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Airborne micro-organisms: survival tests with four viruses

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INTRODUCTION

Although the literature contains numerous reports of the influence of relative humidity (R.H.) and temperature on the survival of airborne bacteria far less is known about viruses. Influenza virus has been the subject of papers by Edward, Elford & Laidlaw (1943); Lester (1943); Loosli, Lemon, Robertson & Appel (1943*a*); Loosli, Robertson & Puck (1943*b*); and Shechmeister (1950); and recently Hemmes, Winkler & Kool (1960) have reported work with influenza and poliomyelitis viruses.

However, the quantitative aspects of some of this earlier work leave much to be desired. Variations in technique which can have considerable bearing on the results are the type of cloud chamber used; presence or absence of light; methods of generating, sampling and assessing aerosols; failure to differentiate between physical and viable decay; and the degree of control over temperature and R.H. To enable reasonable comparisons to be drawn between the survival of a number of micro-organisms, tests should be carried out in strictly controlled conditions.

The recent development of more accurate viral assay techniques; the introduction of the rotating drum for long-term cloud holding (Goldberg, Watkins, Boerke & Chatigny, 1958); and the use of tracer methods for measuring physical decay in aerosols (Harper, Hood & Morton, 1958) make it possible to carry out studies which should be readily repeatable in other laboratories.

This paper reports the influence of R.H. on the survival of four viruses: vaccinia, influenza, Venezuelan equine encephalomyelitis (VEE) and poliomyelitis. Tests with poliomyelitis were carried out between 21 and 24° C.; the other viruses were examined at this and two additional temperature ranges: 7–12° C. and 32–34° C.

MATERIALS AND METHODS

Cloud generating and holding equipment

Aerosols were generated with a Collison atomizer in a Henderson (1952) apparatus modified to operate over a range of R.H. Aerosols were sampled immediately after generation (age about 1 sec.) and then stored in a 75 l. rotating stainless steel drum. The drum and contained cloud were rotated at 3 r.p.m. to reduce physical loss by sedimentation (Goldberg *et al.* 1958).

Subsequent samples were collected from the drum up to 23 hr. after generation. Control of temperature and R.H. in the apparatus was very good and precautions

were taken to ensure that the whole system was at the same temperature and R.H. throughout the period of an experiment.

Aerosol sampling

Aerosol samples were collected in 11 l./min flow-rate impingers (May & Harper, 1957) in 10 ml. of the appropriate collecting fluid. Samplers were operated for between 15 and 120 sec. depending on the expected cloud concentration. During sampling, replacement air was admitted to maintain a constant pressure in the drum; this air was drawn from the Henderson apparatus, adjusted to the same temperature and R.H. as the test aerosol.

Determination of viability of airborne particles

The concentration of viable particles in an aerosol is reduced by physical loss and viable decay. To study viable decay it is necessary to distinguish between the two processes. The method used was to mix the test particles with tracer material which was subject only to physical loss, and measure the ratio of test to tracer in suspension and cloud samples.

For the VEE suspension, the tracer was formalin killed *Pasteurella tularensis* cells which had been labelled by growing on a medium containing ^{32}P. Vaccinia influenza and poliomyelitis suspensions were traced with ^{32}P solution adjusted to pH 7·0 (Harper *et al.* 1958). In all tests the level of radioactivity in suspensions was about 10 μcuries/ml., a level of radioactivity found to be without effect on the infectivity of vaccinia virus stored for 48 hr. at 4° C. or poliomyelitis stored for several weeks, and believed to be without effect on the airborne test particles. (In bacterial experiments using a similar level of radioactivity it was calculated that the airborne cells received a radiation dosage of less than 0·001 LD 50).

The test/tracer ratio found in a suspension was regarded as equal to 100 % viability, and cloud sample ratios were expressed as a percentage of this initial ratio.

