STUDIES ON AIR-BORNE VIRUS INFECTIONS

I. EXPERIMENTAL TECHNIQUE AND PRELIMINARY OBSERVATIONS ON INFLUENZA AND INFECTIOUS ECTROMELIA

BY D. G. ff. EDWARD, W. J. ELFORD AND (THE LATE) P. P. LAIDLAW
From the National Institute for Medical Research, London, N.W. 3

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INTRODUCTION

The circumstances of modern warfare, particularly aerial warfare necessitating the herding together of large numbers of people in air-raid shelters, have made air-borne infection an urgent problem. It was for this reason that soon after the outbreak of war an experimental investigation of the problem was begun under the direction of one of us (the late Sir Patrick Laidlaw) at the National Institute for Medical Research. In any list of the infective agents which are regarded as being commonly transmitted from person to person through the air, certain viruses take a prominent place. When we remember the pandemic with its huge mortality at the end of the last war, our thoughts immediately turn to influenza for an example of air-borne infection. Yet, owing to experimental difficulties, there have been few investigations on viruses dispersed in the air. Our knowledge regarding air-borne infection and the behaviour of infective agents suspended in the air has been largely based on experiments with pathogenic and non-pathogenic bacteria.

Trillat & Beauvillain (1937), however, have shown that ferrets can be infected with influenza by placing them in an atmosphere in which a suspension of the virus has been atomized. Infection was not only due to inhalation of virus, but could occur through the eyes. Previously, Wells & Brown (1936) had described experiments in which suspensions of influenza virus were atomized in a large tank and the air sampled at intervals afterwards, using a Wells air centrifuge with a suitable fluid in the rotating cylinder to collect the virus. Samples of the fluid were tested for infectivity by the intranasal inoculation of ferrets and, in a few instances, also of mice. Positive evidence was obtained indicating

that the virus persisted in the air of the tank up to 1 hr., but not later. A few experiments suggested that the virus suspended in the air could be killed by ultra-violet light.

Influenza virus A has been studied in greatest detail in the experiments to be described in this paper, for it was found that mice could be regularly infected with both the P.R.8 and W.S. strains by allowing them to breathe air into which the virus had been atomized. The course of the disease and the post-mortem appearances of the lungs were exactly the same as those following intranasal inoculation. No histological examination of the lungs has been made, although for detailed study of the histological changes produced by the virus this method of infection by natural inhalation is obviously preferable to instilling a liquid suspension of the virus down the respiratory tract.

The inhalation method has also been successfully used to infect mice with infectious ectromelia. The disease was fatal and at post-mortem generalized lesions were found. Spleen and liver exhibited typical macroscopical and microscopical lesions, and there was an extensive pneumonia, inclusion bodies being observed histologically in the bronchial epithelium. The pathological changes produced in mice by ectromelia following intranasal inoculation have been recently described by Kikuth & Gönnert (1940). Final confirmation that the mice in these experiments were infected with ectromelia was obtained by successful passage from suspensions of the organs, the typical disease being produced by inoculation into the pads of the feet.

Experimental methods

(1) *Strain of virus used.* Aerosols of influenza A virus have been studied mostly, a mouse-adapted strain of P.R.8 being used. Batches of mice were inoculated intranasally and then sacrificed 3 days later. Their lungs were pooled and, after being minced finely, were ground with powdered Pyrex glass in equal parts of nutrient broth and physiological saline to give a 5 % suspension. This was centrifuged for 5–10 min. at about 3000 r.p.m., and the supernatant fluid provided a highly active suspension of virus.

(2) *Spray used.* Atomization of the virus suspension has been achieved with an Aerograph Pencil Spray working at 35 lb. sq. in. pressure and a selected setting of the jet. The mist produced is very heterodisperse, so steps were taken to trap and remove the larger droplets in the following manner: A piece of thick rubber sheeting was stretched across the neck of a small filter-flask and firmly tied on. The nozzle of the aerograph pencil was introduced through a small circular hole in the centre of the rubber, so that an air-tight joint was formed (see Fig. 1, inset). The mist impinged on the bottom of the flask and all but the very fine droplets were trapped.

