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Inactivation of Respiratory Syncytial Virus in Aerosol

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SUMMARY

Aerosols of respiratory syncytial virus were kept at different relative humidities (RH). Viability was studied by a plaque method. Virus recoveries 1 min. after aerosolization were highest at high RH. The stability of the aerosol was maximal at 60 % RH. The total decay after 61 min. varied from 1·1 log. p.f.u. to 2·5 log. p.f.u., with peak values at 30 % RH (2·3 log. p.f.u.) and 80 % RH (2·5 log. p.f.u.), suggesting two processes with different kinetics. Oxidation was excluded as a cause of inactivation by experiments in pure nitrogen.

INTRODUCTION

The inactivation of bacteria and viruses in aerosols is dependent on many factors. The relative humidity (RH) of the ambient air is often very important. The composition of the spray fluid is a second factor, sometimes in relation to RH.

Respiratory syncytial virus, an important respiratory tract pathogen, was aerosolized from partially purified suspensions. The inactivation in air at 20·5° was determined at various relative humidities.

METHODS

Preparation of the virus suspension

A lyophilized stock of the LONG strain of respiratory syncytial virus was prepared with 50 % (v/v) skimmed milk as a stabilizer. All ampoules contained virus of the same passage, grown under the same conditions. Virus from this stock was passed twice in HeLa cells before use in the experiments. The virus was grown in Eagle's medium with the addition of 0·14 % $NaHCO_3$, 3 % calf serum and antibiotics; the pH was about 7·4. The freshly harvested culture fluid was clarified by low-speed centrifugation and sedimented in the ultracentrifuge (Spinco model L, rotor type 50, 60 min. 50,000 rev./min.). The pellets from 100 ml. were resuspended in 1·2 ml. of distilled water. The Na^+ concentration of this suspension was 5·4 mM, indicating a 23-fold reduction of the content of non-virus soluble material as compared to the original suspension. One ml. contained 2 mg. of protein and 0·28 mg. of nucleic acid.

Generation and sampling of the aerosol

Exactly 1 ml. of this partially purified suspension was aerosolized into a static system with a FK 8 direct type atomizer with an airstream under a pressure of 5 atmos.

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Under these conditions about 10^{10} droplets with a mean diameter of 4·9 μm and with less than 10 % of them greater than 10 μm were generated, as determined by the absolute method of May (1945). The virus suspensions contained about 10^7 p.f.u./ml. and always less than 10^8 p.f.u./ml.; the aerosols thus contained very few droplets with more than one virus particle. The virus suspension was titrated just before aerosolization.

The static system consisted of a stainless steel cylindrical drum with a capacity of 2000 l. The temperature of the air in the drum was kept at 20·5° by means of a water-jacket. Air, withdrawn by sampling, was automatically replaced by sterile air of the same RH and temperature. The temperature and the RH of the air in the drum were recorded continuously and were constant within ±0·2° and ±2·0 % respectively. In this system the physical fallout was as defined earlier by Strasters & Winkler (1966). With suspensions of bacterial spores the fallout was 0·1 log./hr which is negligible in comparison with the effects discussed in the present work.

The aerosol was sampled with a modified multistage liquid (MSL) impinger (May, 1966). As most of the virus was found in the bottom compartment of the MSL impinger and only very small quantities were collected in the upper parts, a one stage impinger with the dimensions of the bottom compartment of the MSL impinger was used. Samples of 100 litres of air were taken at 0 min. 20 sec., 15 min. 20 sec., 30 min. 20 sec., 45 min. 20 sec., and 60 min. 20 sec. after aerosolization. A critical capillary in the suction line was used to provide a constant airflow of 55 l./min. through the impinger, the sampling time being 109 sec. The impinger was filled with 10 ml. of sterile phosphate buffered saline. No antifoam agent was added. The impinger fluids were titrated immediately after sampling. The same impinger and critical capillary were used throughout the work.

