, 1957). Guinea-pigs (400–450 g.) were randomized: they were exposed for 4 min. and were housed separately because we have observed transfer of radioactive contamination. At appropriate times, they were killed and the whole lungs (with vessels, including the bronchi) were removed and digested in nitric acid. The radioactive count was made in M. 6 liquid counters (Harper & Morton, 1953). Animals exposed to *Br. suis* and held for 21 days were examined for brucellosis before removing the lungs: gross pathology of spleen, liver, and cervical and bronchial lymph nodes was noted, and cultures were made on fortified tryptose agar of the spleen and in certain cases of lymph nodes also. This procedure is a thorough diagnosis (Harper, 1955).

Cloud samples were taken at several times during the animal exposures and were assessed for *Br. suis* by serial dilution of an aliquot and surface plating on fortified tryptose agar. Radioactive assessments were made by digesting the remaining sample in nitric acid and counting in M. 6 GM counters. The cloud concentrations (of both radioactivity and *Br. suis*) were each averaged to give the dosages to which the animals had been exposed; the cloud concentrations were satifactorily constant throughout each experiment.

We did not take measurements of 'initial retention' immediately after exposure,

but chose, instead, 1 day later. If there were a significant part of the retention subject to rapid removal by ciliary action, which affects all but the alveolar retention, an immediate determination would be undesirable for two reasons: (i) individual measurements would be likely to vary unduly, since ciliary action continues after death and the time between exposure and dissection cannot readily be exactly standardized, and (ii) it is probable anyway that the organisms which are being swept out on the mucous film are less important in infection.

RESULTS

The measured radioactivity (corrected for radioactive decay) is conveniently expressed in terms of ml./min. of cloud retained, by calculation from the measured radioactivity for unit volume of cloud.

Table 1

(Expt. 1. 40 guinea-pigs were exposed: twenty were killed 1 day after exposure and twenty on the 21st day.)

Day killed	Cloud retained (ml./min.)	Mean
1	64, 62, 49, 47, 45, 40, 39, 38, 37, 35, 35, 33, 32, 30, 29, 26, 24, 18, 17, 17	36
21	19, 18, 18, 17, 12, 11, 10, 10, 9·5, 8·9, 8·9, 8·6, 8·0, 7·3, 7·2, 6·5, 5·9, 3·8, 3·7, 2·9	9·8

Table 2

(Expt. 2. Sixty guinea-pigs were exposed and ten were killed within a few seconds and rapidly dissected: the remainder were killed in groups of five at intervals up to the 35th day.)

Day killed	Cloud retained (ml./min.)	Mean
0	82, 82, 74, 72, 61, 51, 44, 38, 28, 17	55
1	39, 38, 35, 33, 13	32
4	65, 39, 33, 26, 26	38
7	47, 37, 28, 25, 24	32
11	26, 20, 17, 16, 14	19
14	12, 10, 9·8, 8·7, 8·2	9·7
17*	19, 13, 12, 7·4	12·9
22	10, 5·5, 4·7, 3·8	5·4
25	7·8, 5·9, 5·7, 4·7, 3·6	5·5
28	9·1, 5·2, 4, 3·8, 3·5	5·1
35	3·3, 2·7, 2 5, 1·3, 0·7	2·1

* One abnormally high result of 35 ml./min. has been omitted from day 17; radioactive contamination of the sample may have occurred.

DISCUSSION

The measurements of initial and residual lung retention are summarized in Table 5 and plotted in Fig. 1.

Three things are obvious. First, that the loss of spores from the lungs conforms to the simple exponential law (Fig. 1, Expt. 2); secondly, that the agreement between experiments is good (Fig. 1); thirdly, that the coefficient of variation

shows no marked change with the passage of time (Table 5): this means that the variation at any subsequent time is probably the result of variation in initial retention rather than variation in rate of loss from the lung. It follows from these observations that the basic idea of estimating initial retention from measurements taken later is probably sound.

