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Factors in the Inactivation of Encephalomyocarditis Virus in Aerosols

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Encephalomyocarditis virus in aerosols is inactivated rapidly at relative humidities below 50%. In glycerol-water mixtures a similar decrease of infectivity occurs when the glycerol concentration exceeds 78% (wt/wt), corresponding to a relative humidity of 50%. The decay in aerosols does not involve oxygen or surface-dependent factors. Variation of temperature shows the inactivation to be a low-energy process with an activation enthalpy of 15 kcal per mol. The damage could be ascribed to dehydration of the virion, presumably proceeding to removal of structurally essential water molecules. This might trigger irreversible changes in the protein coat, resulting in disintegration of the virion.

The mechanism of inactivation of viruses in aerosols is largely unknown. With certain bacteriophages, Trouwborst et al. showed the role of surface-dependent factors (25). Webb postulated removal of bound water molecules from the virion as the cause of inactivation (27).

Picornaviruses generally are most extensively inactivated at low relative humidity (RH) (1, 3, 6, 13). Several workers demonstrated that in this process the ribonucleic acid (RNA) of the virus retains its infectivity (6, 7, 10). In earlier studies it was shown that the decay of encephalomyocarditis (EMC) virus infectivity in aerosols at low RH is paralleled by changes in the protein coat. These changes lead to loss of hemagglutination activity, loss of antigenic structure, and extrusion of the RNA in an infectious form (6, 8). The trigger of this process is investigated in the present work.

Two mechanisms were studied: desiccation and surface-located inactivation. The first hypothesis was tested in a system involving dehydration only, namely, in glycerol-water mixtures. The second possibility was investigated by spreading a virus suspension as a thin film over a glass wall.

Other factors that were varied include temperature, presence of oxygen, composition of spray medium, and presampling rehumidification. Also, attempts were made to obtain a virus population with a higher aerosol stability.

MATERIALS AND METHODS

Virus techniques. Methods of growth and titration of EMC virus were described previously (8). To purify the virus it was sedimented from infected tissue culture suspensions in an ultracentrifuge. Further steps were butanol extraction (23), ECTEOLA-cellulose chromatography (17), CsCl gradient equilibrium centrifugation, and dialysis against distilled water. The recovered infectivity was about 5%. Bacteriophages MS2 and T3 were grown, purified, and titrated as in earlier studies (24, 26).

Glycerol, OED, and saliva. Glycerol (Merck, Darmstadt, bidistilled and p.a.) was dried over P_2O_5 and mixed with virus in phosphate-buffered saline (11). The glycerol concentration was determined refractometrically and the RH was calculated for the atmosphere in equilibrium with the glycerol solution concerned (16).

OED-70 paste was obtained from Nikken Chemicals Co., Ltd., Tokyo. It is a mixture of equal quantities of oxyethylene docosylether and oxyethylene octadecylether, containing a small amount of carboxymethylcellulose, and made up with water to a thick paste (20). The OED content of this paste was set on 100%. Immediately before use it was suspended in hot water by vigorous shaking, cooled and added to the virus suspension. Even in 1% OED, EMC virus was stable on standing for at least 1 h at room temperature. On spraying, OED forms a coat around the droplets (20).

Human saliva was used without antibiotics or any other treatment and had a pH of 7.2. A crude virus suspension was diluted ten-fold in the saliva before spraying. The virus infectivity in saliva was stable on standing at room temperature for several hours. Virus in saliva-containing fluids was titrated with the same plaque technique as virus in bacteria-free media.

Neutralization of EMC virus with antibodies. Rabbits were immunized with live EMC virus, concentrated 100 times by ultracentrifugation. A $1:10^4$ dilution of the rabbit serum obtained neutralized 50% of EMC virus infectivity, as measured by plaque formation. For aerosol experiments virus preparations containing 1.6×10^8 plaque-forming units per ml were mixed with 7 volumes of rabbit immune serum diluted 1.2×10^3. After incubation at 37 C for 30 min

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the virus titer was reduced to 50 plaque-forming units per ml. To reactivate the infectious virus the suspension in phosphate-buffered saline was cooled in ice water and mixed with 2 volumes of cold HCl solution (pH 1.7) to produce pH of 2.0. After 5 min at 0 C the acid was neutralized with 0.1 N cold NaOH. The acid treatment reduced the infectivity by 50%, and the combined antibody-acid procedure had a mean yield of 20% of the original infectivity.