Titration of virus infectivity

Virus was titrated by plaque assay. HeLa cells were grown in medium containing 0·5 % lactalbumin hydrolysate, 0·056 % sodium bicarbonate, 15 % inactivated calf serum and antibiotics in Hanks's salt solution, pH about 7·4. Glass Petri dishes with a diameter of 6 cm. were seeded with 8 ml. growth medium containing 2 to 2·5 × 10^6 cells and incubated in a humid atmosphere with 5 % CO_2. After 2 days the medium was changed. On the third day the medium was removed and the monolayers were inoculated with 0·3 ml. (ten drops from standardized Pasteur pipettes) of the virus suspensions diluted in phosphate buffered saline. Immediately thereafter the dishes were placed in the CO_2 incubator for one hour at 37°. The overlay medium, consisting of 0·5 % lactalbumin hydrolysate (NBC), 5 % inactivated calf serum, 0·1 % yeast extract (Difco), 0·11 % sodium bicarbonate, 0·7 % agarose (l'Industrie Biologique Française S.A.), and antibiotics in Hanks's salt solution (Oxoid), was kept in tubes with 8 ml. in a waterbath at 42°. Immediately after the adsorption period the contents of the tubes were poured cautiously on the monolayers. After solidification of the agar the dishes were sealed in 'Cellotheen' bags (Union Chimique Belge, Ghent) and incubated upside down at 37°. A second overlay was added after 5 days. After 11 days 5 ml. of an overlay medium containing 0·01 % neutral red, 0·8 % agarose and antibiotics in Hanks's salt solution was poured in the dishes. On the 12th day plaques varying in diameter from 0·5 to 2 mm. had developed. The contrast with the normal cell sheet allowed counting by eye. Significant confluency occurred only in dishes

showing more than 300 plaques. After another day at room temperature the plaques were counted again. An increase of the plaque number of up to 10 % was sometimes found.

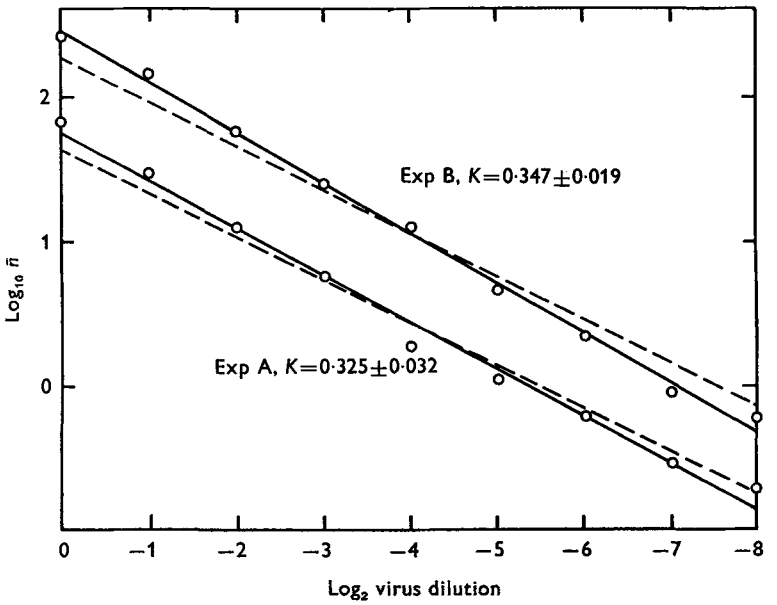

Fig. 1. Relationship between plaque count and virus dilution when the same pipette (expt B) or different pipettes (expt A) are used for inoculation of the successive dilutions. The calculated regressions are drawn as full lines. The theoretical relationship for plaque count inversely proportional to dilution is drawn as interrupted lines. $\bar{n}$ = mean count of ten dishes. K = regression constant with confidence limits.

To test the accuracy of the assay, nine twofold dilutions of virus suspensions A and B were titrated using five Petri dishes per dilution. Both experiments were performed in duplicate. In both experiments a straight line with a regression near the theoretical value of $K = 0{\cdot}301$ was fitted to the data, showing that the plaque count was inversely proportional to the dilution (Fig. 1). The narrower range of K in experiment B was due to more rigid standardization of the inoculated volumes. The standard deviation was less than 20 % for counts between 5 to 50 plaques, less than 10 % for higher counts.

