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The inactivation of poliovirus in aerosols

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INTRODUCTION

Many viruses are far less stable in aerosols than in aqueous solutions. For poliovirus the kinetics of the decay in air have been described by Hemmes, Winkler & Kool (1960, 1962) and Harper (1961, 1963). The mechanism, however, remained obscure.

The only pertinent theory on virus death in air is that of Webb, Bather & Hodges (1963). They developed from indirect evidence the concept that the primary event, causing the decay of bacteria and viruses in air, is the withdrawal of stabilizing water molecules from the nucleic acid of the organism. The nucleic acid structure then collapses and irreversible secondary reactions may occur.

We have tried to obtain direct evidence on the mechanism of the decay of poliovirus in aerosols by studying among other things the biological activity of infectious RNA extracted from aerosolized poliovirus.

MATERIALS AND METHODS

Virus

Poliovirus type 1, strain LSc2ab, was grown and titrated by the plaque method on monolayers of human amnion cells, line U (Doorschodt, 1961). The growth medium consisted of lactalbumin hydrolysate 0·5 % and calf serum 5 % in Hanks's balanced salt solution. The virus was concentrated 20–200 times by means of ultracentrifugation. Before spraying, the virus was diluted 1/2 in Dulbecco's phosphate buffered saline (PBS) containing 1 % peptone.

Infectious RNA (iRNA) was extracted, after concentration by ultracentrifugation, from the collection fluids with the conventional phenol method (Alexander, Koch, Mountain & van Damme, 1958). The extracts contained 1·5 M-NaCl and had a pH of 8. With these extracts human amnion cell monolayers were infected and processed for plaque formation.

The aerosol equipment

Virus suspensions of 1 ml. were sprayed directly in 4 sec. with an atomizer of the type FK 8 by a stream of pure nitrogen. The diameter of the produced droplets ranged from 3 to 5 μ with 10 % larger than 10 μ. The mean 'physical fall-out' corresponded to a K-value (see below) of 0·1 per hr. and was neglected

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in the calculations of biological decay. The aerosols were generated in air at 20° C. in an experimental room of 2000 l. schematically represented in Fig. 1.

Air samples were taken with conventional capillary impingers (Rosebury and others, 1947) at a rate of 10 l./min. Sampling time was 5 min.; the first sample started 1 sec. after the end of the spraying period. As a collection fluid 10 ml. PBS with 1 % peptone (Difco Bacto-peptone) and 0·1 % antifoam AF (Dow Corning Corp. U.S.A.) was used.