Preparation of suspensions

Vaccinia virus

A strain of virus originally obtained from Prof. A. W. Downie, Liverpool University, was used. The suspension was made by gently scraping vesicle fluid from infected rabbit skin and suspending in diluting fluid (see below). After lightly centrifuging, the suspension was diluted 1:7 in the same fluid and stored in a solid CO_2 cabinet in 1 ml. amounts. One container was used for each aerosol experiment, being diluted 1:20 before spraying. This gave suspensions containing 0·08 % solids and about 5×10^7 pock-forming units (p.f.u.)/ml.

Influenza virus

A mouse passaged strain of influenza virus Type A, strain PR 8, used in this establishment was chosen as it had been found to be very stable both in suspension and aerosols.

Ten-day embryonated eggs were inoculated intra-allantoically with 0·1 ml. of a 10^{-5} dilution of frozen mouse lung suspension made in 10 % broth-saline con-

taining 1000 units penicillin and 100 μg. streptomycin per ml. After 2 days incubation at 37° C., eggs were held at 4° C. for 2 hr. Allantoic fluid from all eggs giving a positive haemagglutinin reaction was pooled, filtered through muslin, and diluted 1:8 or 1:10 in previously chilled casein McIlvaine's buffer (see below). The diluted virus was stored in 20 ml. amounts in a solid CO_2 cabinet. One container was used for each aerosol experiment, being sprayed without further dilution. Suspensions prepared in this way contained 0·4% solids and about 1×10^7 membrane piece 50 per ml. (MP 50/ml.: titre giving positive haemagglutination with 50% of membrane pieces inoculated) when assessed by the method of Fazekas de St Groth & White (1958).

The two batches of virus suspension prepared for this work were tested for the presence of incomplete (i.e. non-infectious) virus (von Magnus, 1951) by determining the ratio of egg infective doses (EID 50) to haemagglutinin titre. The ratios found, $10^{5.5}$ and $10^{6.0}$, indicated that these preparations did not contain large amounts of incomplete virus.

Venezuelan equine encephalomyelitis virus

A chick embryo preparation with a titre of about 1×10^{10} mouse I.P. LD 50 (MIPLD 50) per ml. was made available by the U.S. Army Chemical Corps, Fort Detrick, Frederick, Maryland, U.S.A. This suspension was stored in 35 ml. amounts in a solid CO_2 cabinet. One container was used for each aerosol experiment, being sprayed without further dilution.

Poliomyelitis virus

Type I (Brunhilde) virus was grown in a stirred suspension of ERK-D cells in Earle's saline, without glucose, in an atmosphere of 5% CO_2, 75% O_2 and 20% air, for 21 hr. at 37° C. After standing at 4° C. for 48 hr. the clear supernatant fluid was removed and distributed in 20 ml. amounts. Two batches of suspension were prepared; one was stored at 4° C. and the other in a solid CO_2 cabinet. These preparations had titres of about 5×10^8 plaque forming units/ml. and contained 1·3% and 5·9% solids respectively. One container was used for each aerosol experiment, being sprayed without further dilution.

Collection and dilution fluids

Collection fluids for the test viruses were chosen after preliminary aerosol tests with fluids known to be satisfactory for maintaining viability in suspensions.

Vaccinia virus

Samples were collected and diluted in McIlvaine's citric acid/di-sodium phosphate buffer (Clarke, 1928) containing 1% dialysed horse serum. The buffer, at 0·002 M concentration and pH = 7·2, contained penicillin 500 units and streptomycin 50 μg./ml.

Influenza virus

Samples were collected in 0·2% dialysed casein in 0·008M McIlvaine's buffer (pH = 7·2) and diluted for assessment in the standard medium of Fazekas de St Groth & White (1958).

Venezuelan equine encephalomyelitis virus

Samples were collected in 20% egg yolk in Sørenson's buffer (pH = 7·6) and diluted for assessment in Difco heart infusion broth (pH = 7·4).

Poliomyelitis virus

Samples were collected in phosphate buffered saline (pH = 7·4) containing 1% horse serum and diluted for assessment in the same fluid without horse serum.