(3) *Experimental set-up.* The actual set-up of the apparatus varied from one experiment to another according to its purpose. That shown in the diagram is a representative one and could be used for the simple exposure of mice to an aerosol and for titration of that aerosol. In other experiments slight modifications would be necessary. The aerosol, made by the spray (inset), was collected in a large aspirator bottle over either water or a saturated solution of calcium chloride containing a little mercuric chloride as a non-volatile antiseptic. Length of time of spraying depended on the size of the aspirator bottle. For one of 12 l. capacity 2 or 3 min. was sufficient. The air displaced during filling escaped through a funnel covered with gauze or a double layer of muslin, and dipping into a solution consisting of 5 % lysol and 1 % hexyl-resorcinol in 50 % spirit, which had proved an effective trap for bacterial aerosols (Elford & Todd). Mixing of the aerosol

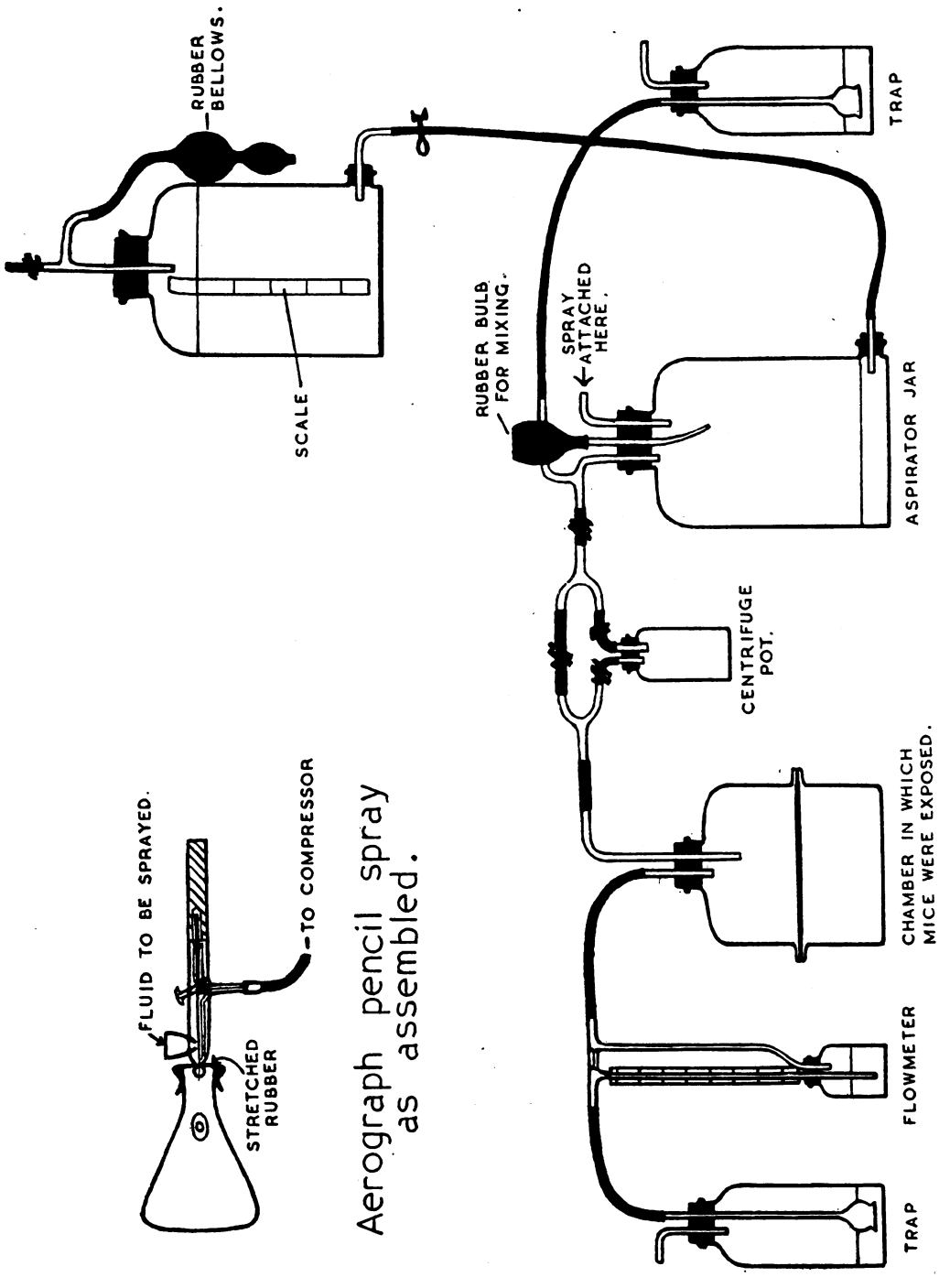

Fig. 1. Apparatus for experimental studies on air-borne infection.