Aerosol and related techniques. Aerosol techniques were described previously (8). From 1 ml of liquid the spray gun produced about 10^{10} droplets with a mean initial volume of 3,000 μm^3, corresponding to a diameter of 18 μm; 90% of the volume then is in droplets with diameters between 8 and 26 μm. The size distribution data were obtained with a modification of May's absolute method (18). One milliliter of neutral red in saturated LiCl solution was nebulized. During this period a glass object slide was exposed for a short time to the aerosol jet at a distance of 10 cm from the orifice of the gun. The slide was coated with a thin film of a mixture of 1 volume of vaseline and 3 volumes of paraffin oil. The included droplets were measured microscopically with an ocular micrometer. The aerosol was stored in a tank of 2,000-liter capacity.

The prehumidification apparatus was constructed after Cox (5), using a raised Porton impinger (19) for sampling. The rotating bulb test was described before (26). The activation enthalpy was calculated with the Eyring equation (28).

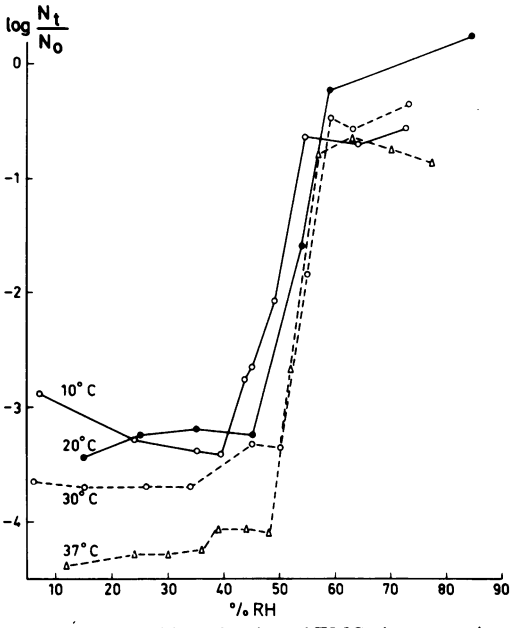

FIG. 1. *Aerosol inactivation of EMC virus at various temperatures. Virus was sprayed from Hanks balanced salt solution. Samples were taken from 30 to 35 min thereafter.*

RESULTS

Influence of RH, temperature, oxygen, and antibodies. The effect of changing temperature between 10 and 37 C on survival of EMC virus in aerosol was small (Fig. 1). Figure 1 also demonstrates that inactivation depended on relative rather than on absolute humidity: at all four temperatures tested the transition between low and high stability occurred from 45 to 60% RH; at any RH the absolute humidity at 37 C was five times higher than at 10 C. From survival-time curves the activation enthalpy for the first 5 min at 25% RH was estimated on about 15 kcal per mol, using Eyring's equation (28).

Figure 2 shows that the recovery at 20 C could not be raised by presampling rehumidification; the recovery of bacteriophage T3 responds to such a treatment (12) and is presented as a control of the technique. No different survival of EMC virus during the first 30 min after spraying was found when changing the atmosphere from air to nitrogen with 0.01% oxygen. Also, coating the virus with antibodies prior to atomization (see Materials and Methods) did not alter the aerosol decay curve. It could be argued that the virus-antibody complex could be disrupted by the physical stresses of spraying. Experiments at high RH, where virus is relatively stable, showed that such a dissociation indeed occurred but involved only 1% or less of the virus population.

Influence of medium components. Addition of 3% calf serum to the spray medium did not change the inactivation rate of EMC virus in aerosols. To investigate the role of components of the crude virus suspensions virus was purified. The general pattern of survival versus RH was the same before and after purification (Fig. 3). Quantitative differences were observed. At 45% RH the survival was 2 logs lower after purification; it is remarkable that at still lower RH the survival was higher than at 45% RH, a phenomenon also observed with crude virus preparations, though less pronounced. Addition of Hanks salts did not restore the original level of survival. The inactivation of EMC virus after spraying from saliva is depicted in Fig. 3 also. The curve resembles that found with suspensions in distilled water, only at low RH the decay in saliva was about 1 log larger than in water. Saliva from two other subjects yielded the same results.

EMC virus in glycerol. In Fig. 4, survival of unpurified EMC virus in glycerol is plotted against the RH equivalent of the glycerol-phosphate-buffered saline mixtures. For comparison the curve for the same virus preparation (without glycerol) in aerosols has been superimposed.