Assessment methods: radioactive

Assessments were carried out on nitric acid digests of samples (Harper *et al.* 1958), in the M6 liquid counter described by Veall (1948) using conventional scaling and timing equipment. The 95% confidence limits of the radioactive counts ranged between ±2% and ±7%.

Assessment methods: viable

Vaccinia virus

Serial 1:2 or 1:3 dilutions of samples were assessed by the method of Westwood, Phipps & Boulter (1957). Between 5 and 10 eggs were used for each dilution; these were arranged so that at least one level usually gave pock counts of acceptable numbers (30–70). In some experiments where suspension counts were low or viable decay was rapid, pock counts outside this range had to be accepted. Weighted means were used to arrive at the number of p.f.u./ml. of sample.

Influenza virus

Serial 1:2 or 1:4 dilutions of samples were assessed by the membrane piece method of Fazekas de St Groth & White (1958). Twenty membrane pieces were used for each dilution level. Titres were determined by probit analysis and expressed as MP50/ml. The 95% confidence limits differed from the most probable values by factors ranging from 1·4 to 1·6.

Venezuelan equine encephalomyelitis virus

0·25 ml. amounts of serial 1:3 or 1:10 dilutions were injected intraperitoneally in mice weighing between 10 and 13 g. The mice, which were all from the same breeding stock, were free from intercurrent infection. Ten mice per dilution level were used for assay of cloud samples, 25 mice per dilution for suspension assays. Mice were examined daily and deaths recorded for up to 12 days after injection. The few mice found dead during the first 2 days (less than 0·2% of deaths) were excluded from the results. Over 99% of deaths occurred between the 4th and 9th day after injection. LD50 values were determined by probit analysis, and ex-

pressed as mouse I.P. LD 50 (MIPLD 50)/ml. In thirteen out of fifteen tests, the 95 % confidence limits differed from the most probable values by factors ranging from 2 to 5; in the other two tests the limits differed by factors of 6 and 8.

Table 1. *Viability of airborne virus 0–23 hr. after spraying*

Temp. (°C.)	R.H. (%)	No. of tests	Percentage viable at given times (hr.) 0*	½	1	4	6	23
			(*a*) Vaccinia					
10·5–11·5	20	1	94	68	78	82	79	81	66
	50	1	94	90	90	83	92	77	59
	82–84	2	97	81	71	79	59	60	27
21·0–23·0	18–19	2	97	86	80	66	46	45	15
	48–51	3	93	82	83	86	57	50	12
	82–84	3	112	96	73	66	24	18	Trace
31·5–33·5	17–19	2	80	67	67	61	51	33	13
	50	2	74	76	68	51	26	15	Trace
	80–83	2	88	88	54	36	5·9	1·2	Trace
			(*b*) Influenza					
7·0–8·0	23–25	3	88	87	80	78	68	63	61
	51	3	66	49	75	61	39	42	19
	82	3	126	120	71	70	39	35	3·0
20·5–24·0	20–22	5	75	77	65	64	74	66	22
	34–36	3	86	93	58	59	66	53	14
	50–51	3	84	62	49	29	6·4	4·2	Trace
	64–65	3	77	45	29	15	6·6	3·2	N.D.
	81	4	67	55	22	13	6·4	5·0	Nil
32·0	20	3	87	70	56	45	18	17	1·3
	49–50	3	98	45	22	13	2·7	0·7	Nil
	81	3	91	50	15	6·6	Trace	Trace	Nil
			(*c*) Venezuelan equine encephalomyelitis					
9·0–9·5	19	1	69	54	25	20	27	50	26
	48	1	100	86	16	24	29	24	11
	86	1	105	90	57	119	100	67	6·2
21·0–23·0	19–23	2	23	17	19	14	11	7·5	1·7
	50	2	35	28	21	14	7·8	5·2	0·1
	81–86	2	92	63	82	26	16	4·0	0·1
32·0–33·0	19	2	27	25	18	9·9	6·9	3·1	0·17
	48	2	25	22	8·5	6·1	*ca.* 1·0	0·1	Trace
	81–85	2	132	80	33	17	*ca.* 2·0	Trace	Nil
			(*d*) Poliomyelitis					
20·5–23·5	18–23	4	19	10	5·5	5·9	3·4	3·3	1·1
	35–36	4	19	7·4	6·4	6·7	5·6	5·3	0·9
	49–51	5	66	0·6	0·16	0·06	0·03	Trace	Trace
	64–65	5	96	96	91	94	61	55	10
	80–81	5	120	131	112	124	111	105	85