was effected by squeezing several times a rubber dental bulb attached to a glass tube which on passing through the bung tapered to a jet. Care to avoid the formation of mist through sudden pressure changes was found to be most important in humid atmospheres, and a toy rubber balloon was generally attached to the reservoir to have an equalizing effect. The aerosol could be shut off in the bottle by closing the clips and then left to age for any desired period of time before testing. Except in experiments designed to test the persistence of infectivity, it was usual to allow the aerosol to age 15 min., so that the larger particles might settle out. Then by adjusting the relative positions of the two aspirator bottles, and using the rubber bellows when necessary, a sufficient volume (rate of flow usually regulated to 2·5–3 l./min., as indicated by the flowmeter) of aerosol was displaced into the desiccator containing six mice and a gauze-covered tray of soda lime. In this way the mice could be exposed to the aerosol for periods up to 30 min. At the end of the period of exposure fresh air was drawn through the desiccator to sweep out the remaining virus particles. The desiccator was then taken on to the balcony outside the laboratory, there opened, and the mice transferred to boxes.

(4) *Estimation of virus content of aerosol.* The method employed for measuring the concentration of virus in the aerosol was to centrifuge the aerosol in a pot of known volume, containing a measured amount of broth, at 2000 r.p.m. for 30 min. The pot with a capacity of 225 c.c. was arranged so that the first litre of aerosol passed through it and the rest could be by-passed. After filling, 2·25 c.c. of broth was run in gently through the inlet tube; then both inlet and outlet tubes were closed with rubber bungs and the pot centrifuged. Thus a hundredfold concentration was effected if all the virus was deposited into the broth. Serial tenfold dilutions of this broth were inoculated intranasally into batches of mice so that the virus concentration in the aerosol could be estimated in terms of the number of minimal infecting doses (M.I.D.). In many of the early experiments the broth was added to the centrifuge pot before filling with aerosol, and subsequent comparison with the procedure described above showed that the amount of virus trapped by the fluid during filling, although just detectable, was not sufficient to affect significantly the result of the animal titration.

The suspension of virus sprayed in each experiment was also titrated in mice. The latter were of a mixed strain, bred at the Medical Research Council's Farm Laboratories, Mill Hill. Animals were chosen weighing between 14 and 16 g., and titrations were done in the usual way by instilling 0·05 c.c. of tenfold dilutions intranasally under ether anaesthesia into batches of three mice. The lungs of mice which died were examined and survivors were killed on the tenth day and examined post-mortem. The presence and amount of consolidation were noted.

(5) *Precautions employed.* The use, in this experimental procedure, of a slight positive pressure to circulate the aerosol, entails a risk of infected particles escaping into the laboratory air through leaks in the apparatus. Therefore, it is imperative to test and check up the assembly carefully before an experiment, and to wire or tie on all bungs and joints such as those of the desiccators. At the end of an experiment the apparatus was rendered safe before dismantling by replacing the infected air by fresh air, sucked through the apparatus, by a water pump.

It is a well-established fact that exposure to bright daylight increases the rate at which sprayed organisms die off in the air. Our experiments have always been carried out on a bench away from the window, and where the light was of low intensity.

STABILITY OF THE AEROSOLS

The length of time during which aerosols of P.R.8 virus retain their infectivity for mice has been investigated. Using the arrangement of apparatus already described, aerosols were sampled immediately and at chosen intervals after atomization, (*a*) by filling centrifuge pots and then titrating the virus deposited in broth by centrifugation, and (*b*) by direct exposure of mice in desiccators. The results of two typical experiments are given in Table 1. In one, the aerosol was collected over water, so that the rate of drying of the atomized particles was minimized; in the other, more rapid and complete drying of the particles was obtained by collection over saturated calcium chloride solution, so that the particles were in effect droplet nuclei, such as have been described by Wells (1934).

Table 1. *Persistence of infectivity of ageing aerosol*

Age of aerosol in min.	Direct exposure of mice to aerosol for 10 min.	Intranasal inoculation of mice with broth into which virus had been deposited from aerosol by centrifugation		
		Dose = 2·5 c.c. of aerosol	Dose = 0·25 c.c. of aerosol	Dose = 0·025 c.c. of aerosol
		(1) Over water		
1		5, 5, 5	5, 5, 2	1, 0, 0
15		5, 5, 5	2, 2, 1	1, 0
30		5, 3	1, 1, 0	0, 0, 0
60	5, 2, 2, 2, 1, 1	2, 2, 1	0, 0, 0	0, 0, 0
120		0, 0, 0	0, 0, 0	
180	0, 0, 0, 0, 0, 0	0, 0, 0	0, 0	
300		0, 0, 0		
		(2) Over saturated calcium chloride		
1		5, 5, 5	5, 5, 5	1, 0
15		5, 5, 3	3, 3, 3	3, 3, 0
30		5, 5, 5	2, 2, 1	1, 0, 0
60	5, 5, 5, 5, 5, 5	5, 5, 5	3, 2, 1	0, 0, 0
120		5, 5, 3	0, 0, 0	
180	5, 5, 4, 4, 3, 3	2, 1, 0	0, 0, 0	
300		2, 0, 0		