Table 3

(Expt. 3. Sixty guinea-pigs were exposed to a mixed cloud of *Brucella suis* and radioactive spores. Ten were killed after 1 day and the lungs assessed for radioactivity: the remaining fifty were examined for brucellosis and assessed for radioactivity on the 21st day.)

Day killed		Cloud retained (ml./min.)	Mean
1		51, 50, 47, 47, 45, 41, 20, 19, 19, 16	36
21	Infected	17, 17, 13, 13, 12, 10, 9·3, 9·2, 9, 8·4, 8·1, 8·1, 7·9, 7·5, 5·9, 5·2, 5·2, 3·7, 3·4, 3	8·6
	Non-infected	12, 11, 11, 11, 11, 10, 10, 10, 9·4, 8·3, 8·2, 8·1, 7·9, 7·7, 6·3, 6·2, 6·1, 5·9, 5·4, 5·4, 5·3, 5·2, 4·8, 4·5, 4·3, 4·3, 3·7, 3·6, 3·3	7·3

Note: one very high result, due probably to radioactive contamination, has been omitted from the day 21 results.

The *Brucella suis* suspension was 6 days old, and the calculated retention in day 1 guinea-pigs was 10·1 cells.

Table 4

(Expt. 4. Expt. 3 was repeated.)

Day killed		Cloud retained (ml./min.)	Mean
1		63, 59, 55, 55, 55, 51, 41, 32, 32, 17	46
21	Infected	20, 20, 11, 9·8, 8·9, 6·8, 4·6	11·6
	Non-infected	18, 18, 18, 17, 16, 16, 15, 15, 14, 14, 14, 14, 13, 13, 13, 12, 12, 11, 11, 9·8, 9·6, 9·2, 8·9, 8·9, 8·4, 8·3, 8·2, 8, 7·8, 7·6, 7·1, 7·1, 7, 7, 6·8, 6·8, 6·2, 5·5, 5·1, 4·7, 4·6, 4·4, 4·2, 3·8	10·3

Note: The infection level was lower than had been intended.

The *Brucella suis* suspension was 83 days old, and the calculated retention in day 1 guinea-pigs was 8·8 cells.

The accuracy with which the mean residual retention in a group after a time lapse can be used to estimate the initial retention depends on the variance of the slope of the retention/time graph. The slopes of the individual lines in the four experiments are given in Table 6.

These regression coefficients agree sufficiently well with one another (probability = 20% approximately) and therefore a common slope can be established which can be used to estimate the initial mean retention from the mean retention at a later time, with an accuracy dependent on the variance of the slope. We have already observed that the variance of individual retentions *within* each group does not change with time, so we can also estimate the variance of the initial retentions about their mean.

Table 5. *Summary of Experiments* 1–4, *showing mean retention in each group*

Expt. no.	Day	No. of guinea-pigs	Mean retention (ml./min.)	C. of V.	% of day 1
2	0	10	55	40	172
1	1	20	36	36	(100)
2	1	5	32	30	(100)
3	1	10	36	39	(100)
4	1	10	46	31	(100)
2	4	5	38	38	119
2	7	5	32	27	100
2	11	5	19	22	59
2	14	5	9·7	14	30
2	17	4	12·9	32	40
1	21	20	9·8	45	27
3	21	49	7·9	42	22
4	21	50	10·5	42	23
2	22	5	5·4	45	17
2	25	5	5·5	25	17
2	28	5	5·1	41	16
2	35	5	2·1	45	7

Note: C. of V. is coefficient of variation—root mean square deviation expressed as percentage of arithmetic mean.