* Samples collected *ca.* 1 sec. after spraying.
N.D. = not done.
Trace = Samples containing viable virus in amounts too small for accurate assay.

Poliomyelitis virus

Infectivity titrations were carried out with ERK-D cells using a modification of Cooper's (1955) plaque technique. 0·4 ml. volumes of dilutions, arranged so that plaque counts ranged between 20 and 150 per plate, were assessed in either 2 or 4 plates per dilution depending on the availability of cells. Where the level of viability was below 1 % the number of plaques on plates inoculated with undiluted impinger fluid ranged between 1 and 50. Results were expressed as plaque forming units/ml.

RESULTS

Suspensions were usually titrated before and after spraying to determine whether refluxing in the atomizer resulted in any change in titre. With vaccinia, influenza and poliomyelitis no significant differences were found. The results with VEE were not accurate enough to detect small changes in titre.

Tests with airborne viruses were carried out at several R.H. levels at each of the three temperature ranges studied. Most test conditions were used on two or more occasions for each virus. Table 1 shows the arithmetic means of results from replicate tests along with the results from single runs which could not be repeated because the apparatus and holding room were being rebuilt.

DISCUSSION

Aerosol viabilities reported in this paper are based on the ratio of virus titre to radioactive count in suspension and cloud samples, and can be criticized on the ground that test and tracer materials were not physically identical (Harper *et al.* 1958). However, the high proportion of initial cloud viabilities around 100 %, absence of negative viable decay (i.e. measured viability increasing as holding time increases), and the finding of viabilities over 50 % in clouds 23 hr. old, all suggested that physical decay rates of test and tracer materials (in the size range collected by the impinger) were not very different. Dimmick (1960) found no significant differences between the physical decay rate of bacterial aerosols traced with either labelled bacteria or ^{32}P solution. In any event the magnitude of the differences in viable decay rates in different holding conditions were such that the general conclusions reached remain valid.

When viability is measured in aerosols about 1 sec. after the wet droplets have mixed with 25 volumes of secondary air, it is a measure of the resistance of the virus to the rapid changes taking place during equilibration with the test atmosphere. With vaccinia and influenza virus initial viabilities were high in all the test conditions; analysis of variance showed that initial viability was not dependent on either temperature or R.H. With VEE and poliomyelitis, where the less precise results were not tested statistically, there was a tendency for initial viability to be dependent on both temperature and R.H., suggesting that these viruses were more sensitive to the process of becoming airborne.

The influence of temperature on viable decay in stored aerosols was similar for three viruses (VEE, vaccinia and influenza; poliomyelitis was tested at only one temperature); all survived better at the lowest temperature tested. In this respect

the viruses behaved like most bacteria so far examined. The influence of R.H. on stored clouds was also similar for these three agents; all showed better survival at the lowest R.H. (17–25 %) tested. Poliomyelitis showed the opposite picture, survival being best at the highest R.H. (80–81 %) tested. The opposite influence of R.H. on the survival of influenza and poliomyelitis viruses has been reported by Hemmes *et al.* (1960).