Resulting infection is expressed in terms of a numerical index. Thus, 5 represents death with influenzal consolidation of lung. Among survivors killed on the tenth day, almost complete consolidation is represented by 4, consolidation affecting three-quarters of the lung by 3, half by 2, and a quarter or less by 1.

The experiments with an aerosol over water demonstrated that the loss of infectivity at the end of the first 15 min. was slight, at 30 min. nearly 90 %, and at 1 hr. it approached 99 %. Virus could not be detected in the centrifuged sample at 2 hr., nor by exposure of mice at 3 hr. On the other hand, the dry aerosol was much more persistent. After 1 hr. there had been barely a tenfold decrease in infectivity. At 3 hr. about 1 % of virus remained and its presence could be still demonstrated in the centrifuged sample at 5 hr.

In another experiment a series of pairs of pots were filled with an aerosol of P.R.8 and broth added. At intervals one pot of each pair was centrifuged and the total viable virus deposited in the broth titrated. The virus remaining suspended in the atmosphere of the other pot was swept out and replaced by air drawn in with a water pump, and the virus that had sedimented in the broth titrated. The experiment suggested that all, or nearly all, the virus particles had settled out in less than 60 min. It appears, therefore, that the disappearance of infective particles in an atmosphere of high humidity is largely due to this natural settling.

The data in Table 1, interpreted as representing rate of settling, led to the following estimations of the mean particle size in the aerosol:

(i) Aerosol over water ... Particle diameter = 2·3μ approx.
(ii) Aerosol over saturated $CaCl_2$ ... Particle diameter = 1·3μ approx.

The particle size of influenza virus as contained in a liquid extract of infected mouse-lung tissue averages 100 mμ (Elford, Andrewes & Tang, 1936), so the above figures for the size of the aerosol particles serve to emphasize the fact that the very considerable amount of tissue protein present in the original virus suspension largely determines the size of the nuclei in the aerosol. There may, of course, be more than one virus particle in some of the nuclei. Unfortunately, we have not yet obtained corresponding data for the rate of settling of particles in an aerosol prepared from washed preparations of influenza virus.

Filtration experiments, in which an aerosol of influenza virus (from unwashed suspensions) prepared over saturated calcium chloride solution was passed through dry 'gradocol' membranes of known porosities at the rate of 120 c.c./sq. cm./min., and mice exposed to

Table 2

Membrane porosity in μ	Filtrate tests	
	Exposure time min.	Reaction of mice
1·2	3	Mice quite normal
2·3	5	Mice quite normal
4·2	3·5	2 out of 3 mice showed small lung lesions—threshold dose
Control unfiltered	3	All three mice succumbed to influenzal pneumonia

the filtrate yielded the results summarized in Table 2. The evidence of filtration therefore would appear to confirm the relatively high value for the particle size of the influenza virus aerosol as prepared in the experiments.

Concentration of virus in the aerosol

The concentration of virus in the atmosphere produced by a constant method of atomization appears to depend directly on the titre of the suspension atomized. With P.R.8 the latter has remained fairly constant throughout the experiments, averaging between 2×10^7 and 2×10^8 M.I.D./c.c. In the same way the concentration of virus in aerosols made under similar conditions has remained more or less constant. For example, an aerosol after standing 15 min. over saturated calcium chloride solution usually contained one M.I.D. in 0·05 c.c. The maximal concentration of virus in the atmosphere is thus not high. Filtration of the suspension, prior to its being atomized, usually reduces the available concentration of virus, so that the resulting aerosols are too weak in infectivity to be studied satisfactorily. In the same way experience with other viruses has shown that only those, of which suspensions are obtainable with concentrations at least as high as those of P.R.8, can be successfully examined with this technique.

For the quantitative estimation of the direct infectivity of aerosols for mice, two procedures have been followed: (*a*) exposure for a fixed time to dilutions of the aerosol, and (*b*) exposure for progressively shorter times to full strength aerosol.