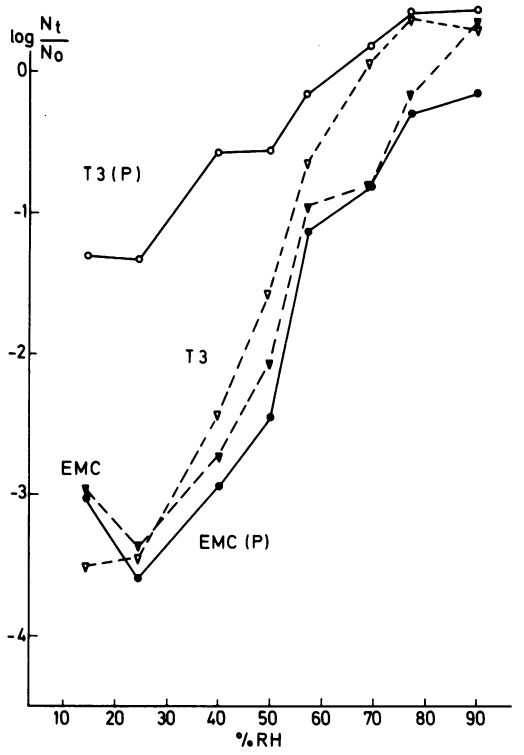

FIG. 2. *Effect of presampling humidification on recovery of EMC virus and phage T3 from aerosols. The viruses, suspended together in phosphate-buffered saline with 1% peptone, were sprayed and sampled after 30 min simultaneously with Porton impingers with (P) and without prior passage through a humidifying chamber. Symbols: ▼, EMC virus without prehumidification; ●, EMC virus with prehumidification (P); ▽, phage T3 without prehumidification; ○, phage T3 with prehumidification (P).*

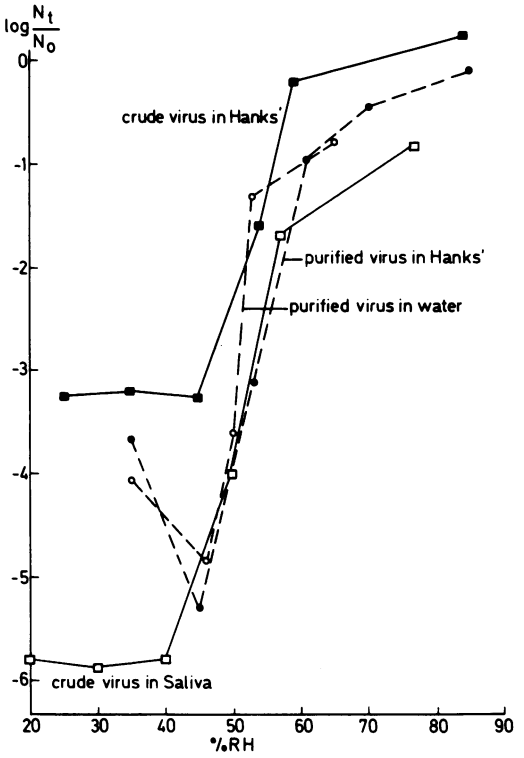

FIG. 3. *Influence of medium on survival of EMC virus in aerosols. Samples were taken from 30 to 35 min after nebulization.*

EMC virus was labile at glycerol concentrations above 78% (wt/wt), correlating with 50% RH. The curves for glycerol and aerosol are, above 35% RH, similar in a qualitative and quantitative way. Around 25% RH there is an extra lability region in glycerol, as occurs at 45% RH with purified virus preparations.

Sensitivity of EMC virus to surface exposure. In contrast to EMC virus, the structurally closely related bacteriophage MS2 is unstable in aerosols at high RH, presumably because of surface-dependent factors (24). To be sure that differences in the spraying fluids could not be incriminated in this discrepancy, the two viruses were, after purification, sprayed from the same suspension in 0.1 M NaCl.

Figure 5 shows that at low RH the decay of the two viruses was the same. At high RH phage MS2 was subject to rapid inactivation, whereas EMC virus was stable. After spraying from 1 M NaCl at 90% RH the difference was even more pronounced.

Figure 6 gives the experiments with the surface-active agent OED at 30% RH. At 0.003% OED the inactivation shortly after spraying was reduced; with 0.03% this initial loss was even completely abolished. When turning to the air samples at 30 to 35 min, however, only marginal protection by OED was noticed with concentrations up to 0.5%.