With influenza and poliomyelitis viruses the influence of R.H. was examined in more detail in the temperature range 21–24° C. There was a sudden increase in the viable decay rate of influenza virus when the R.H. was raised above 35 %. At R.H. of 50, 65 and 80 %, viable decay of this virus proceeded at closely similar rates. However with poliomyelitis virus, where the influence of R.H. was reversed, the sudden increase in viable decay rate when the R.H. was lowered to 50 % was followed by improved survival at R.H. of 35 % and 20 %, though viable decay rates were still more rapid than at R.H. above 50 %. Hemmes *et al.* (1960), working with these two viruses, showed increased viable decay rates around R.H. 50 % but did not find any improvement in decay rates of poliomyelitis virus at lower R.H.

The results in Table 1 show that airborne viral particles remain viable for a considerable time in favourable conditions, and that these conditions are not the same for the four viruses tested so far. Tests with other viruses may show that there is as much variety in their sensitivity to R.H. as with bacteria.

Two factors which could modify the results reported here are: (*a*) the method of preparing suspensions, and (*b*) the influence of light. Unpublished work in this establishment has shown that the influence of R.H. on the viable decay of some airborne bacteria can be very much modified by the use of different growth media in preparing suspensions. The response of viruses to R.H. may also be susceptible to modifications by the use of different growth conditions and suspending fluids, but this has not been tested. No reference to the influence of light on the viability of airborne viruses has been found, but Skinner & Bradish (1954) have reported the sensitivity of some virus suspensions to exposure to light during laboratory assays. It seems possible that light may be a potent modifying factor in the survival of airborne viruses as is the case with bacteria. The tests reported here were all carried out in the dark.

SUMMARY

Airborne virus particles of vaccinia, influenza, Venezuelan equine encephalomyelitis, and poliomyelitis have been tested for viable survival in the dark at controlled temperatures and relative humidity (R.H.) for up to 23 hr. after spraying.

Viable survival at each R.H. level was better at lower temperature than at higher temperature. Poliomyelitis virus showed best survival at high R.H.; the other three viruses survived best at low R.H.

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REFERENCES

CLARKE, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed. p. 214. London: Baillière, Tindall and Cox.
COOPER, P. D. (1955). *Virology*, **1**, 397.
DIMMICK, R. L. (1960). *J. Hyg., Camb.*, **58**, 373.
EDWARD, D. G. FF., ELFORD, W. J. & LAIDLAW, P. P. (1943). *J. Hyg., Camb.*, **43**, 1.
FAZEKAS DE ST GROTH, S. & WHITE, D. O. (1958). *J. Hyg., Camb.*, **56**, 151.
GOLDBERG, L. J., WATKINS, H. M. S., BOERKE, E. E. & CHATIGNY, M. A. (1958). *Amer. J. Hyg.* **68**, 85.
HARPER, G. J., HOOD, A. M. & MORTON, J. D. (1958). *J. Hyg., Camb.*, **56**, 364.
HEMMES, J. H., WINKLER, K. C. & KOOL, S. M. (1960). *Nature, Lond.*, **188**, 430.
HENDERSON, D. W. (1952). *J. Hyg., Camb.*, **50**, 53.
LESTER, W. (1943). *J. exp. Med.* **88**, 361.
LOOSLI, C. G., LEMON, H. M., ROBERTSON, O. H. & APPEL, E. (1943*a*). *Proc. Soc. exp. Biol., N.Y.*, **53**, 205.
LOOSLI, C. G., ROBERTSON, O. H. & PUCK, T. T. (1943*b*). *J. infect. Dis.* **72**, 142.
MAGNUS, P. VON (1951). *Acta path. microbiol. Scand.* **28**, 278.
MAY, K. R. & HARPER, G. J. (1957). *Brit. J. industr. Med.* **14**, 287.
SHECHMEISTER, I. L. (1950). *J. Infect. Dis.* **87**, 128.
SKINNER, H. M. & BRADISH, C. J. (1954). *J. gen. Microbiol.* **10**, 377.
VEALL, N. (1948). *Brit. J. Radiol.* **21**, 347.
WESTWOOD, J. C. N., PHIPPS, P. H. & BOULTER, E. A. (1957). *J. Hyg., Camb.*, **55**, 123.