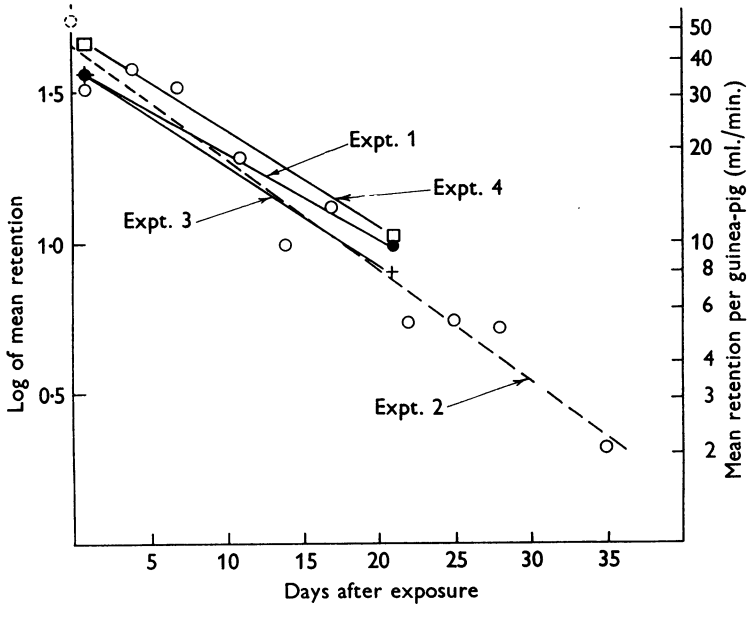

Fig. 1. Retention of airborne radioactive bacterial spores in lower respiratory tract of guinea-pig, measured at various times after inhalation. ○ is zero time, Expt. 2, (not used in calculations).

We need not rely entirely on measurements made at the termination of an experiment; we can choose to expend a number of the exposed animals at the beginning of the holding period. This must be done if the slope is unknown, or may be done to collect further results for a slope already established. From the results in Tables 1–4 we can calculate the consequences of such a procedure. Suppose that we expose sixty guinea-pigs and sacrifice n at day 1 and $60-n$ at day 21; then the variance at day 21 can be combined with the day 1 observations to determine the fiducial limits applicable to the observed initial retention (Table 7).

Table 6. *Slopes of retention/time graphs, Experiments* 1–4

Expt.	Slope
1	$-0{\cdot}029 \pm 0{\cdot}003$
2	$-0{\cdot}038 \pm 0{\cdot}002$
3	$-0{\cdot}033 \pm 0{\cdot}003$
4	$-0{\cdot}033 \pm 0{\cdot}003$

Table 7

(60 guinea-pigs exposed. Retention measured in n at day 1 and $60-n$ at day 21. Fiducial limits on initial retention calculated using variance = 0·0378 based on Expts. 1–4.)

n	Ratio of upper to lower 95 % fiducial limits
5	2·19
10	1·74
15	1·57
20	1·48
25	1·42
30	1·37

It will be seen that $n = 10$ gives a substantial increase in accuracy over $n = 5$, but that there is no great advantage in further increasing n. We can calculate what these limits mean in terms of infection: if sixty guinea-pigs are exposed and ten are sacrificed initially, the upper and lower 95 % fiducial limits on the observed mean retention are in the ratio 1·74/1. Experimental experience indicates that if the mean retained dose is an ID_{50}, the upper and lower percentage infections corresponding to this ratio would be about 40 and 60 %: the same conclusion follows from application of the Druett–Peto hypothesis (Druett, 1952; Peto, 1953), or from insertion of the limits on a probit/log dose regression line of slope 2. This gives an idea of the present accuracy of the technique which is sufficiently encouraging: it is to be expected that accumulation of further results will lead to greater accuracy.

There are three ways in which this proposed method of calculating initial retention from later measurements might be invalidated. In the first place, the retention of bacterial spores might differ from that of smaller pathogenic cells like *Br. suis*: this would mean that the two kinds of cell were not retained in the same ratio as that measured in the cloud. To check this, two experiments were done in which guinea-pigs were exposed to a cloud of radioactive killed *Br. suis* (Table 8), and killed immediately

These results with *Br. suis* agree well with normal experience using bacterial spores: typical results from our records for groups of four or six guinea-pigs are (ml./min. retained in lung, immediate determination): 76, 75, 75, 73, 69, 67, 66, 64, 55, 51—weighted mean 68 ml./min.