Applying another system purified EMC virus was subjected together with phage MS2 to the rotating bulb test (26). Here an aqueous virus suspension is spread as a thin film over a glass wall by rotating a small volume in a large spherical flask. As in the combined aerosol experiment phage MS2 lost infectivity very quickly (3 logs in 60 min), whereas EMC virus was completely stable for at least 60 min (Fig. 7).

Attempts to obtain a virus population with increased aerosol stability. EMC virus was aerosolized at 45% RH. After 15 min an air sample was taken and resprayed at 45% RH.

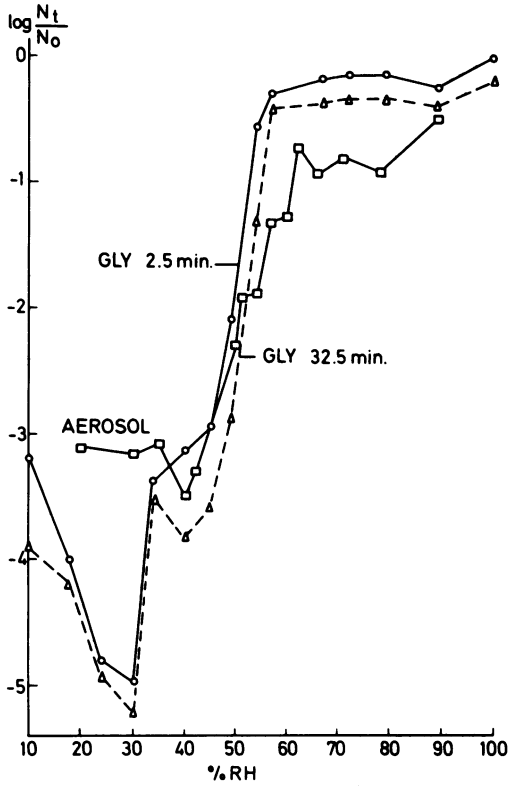

FIG. 4. *Inactivation of EMC virus on standing in glycerol solutions. EMC virus was diluted in mixtures of glycerol and phosphate-buffered saline in various ratios, corresponding to various relative humidities. Samples were taken from the solutions at 2.5 min* (○) *and at 32.5 min* (△) *after addition of virus. For comparison the survival curve of unpurified EMC virus in aerosols is also plotted* (□); *samples were taken from 30 to 35 min.*

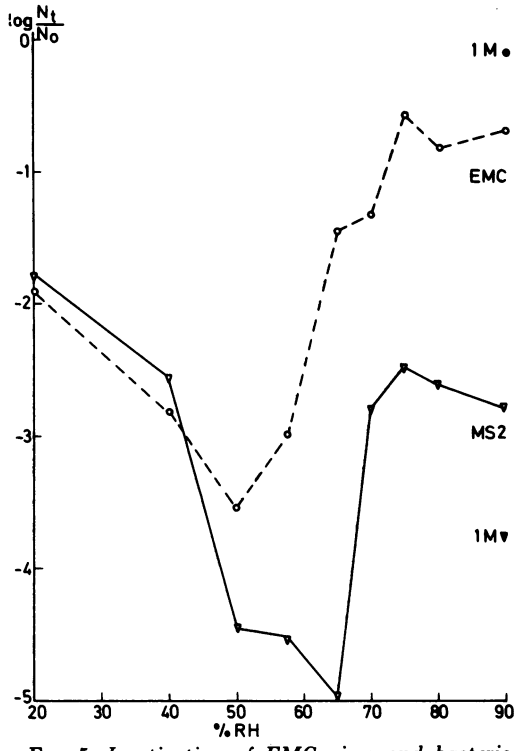

FIG. 5. *Inactivation of EMC virus and bacteriophage MS2 in aerosols. The purified viruses were both diluted into the same batch of 0.1 M NaCl and nebulized. The aerosols were sampled from 30 to 35 min and titrated for EMC virus* (○) *and for phage MS2* (▽). *At 90% RH two additional experiments were performed in 1 M NaCl. Symbols:* ●, *EMC virus;* ▼, *phage MS2.*

The resulting inactivation curve was the same as that of the original virus, sprayed from collection fluid. In another attempt to select a resistant variant, EMC virus was nebulized at 45% RH and sampled 60 min thereafter. The recovered virus was grown in L cells to high titer, sprayed again, etc. This procedure was carried out sequentially for 13 times. The virus preparation obtained did not show increased survival in aerosols.