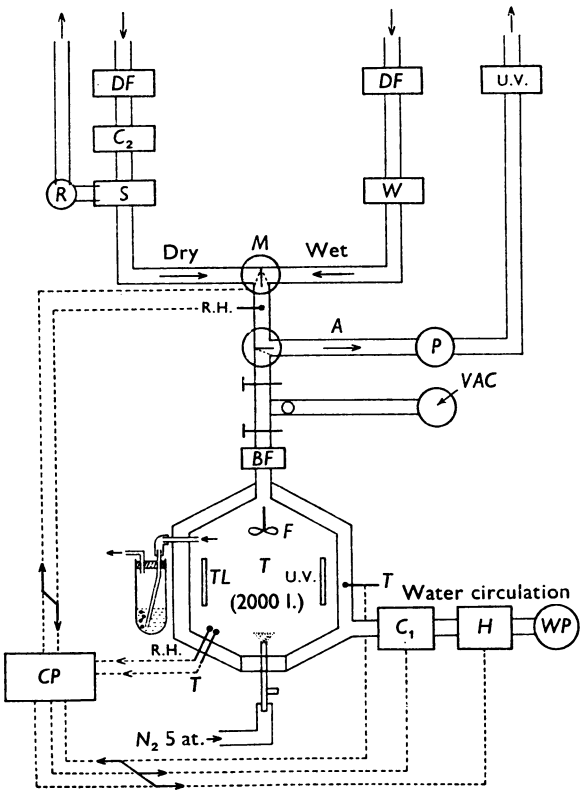

Fig. 1. Scheme of apparatus for producing, storing and sampling of aerosols. Tank T has a double wall, in which thermostatically heated water circulates to maintain a temperature of 20° C. (pumping by WP, heating by H, cooling by C_1). By means of pump P fresh air (filtered by dustfilters DF) is dried over silicagel S (to regenerate with R) or humidified over a water surface W. These two streams are mixed in the mixing valve M, giving an air stream A of the desired relative humidity (R.H.). After evacuation, the tank can be filled with sterile air of the required R.H. through the bacteria retaining filter BF. The continuous air stream A also replaces the air withdrawn during sampling. The composition of the air in the tank is kept uniform by a fan F and can be sterilized by ultraviolet radiation. Temperature and R.H. are recorded and regulated automatically by the control panel CP. The apparatus was designed by Dr K. C. Strasters and Prof. Dr K. C. Winker and built by 'Lucht- en Droogtechniek N.V.' in Rotterdam.

RESULTS

Influence of the relative humidity on the decay rate

An organism sprayed in air is exposed to the stress of spraying, to the quick evaporation till the droplets are in equilibrium with the ambient air and to the decay in the stable aerosol during storage. The mechanisms of inactivation during these three phases might differ. For this reason the inactivation during the first minutes (till the end of the first sample) is treated separately from the loss during storage of the stable aerosol.

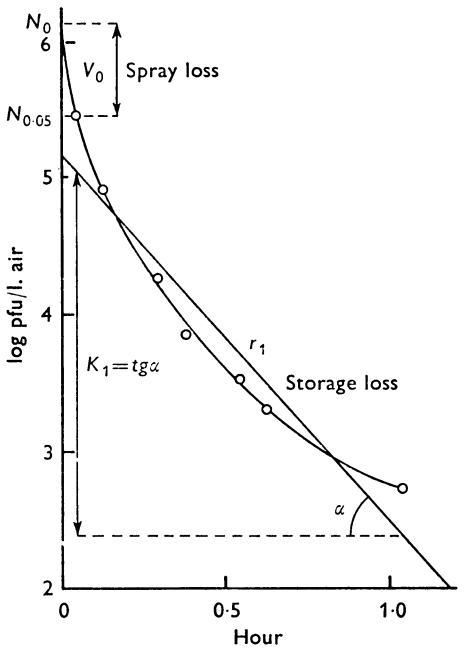

Fig. 2. Inactivation of poliovirus in air with 45% relative humidity. Symbols as in Fig. 3; r_1 = regression line over the first hour.

Figure 2 shows the outcome of a typical experiment and demonstrates the symbols used in this report. The spray-loss V_0 is defined as the logarithm of the quotient of the calculated virus concentration in the aerosol (no decay) and the actual concentration as deduced from the titre of the first sample. The rate of loss during storage, K, is recorded as the logarithm of the concentration reduction factor over an hour.

K-values were calculated with the method of least squares from the experimental titres. As the decay rate is slowing down with time, K is dependent on time. Nevertheless, the K-values of experiments with the same sampling schedule can be used for the comparison of inactivation rates under different conditions.

The influence of relative humidity (R.H.) is summarized in Fig. 3, in which spray-loss V_0 and storage-loss-rate K are plotted against R.H.

The spray loss was large at low R.H. and decreased with rising humidity, being

minimal above 55% R.H. The storage-loss-rate was low below 35% and above 70% R.H. but high between 40 and 55% R.H. This range of maximal storage-loss-rate coincides with the range of decrease of the spray-loss.

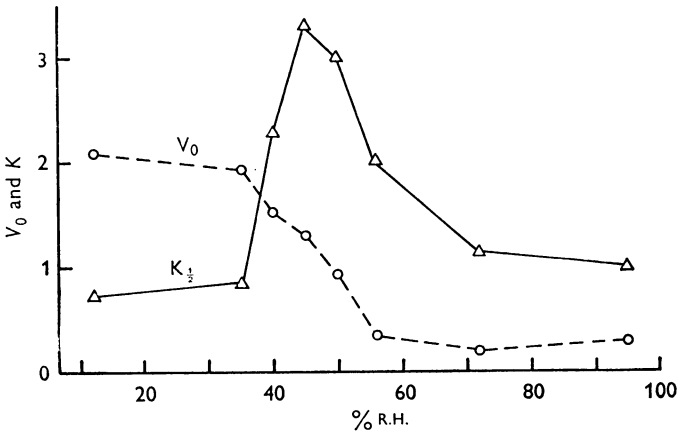

Fig. 3. Inactivation of poliovirus in air in relation to the relative humidity (R.H.). $V_0 = \log N_0 - \log N_{0\cdot04}$ with N_0 = calculated titre (no decay) and N_t = actual titre after t hr. $K_t = (\log N_{0\cdot04} - \log N_t) \times 1/(t - 0\cdot04)$.