In the actual aerosol experiments three serial tenfold dilutions in PBS were inoculated on five to ten dishes each. For practical reasons different pipettes were used. The virus content was calculated from the mean plaque count for the dilution showing the highest acceptable number of plaques. Infectivities were expressed as log. p.f.u./ml.

Calculations

The maximum number of p.f.u. theoretically present in 100 l. of air immediately after aerosolization (n_0) was calculated from the initial infectivity of the 1 ml. of virus suspension aerosolized completely into 2000 l. of air. The number of p.f.u. present in 100 litres of air at the mid-time of each sampling period (n_t; t = 1, 16, 31, 46 or 61 min.) was calculated from the infectivities of the impinger fluids. A correc-

tion was used for dilution of the cloud by previous withdrawal of samples. Evaporation of impinger fluids appeared to vary from 0·4 to 1·0 ml. per sample according to the RH of the air. The results were not corrected for this factor, which did not influence the inactivation rate.

RESULTS

Aerosol stability was investigated at 20, 30, 40, 50, 60, 70, 80 and 90 % RH. Five to nine experiments were performed at every RH tested (Fig. 2).

At 20, 40, 50 and 60 % RH straight inactivation lines were found. At 30, 70, 80 and 90 % RH the inactivation rates appeared to decrease with time, especially from 1 to 16 min. after aerosolization.

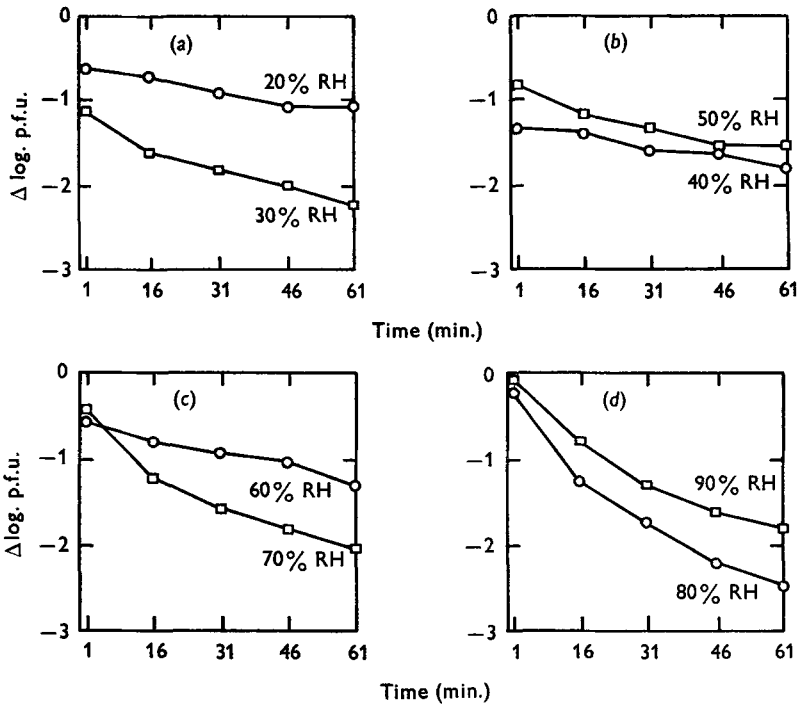

Fig. 2. Graphs (*a*—*d*). Mean inactivation of RS virus in aerosols at different relative humidities (RH). The inactivation (Δlog p.f.u.) at each time is expressed as the log. difference between the maximum virus content of the air at time zero and that shown by sampling at the indicated time. The former is calculated from the initial infectivity of the virus suspension aerosolized.

Mean inactivation rate constants for the period from 1 to 61 min. (K_{1-61}) and for the period from 16 to 61 min. (K_{16-61}) were calculated from the individual data (n_1-n_{61}) by the method of least squares. K is expressed as Δlog. p.f.u./hr. From n_0 and n_1 the mean inactivation in the first minute (v_{0-1}) was calculated as Δlog. p.f.u. (Table 1).