DISCUSSION

The inactivation of a virus in aerosol experiments could occur in several stages. With EMC virus, fragmentation of the liquid at spraying does not lead to appreciable damage, in view of the high recoveries in moist air. The same applies to the shearing forces in the operating impinger. Consequently the (primary) damage to the virion occurs in the aerosol or at the entry in the collection fluid.

It appears that neither oxygen nor medium components can be held responsible for the inactivation. Purification even led to decreased aerosol stability of the virus (Fig. 3), as was also found by Benbough with poliovirus (4).

Bacteriophage MS2 structurally resembles EMC virus. Both are small (diameter, 25 to 30 nm), lipid-free, icosaeder-shaped viruses containing single-stranded RNA. It is remarkable, therefore, that phage MS2 is sensitive to surface-located forces (24, 26), whereas EMC virus appears not to be so as demonstrated by the OED and rotating bulb experiments (Fig. 6 and 7). Perhaps this difference is related to the method of entry of these viruses into the host cell. Probably RNA phages as MS2 disintegrate outside the bacterial cell before penetration of the RNA (21), in contrast to EMC virus which is presumably engulfed by the sensitive cell with-

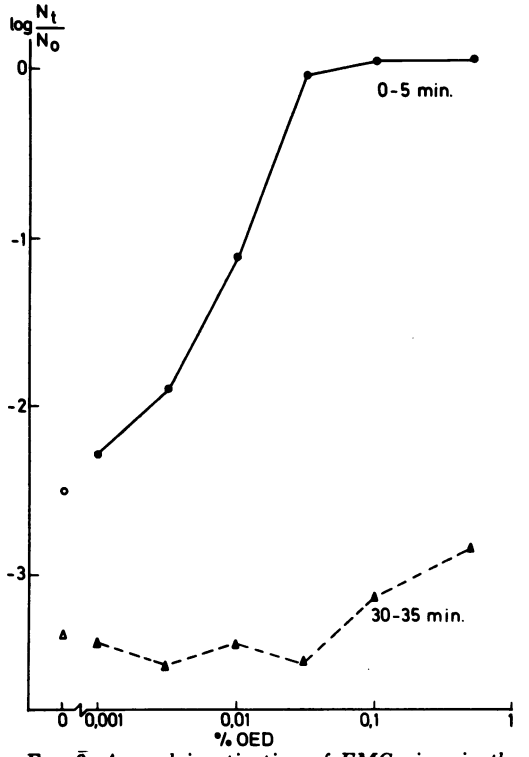

FIG. 6. *Aerosol inactivation of EMC virus in the presence of OED. Aerosols were sampled from 0 to 5 min (●; O, no OED added), and from 30 to 35 min (▲; Δ, no OED added). RH 30%.*

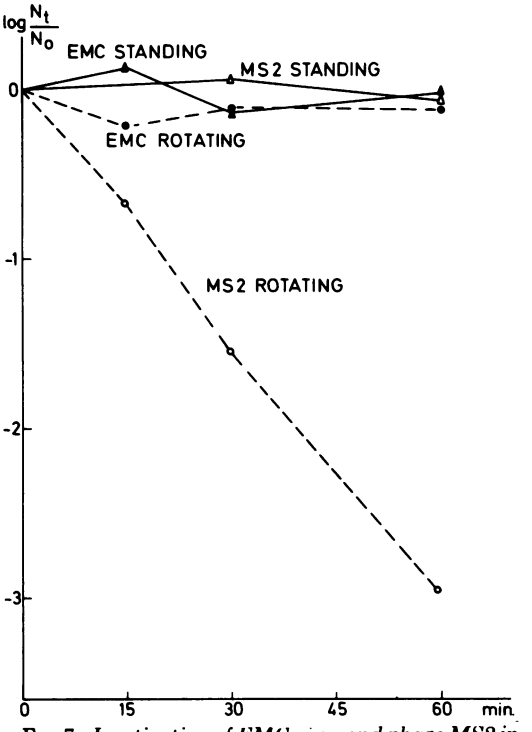

FIG. 7. *Inactivation of EMC virus and phage MS2 in a rotating bulb. The two viruses were rotated together in 1 M NaCl in a large round glass bulb. Samples were titrated on EMC virus and on phage MS2. Similar virus suspensions standing still were run as controls.*

out detectable breakdown.

Concerning aerosols, the findings suggest that the inactivation of phage MS2, but not of EMC virus, is caused by exposure to the large air-water interface.