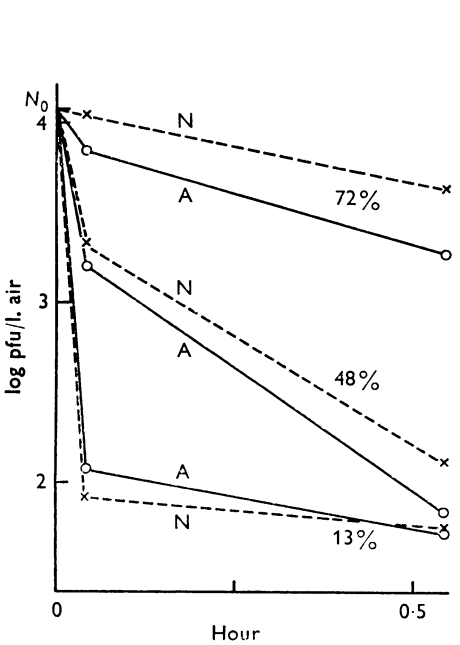

Fig. 4

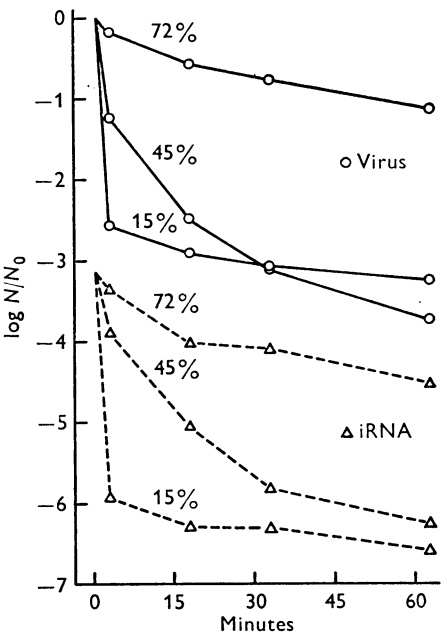

Fig. 5

Fig. 4. Inactivation of poliovirus in nitrogen (N) and in air (A) at 13%, 48% and 72% R.H.; pfu = plaque-forming units.

Fig. 5. Comparison of the decay of poliovirus in air with the inactivation of the RNA in the virus particles. The mean titres of four experiments are plotted against time.

Influence of oxygen

To determine the role of oxidation, poliovirus was nebulized and stored in a nitrogen atmosphere. In Fig. 4 the titres are plotted against time for corresponding experiments in nitrogen (0·1 % oxygen) and in air.

No significant difference was noted between the inactivation of poliovirus in the two gases. Therefore, oxygen is not an important factor in the decay of the virus.

Inactivation of the RNA in the poliovirus particle

Batches of virus containing 3×10^9 pfu/ml. were sprayed in air of low (15–18 %), moderate (45 %) or high (71–72 %) R.H. All aerosols were sampled from 0 to 5, 15 to 20, 30 to 35 and 60 to 65 min. after nebulization. The impinger fluids were titrated for whole virus and for infectious RNA (iRNA).

Table 1 and Fig. 5 represent the results. In Table 1 the 'nucleic acid extraction and infection efficiency' (N.E.) is calculated as the quotient of the titres of iRNA and virus. The titres plotted in Fig. 5 are the mean titres of the four experiments given in Table 1.