The second factor which could upset the method would be loss of ^{32}P from the spores, since we are assuming that the decrease in (corrected) count is entirely due to removal of the whole spores. Radioactive spores from the batch used in Expt. 2 were incubated in water at 37° C., with centrifugation at intervals to measure the radioactivity remaining in the spores; the amount leached out was negligible up to 5 weeks (Table 9).

Table 8. *Guinea-pigs exposed to clouds of radioactive killed* Brucella suis, *and then killed and rapidly dissected*

	No. of guinea-pigs	Mean lung retention (ml./min.)
Expt. 5	9	72
Expt. 6	10	67

One guinea-pig omitted from Expt. 5: it had heavily diseased lungs and gave very low retention.

Table 9. *Radioactive spores in water at* 37° C.; *loss of radiophosphorus*

Days storage	Radioactivity of spores as percentage of total
0	99
1	99
4	98
7	98
11	98
14	97
17	97
22	96
25	94
28	97
35	94

It was possible that the lung might provide a more favourable environment than water for loss of ^{32}P. We could see no way of directly checking this and chose therefore to repeat the experiment with spores suspended in tryptic meat broth (Powell, 1957). The radioactivity of the spores did fall more rapidly, to about 84% at 21 days and the same level at 35 days: this, however, was probably due to a difference in the radioactive spores used, for the same larger loss was found when they were suspended in water. Further, this loss is not enough to invalidate the retention experiments.

The third limitation of the technique, and one which has not been investigated, is the influence of a disease involving substantial lung changes on the rate of removal: it seems likely that plague infection, for example, would considerably modify the slope of the residual retention/time graph (Ames & Nungester, 1949). We do not propose to investigate this at present: in the meanwhile, the method is

reported because of its probable interest to workers on experimental respiratory infection. It could, for example, be extended to estimate total initial retention, by using figures such as we have obtained for the percentage distribution of retained particles (Harper & Morton, 1953).

It is interesting to speculate about the applicability of this method to individual guinea-pigs. We cannot, of course, measure the initial and final retention in the same animal, but it has already been observed that, since the variance within groups does not increase with time, the variance at a later time is probably due to initial variance in retention and is not significantly affected by individual variations in rate of loss. It can therefore be argued that the animal with the highest final retention has the greatest probability of being that with the highest initial retention, and so forth. If, then, we take the final retentions as proportional to the individual initial retentions, it can be seen from Tables 3 and 4 that the mean dose of *Br. suis* was slightly higher in infected than in non-infected guinea-pigs. The scatter in each group is, however, so large that the difference proves not to be statistically significant. It follows from this that the fate of individual animals exposed to a common dosage is not significantly affected by the variation in retained dose over the range observed in our experiments.

Mr Leonard J. Goldberg (Naval Biological Laboratory, Oakland, California) has pointed out to us that the rapid initial loss, against which we had taken the precaution of delaying the measurements of 'initial retention' for 1 day, did not in fact occur, and that this is in accordance with our earlier work (Harper & Morton, 1953). This does not affect the validity of the method, and one may still prefer to measure the 'initial retention' at day 1, which is more convenient if a large number of animals has to be handled; and also some change in experimental conditions might induce rapid early clearance. Goldberg also points out that if there were deposition on ciliated epithelium leading to a more rapid early loss, the method could be used to discriminate between 'ciliated' and 'non-ciliated' deposition. The validity of this would depend on the uniform exponential clearance which we have demonstrated.

SUMMARY

When killed radioactive bacterial spores are inhaled by guinea-pigs and retained in the lung, it is found that the rate of loss up to 5 weeks later is sufficiently constant for the initial retention of a group of animals to be calculated from the measured retention at a later time (say 3 weeks after exposure). If, therefore, the inhaled cloud contained also a pathogen, in known proportion to the spores, the initial retention of the pathogen could be calculated. The experiments apply to groups of animals, but it is believed that the conclusions are valid also for single animals. In experiments with *Br. suis* it was found that the fate, as regards infection, of individual animals within a group exposed to a common dosage was not significantly related to the estimated retained dose, despite the wide variation of individual doses about the group mean.

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