With EMC virus other possible factors include dehydration and rehydration at sampling. Direct evidence for this hypothesis comes from the study of EMC virus suspended in concentrated glycerol solutions. This environment is comparable to aerosols of low RH with regard to desiccation and leads to the same pattern of virus inactivation (Fig. 4). OED retards, but does not prevent, the evaporation of droplets by coating (20). If the dehydration hypothesis is correct, inactivation would be retarded by OED but not prevented, and this is what happens (Fig. 6). It follows also that the ultimate extent of virus inactivation depends on the ultimate water content of the aerosol particle, which is determined by the ambient RH only.

The kinetics of dehydration in aerosols are not known and can not be compared with the kinetics of virus inactivation therefore. The studies of Scheuplein and Morgan (22) on desiccation of thin keratin membranes (10 to 20 μm in thickness) may be relevant. Two phases are distinguished in this work. The first lasted for 10 min, was characterized by rapid water loss, and was considered to reflect evaporation of free water from the surface. In the second phase desorption proceeded slowly and was ascribed to diffusion and elimination of bound water. The second phase continued for several hours. The activation enthalpy for diffusion of water in such membranes is stated as 14 to 15 kcal per mol at 25 C. Under the different conditions these phases might occur in evaporating aerosol droplets in an accelerated way. On spraying, the evaporation of free water does no harm to EMC virus. This appears from the stability in aerosols from NaCl solution at 60 to 75% RH, where no free water is left after a short time. It is only below 45% RH that maximal inactivation occurs with an activation enthalpy of 15 kcal per mol. These data are in accordance with the hypothesis of removal of structurally essential bound-water molecules being the cause of inactivation of EMC virus in aerosols.

Testing for virus infectivity can only be achieved in dilute aqueous solutions. In principle, therefore, dehydration and rehydration cannot be distinguished as causes of inactivation. Evidence concerning this point can be obtained by changing the kinetics of dehydration and of rehydration and measuring which change effects virus inactivation.

With bacteriophage T3, for instance, the recovery from aerosols could be raised 100- or 1,000-fold by means of a presampling humidification device (Fig. 2; 12). This indicates that the irreversible damage to the virion is done during rehydration. On the other hand EMC virus recovery from aerosols was not influenced by changing rehumidification kinetics (Fig. 2), but it was by changing dehydration rates with OED (Fig. 6). This suggests that inactivation occurs during desiccation in aerosol rather than at sampling.

Little can be concluded about the molecular processes leading to the aerosol inactivation. Thermal inactivation of poliovirus at temperatures above 44 C is ascribed to protein denaturation, with an activation enthalpy of 244 kcal per mol (9). With simian virus 40 in aerosols, Akers et al. observed a very marked decrease of stability when raising the temperature from 21 to 32 C (2). With EMC virus the influence of temperature is only slight, however (Fig. 1). The calculated activation enthalpy of 15 kcal per mol points to low-energy processes as diffusion of bound water (22) or the break of one or two chemical bonds. This small event then apparently triggers the breakdown of the whole capsid structure, resulting in loss of hemagglutination activity, loss of affinity for hemagglutination inhibiting antibodies, and release of the virus RNA in a free infectious form, as described earlier (8). Attempts to protect virus by coating with antibodies have failed. This could mean that the loss of antigenic structure (8) is secondary to other changes.

Spread of some picornaviruses occurs partially with respiratory secretions, dispersed as aerosols (15), or shed on surfaces (14). Before the next host is reached enough time will elapse to allow droplets in aerosols or on surfaces to equilibrate with the ambient air. Figure 3 shows that this process can entail rapid inactivation. The saliva components do not protect the virus. Below 40% RH the decay in saliva was even larger than in water. Similar observations were made by Barlow and Donaldson, working with foot-and-mouth disease virus (3).

After inactivation, in aerosol, of 99.9% of EMC virus, the remaining infectivity is more stable than the original virus. In an earlier paper (8) it was shown that this surviving virus fraction sedimented in a sucrose gradient with the same velocity as normal virus. This suggests that no major physical change, such as clumping, is responsible for the enhanced aerosol resistance (8). In this study it was demonstrated that the persistent virus fraction on respraying presents the same inactivation curve as the original population. The fraction is, therefore, not formed by a subpopulation of particles, with a genetically or phenotypically different structure. At very low RH the surviving fraction is larger than at moderately low RH (Fig. 3). This suggests the possibility that this mechanism of survival has to be sought in a more rapid extrusion of water molecules than occurs with the majority of the virus particles.

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