Table 1. *Inactivation in air of poliovirus and of the RNA within the virus particles*

	15–18 % R.H.			45 % R.H.			71–72 % R.H.		
	$K_1(V)$*	$K_1(R)$†	N.E.‡	$K_1(V)$	$K_1(R)$	N.E.	$K_1(V)$	$K_1(R)$	N.E.
1	0·7	—	—	1·9	3·3	5×10^{-4}	0·7	1·4	4×10^{-4}
2	0·8	0·7	16×10^{-4}	2·1	2·2	15×10^{-4}	1·0	1·1	15×10^{-4}
3	0·5	0·5	7×10^{-4}	2·5	1·7	6×10^{-4}	1·1	0·7	4×10^{-4}
4	0·5	0·7	9×10^{-4}	2·7	2·3	7×10^{-4}	0·7	0·8	9×10^{-4}
Mean	0·62	0·62	$9{\cdot}9 \times 10^{-4}$§	2·29	2·37	$8{\cdot}6 \times 10^{-4}$	0·90	1·02	$7{\cdot}8 \times 10^{-4}$

* $K_1 = -\Delta$ log titre per hour calculated with the method of least squares from the four observed titres of the first hour. $K_1(V) = K_1$ of the whole virus.

† $K_1(R) = K_1$ of the infectious RNA (iRNA).

‡ N.E. = 'nucleic acid extraction and infection efficiency' = titre iRNA : titre virus; with not-sprayed virus the N.E. amounts to $5{\cdot}9 \times 10^{-4} \pm 0{\cdot}6 \times 10^{-4}$.

§ The high mean values of N.E. are due to Expt. 2.

Table 1 and Fig. 5 show a close parallelism between the inactivation of the whole virus and the iRNA. Also the N.E. does not differ from that of unaerosolized virus.

Koch (1960) studied the thermal inactivation of poliovirus in fluid media and described that after inactivation at 54° C. many apparently uninfective particles were still infective in hypertonic medium. Hence we titrated our impinger fluids by infecting the monolayer cells in hypertonic as well as in isotonic medium. No significant difference was observed in our system, indicating that few particles with a missing or damaged protein coat and intact RNA were present.

The results obtained suggest that the virus decay is due to inactivation of the RNA.

Influence of preincubation with L-*cystine*

Preincubation with L-cystine protects poliovirus against inactivation at 50° C. (Pohjanpelto, 1958). The amino acid is thought to react with SH-groups of the virus protein, thus stabilizing its structure. Consequently we investigated the decay of poliovirus in air using virus incubated with 50 (occasionally 500) μg L-cystine/ml. for 15 hr. at 37° C. and pH 8.

For these experiments a batch of poliovirus was grown in Hanks's balanced salt solution in the absence of lactalbumin hydrolysate and serum (which contain L-cystine): 'virus-H'. This virus indeed responded to stabilization against heating at 50° C. by L-cystine approximately as reported by Pohjanpelto. Before and after incubation with the amino acid the inactivation factor was $10^4\times$ and $2\times$ respectively after 5 min. at 50° C.

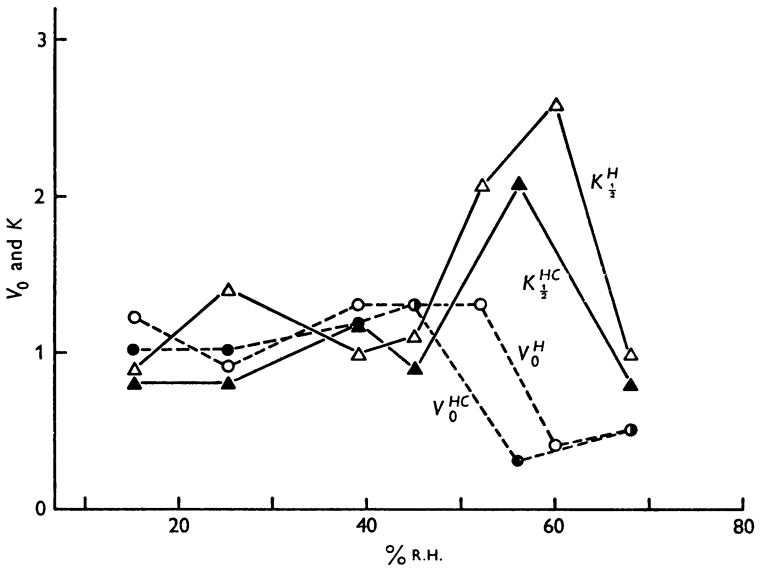

Fig. 6. Decay in air of poliovirus grown in Hanks's balanced salt solution before (*H*) and after (*HC*) incubation with 50–500 μg. L-cystine/ml. for 15 hr. at 37° C. and pH 8. Symbols as in Fig. 3.