As v_{0-1} was negligible at 90 % RH, mechanical inactivation by spraying, which will be independent of RH, could not account for the considerable inactivation which occurred in the first minute at the lower relative humidities. A decrease of the efficiency

of collection at a lower RH cannot explain more than the initial inactivation which is found at 20 % RH. Therefore, it may be concluded that the peak value of v_{0-1} at 30 to 40 % RH was caused by some other mechanism, apparently inactivation in air.

The mean inactivation rate constants showed a definite peak at 80 % and a smaller, second peak at 30 % RH. This was so also when only the inactivation from 16 to 61 min. was considered (Table 1).

These kinetics suggested two mechanisms of inactivation; one causing a maximal loss of activity at 30 to 40 % RH within the first minute after aerosolization and another, maximal at 80 % RH, having a smaller but more prolonged effect.

In order to exclude oxidation as a cause of these hypothetical mechanisms, two experiments were performed at 30 and 70 % RH in an atmosphere of pure nitrogen. Under these conditions v_{0-1} and k_{1-61} did not differ significantly from the mean values in air at these relative humidities (Table 1).

Table 1. *Inactivation of respiratory syncytial virus aerosolized in air at different relative humidities (RH)*

RH (%)	No. of experiments	$v_{0-1} \pm$ SD*	$k_{1-61} \pm$ SD†	$k_{16-61} \pm$ SD‡
20	5	0·62 ± 0·07	0·50 ± 0·13	0·47 ± 0·09
30	6	1·12 ± 0·22	**1·07** ± 0·13	**0·86** ± 0·10
	(1)	(1·25)	(0·91)	
40	5	**1·33** ± 0·13	0·59 ± 0·10	0·66 ± 0·09
50	5	0·84 ± 0·18	0·77 ± 0·25	0·61 ± 0·20
60	6	0·55 ± 0·15	0·70 ± 0·07	0·64 ± 0·11
70	9	0·48 ± 0·12	1·47 ± 0·22	1·03 ± 0·28
	(1)	(0·56)	(1·46)	
80	5	0·22 ± 0·09	**2·20** ± 0·17	**1·49** ± 0·22
90	6	0·09 ± 0·11	1·72 ± 0·09	1·35 ± 0·10

* v_{0-1} Mean loss of activity (Δlog. p.f.u.) in the first minute after aerosolization.
† k_{1-61} Mean inactivation rate constant (Δlog. p.f.u./hr) from 1 to 61 min. after aerosolization.
‡ k_{16-61} Mean inactivation rate constant (Δlog. p.f.u./hr) from 16 to 61 min. after aerosolization.
Peak values are in bold type. The data given in parentheses refer to experiments in a nitrogen atmosphere.

DISCUSSION AND CONCLUSIONS

Aerosol studies have been performed with a number of myxoviruses and paramyxoviruses. For influenza virus, strain PR8, maximal decay rates were found at over 50 % RH by Hemmes, Winkler & Kool (1960) and Harper (1961). Hood (1963) reported maximal inactivation at 50 % RH for strains PR8 and SINGAPORE, the decay rates being 0·8 and 0·6 log./hr respectively. For Newcastle disease virus the maximal decay rate (1·4 log./hr) was found at 35 % RH (Songer, 1967). Measles virus was investigated by de Jong & Winkler (1964) and for this virus maximal inactivation in air (3·1 log./hr) occurred at 68 % RH. For parainfluenza virus type 3, a maximal decay rate of 1·1 log./hr was reported at 80 % RH (Miller & Artenstein, 1967). In these studies spray fluids of different compositions were used. Often the inactivation was studied only at three different relative humidities and the effects of small changes in relative humidity may have escaped observation.

In the present work, the purified suspensions had a low protein content. This may account for the high RH-optimum found; a shift of maximal inactivation to higher

RH was found when serum was excluded from the spray fluid in studies with poliomyelitis virus (de Jong & Winkler, 1968).

A second optimum due to a distinct inactivation process has not been reported previously. This low RH-optimum may be a peculiarity of respiratory syncytial virus.

The present results do not indicate the mechanisms of inactivation although oxidation as a cause of the inactivation of respiratory syncytial virus in air may be excluded.

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