After dilution 1/2 in PBS both virus preparations were subsequently tested for their stability in air (see Fig. 6).

Treatment with L-cystine apparently does not change the rate of inactivation of poliovirus in air with varied R.H. This observation is in accordance with our hypothesis that protein is not primarily involved.

DISCUSSION

The kinetics

The relation between virus inactivation and R.H., as depicted in Figs. 3 and 6, is characterized by (1) a maximal spray-loss at low R.H., (2) stability of the

virus after spraying in dry and in wet air, but quick inactivation at moderate humidity, and (3) coincident increase of the K-value with the decline of V_0.

These results confirm the reports of Hemmes *et al.* (1960, 1962) and Harper (1961, 1963). They are also consistent with a tentative rule, deduced from published data on the survival of viruses in aerosols. This rule states that, after spraying in protein-containing suspensions, lipid-free viruses decay with a higher rate at relatively low R.H.'s, whereas lipoviruses die off quickest in relatively wet air (de Jong, 1967); this rule was already suggested by Buckland & Tyrrell (1962) on the basis of experiments with virus suspensions dried on glass slides.

Further, facts 1 and 2 suggest that the virus is inactivated during the desiccation process but is stable when equilibrium is reached, whatever the degree of water loss. At first sight this seems at variance with the phenomena at moderate R.H. However, in these aerosols the inactivation rate decreases with time and eventually levels off to low values, suggesting that the virus is again stable at equilibrium. Although the rate is slowed down, inactivation continues longer, extending beyond the 5 min. of the first sample. This of course implies a decrease of spray-loss with a corresponding increase of storage-loss. These results would consequently fit into the hypothesis of inactivation during dehydration, provided that evaporation of the aerosol droplets and virus particles to equilibrium conditions could be such a slow process. In this context equilibration means equilibrium between the water vapour in the air, the (bound) water in the droplet or droplet nucleus, and the bound water of the virus particle itself. In the authors' knowledge there are no data available about this process in aerosols.

The presence of serum in the spray fluid will slow down evaporation owing to protein hydration. Maximal K-values will then occur at lower R.H. than in the absence of serum. This is indeed what happens as can be seen by comparing Figs. 3 and 6.

The mechanism

Our experiments provide evidence that the virus nucleic acid is primarily affected. Which process is induced or enhanced by desiccation is not known.

Oxidation is an important factor in the thermal inactivation of poliovirus in aqueous suspension at 50° C. (Pohjanpelto, 1958) but not at 37° C. or lower (Lund & Lycke, 1961; Pohjanpelto, 1962). The experiments (at 20° C.) shown in Fig. 4 exclude oxygen as a harmful agent in air.

Though chemical reactions with medium components cannot be ruled out, denaturation seems the most probable mechanism. This is consistent with thermodynamic data (see Table 2).

The low changes of enthalpy and entropy are characteristic for a single chemical event such as the denaturation of single-stranded RNA and exclude protein denaturation as the first event of inactivation. For poliovirus no data are available, however.

If inactivation of poliovirus in aerosols is a denaturation of the RNA, analogy can be suspected to the thermal decay in aqueous solution, as recently analyzed by Dimmock (1967). Dimmock clearly demonstrated that above 44° C. the antigen

structure of poliovirus changes from type 'N' to type 'H' whereas the amount of extractable infectious RNA is unaffected. On the other hand, below 44° C. only the titre of iRNA decreases on prolonged heating, whereas the N-antigen remains intact. In accordance with these observations L-cystine protects poliovirus completely against inactivation at 50° C., very poorly at 37° C. and not at all at 32° C. (Pohjanpelto, 1962).

Table 2. *Activation-enthalpy and -entropy at several modes of virus inactivation*

	ΔH*	ΔS†	ref.‡
Inactivation lipid free viruses in air	8·3	−42	1
Denaturation single stranded virus RNA	19 to 23	−11 to −19	2
Denaturation protein	33 to 200	19 to 537	3
'Low-temperature' heat inactivation poliovirus	28	7	4
'High-temperature' heat inactivation poliovirus	244	689	4

* ΔH = change in enthalpy in kcal./mol.
† ΔS = change in entropy in cal./mol. °K.
‡ References: (1) own calculations; (2) Ginoza *et al.* (1964) with TMV and phage R 17; (3) Woese (1960); (4) Dimmock (1967).

Our findings agree with the phenomena of heat inactivation at low temperature. We suggest that the decay of poliovirus in aerosols proceeds by the mechanism of thermal inactivation of the 'low temperature type', accelerated by dehydration. The accelerating effect of desiccation is intelligible in terms of the concept of stabilizing water molecules. When such a stabilizing water molecule is removed the chance of its replacement by another water molecule is less likely in a dry environment.

SUMMARY

Poliovirus type 1, strain LSc2ab, was directly sprayed in a static air cabinet at 20° C. and sampled afterwards with impingers. Virus inactivation during spraying was maximal in dry air; during storage maximal decay occurred at moderate humidity.

With regard to the mechanism of the inactivation the following facts have been observed: (1) The RNA in the virus decays simultaneously with the whole virus particle. (2) Oxidation does not play any significant role. (3) Incubation with L-cystine protects the virus against inactivation at 50° C. in aqueous suspension, but not against decay in aerosols.

On the strength of these observations it is concluded that in aerosols denaturation of the viral RNA is the cause of the inactivation of poliovirus. The decay in aerosols has some features in common with the thermal inactivation in aqueous suspensions at room temperature.

The technical assistance of Mr M. Harmsen is gratefully acknowledged.

REFERENCES

ALEXANDER, H. E., KOCH, G., MOUNTAIN, I. M. & VAN DAMME, O. (1958). Infectivity of ribonucleic acid from poliovirus in human cell monolayers. *J. exp. Med.* **108**, 493.

BUCKLAND, F. E. & TYRRELL, D. A. J. (1962). Loss of infectivity on drying various viruses. *Nature, Lond.* **195**, 1063.

DIMMOCK, N. J. (1967). Differences between the thermal inactivation of picornaviruses at 'high' and 'low' temperatures. *Virology* **31**, 338.

DOORSCHODT, H. J. (1961). De betekenis van continue cellijnen voor de virologie. *Boerhaave cursus*, p. 20.

GINOZA, W., HOELLE, C. J., VESSEY, K. B. & CARMACK, C. (1964). Mechanisms of inactivation of single-stranded virus nucleic acids by heat. *Nature, Lond.* **203**, 606.

HARPER, G. J. (1961). Airborne micro-organisms: survival tests with four viruses. *J. Hyg., Camb.* **59**, 479.

HARPER, G. J. (1963). Some observations on the influence of suspending fluids on the survival of airborne viruses. *First Int. Symp. on Aerobiol., Berkeley* 1963, pp. 335.

HEMMES, J. H., WINKLER, K. C. & KOOL, S. M. (1960). Virus survival as a seasonal factor in influenza and poliomyelitis. *Nature, Lond.* **188**, 430.

HEMMES, J. H., WINKLER, K. C. & KOOL, S. M. (1962). Virus survival as a seasonal factor in influenza and poliomyelitis. *Antonie van Leeuwenhoek* **28**, 221.

DE JONG, J. C. (1967). Ph.D. Thesis, University of Utrecht.

KOCH, G. (1960). Influence of assay conditions on infectivity of heated poliovirus. *Virology* **12**, 601.

LUND, E. & LYCKE, E. (1961). The effect of oxidation and reduction on the infectivity of poliomyelitis virus. *Arch. ges. Virusforsch.* **11**, 100.

POHJANPELTO, P. (1958). Stabilization of poliovirus by cystine. *Virology* **6**, 472.

POHJANPELTO, P. (1962). Oxidation in thermoinactivation of poliovirus. *Virology* **16**, 92.

ROSEBURY, T. and others (1947). *Experimental Air-borne Infection.* Baltimore: Williams and Wilkins.

WEBB, S. J., BATHER, R. & HODGES, R. W. (1963). The effect of relative humidity and inositol on air-borne viruses. *Can. J. Microbiol.* **9**, 87.

WOESE, C. (1960). Thermal inactivation of animal viruses. *Ann. N.Y. Acad. Sci.* **83**, 741.