A 1/100 dilution was made by introducing 1·6 l. of an aerosol of P.R.8 collected over water into a sealed tank of 162 l. capacity. Six mice were exposed in this for 1 hr. Three died with influenzal pneumonia, and on the tenth day post-mortem examination of the survivors showed that one had consolidation affecting three-quarters of the whole lungs, and in the other two consolidation affected half the lungs. In another experiment three mice were exposed independently one after the other to full-strength aerosol in a vessel of 100 c.c. capacity. In each case the exposure was for 1 min. Two mice died with influenzal pneumonia, and the third, when killed on the 10th day, had lesions affecting about half of its lungs.

Attempts have been made to compare the susceptibilities of mice to the two methods of infection with influenza, (*a*) exposure to an aerosol, and (*b*) instillation of the virus intranasally under ether anaesthesia. In one experiment, after allowing an aerosol to stand for 15 min. over saturated calcium chloride, the aspirator bottle was emptied except for the last litre of aerosol, a centrifuge pot being filled at the same time. Then 9 l. of air were taken into the aspirator bottle, well mixed, and 9 l. of the mixture passed through a desiccator in which six mice were exposed to this 1:10 dilution for 10 min. Further

Table 3. *Comparison of methods for infection of mice with influenza virus*

Method of infection	Results of tests at successive tenfold dilutions			
	1/1	1/10	1/100	1/1000
		(*a*) Aerosol		
Direct exposure to aerosol for 10 min.	—	5, 5, 5, 5, 5, 5	4, 3, 3, 2, 2, 2	1, 0, 0, 0, 0, 0
	(*b*) Broth containing sedimented virus			
Intranasal inoculation of 0·05 c.c.	5, 5, 1	5, 3, 3	5, 1, 1	—

dilutions were made in the same way, and mice were exposed similarly to 1:100 and to 1:1000 dilutions of the original aerosol. Other mice were inoculated intranasally with known dilutions of the broth containing the sedimented virus from the centrifuge pot. The results (as shown in Table 3) indicated that mice receiving intranasally 0·05 c.c. of fluid, in which the virus from 5 c.c. of original aerosol was concentrated, developed infection of like severity to that shown by mice breathing normally for 10 min. in an atmosphere in which the aerosol had been diluted tenfold. Similarly, the infections produced by doses of one-tenth these magnitudes respectively also corresponded. Other experiments, in which comparisons of the two methods of infection could be made, confirmed this approximate relationship.

Our colleagues, Drs G. L. Brown and F. C. MacIntosh, whose valued co-operation we gratefully acknowledge, have measured the respiratory minute volume of a normal mouse and find it to be about 50 c.c. Using this figure to interpret further the findings of the above experiment, it would appear that 500 c.c. of the tenfold diluted aerosol, when breathed by a mouse, produces an influenzal infection comparable to that resulting when the equivalent of 5 c.c. full-strength aerosol is given intranasally. Assuming that like infections may be attributed to like amounts of virus—probably a valid assumption only for doses that are not excessive, and providing any differences in distribution of the virus reaching the susceptible tissue cells in the two instances do not significantly affect the net degree of infection—it seems that at best not more than 10 % of the virus breathed

actually reaches the lungs. Since, too, it is probable that only a fraction of the virus instilled intranasally penetrates so far, the proportion of breathed virus arriving in the lungs may be as little as 1 %, most of the virus being retained in the nasal and respiratory passages. It is of interest to note in this connexion that the filtering efficiency of the human nose towards air-borne organisms has been experimentally evaluated at between 60 and 90 % according to the particle size (Rooks, 1939).

The infection contracted by a group of mice exposed to an aerosol of influenza virus is noticeably more regular than that following intranasal inoculation. This implies a more constant dose and a more even distribution of virus than with the former mode of infection, where the variable losses of virus notorious in the instillation technique are eliminated. The uniformity characterizing infection produced by the inhalation method has already proved of practical value in investigations for which it is desirable to have a group of animals all infected to a like degree.

Discussion

The method outlined here for the experimental study of infection produced by virus aerosols has been successfully applied to the viruses of vaccinia and herpes in addition to the two viruses dealt with in the paper. It must be constantly borne in mind, however, that all manipulations are being conducted with a slight positive pressure inside the system, probably of the order of 1 in. water pressure, due mainly to the resistance of the liquid traps. Hence the utmost care is necessary to ensure that all joints and connexions are tight, otherwise trouble must be expected through not having the virus aerosol under control. When disconnecting any portion of the apparatus the pressure should be reduced a little below atmospheric value so that air will always tend to enter, rather than virus escape from, the system. While the method we have employed is probably the more convenient when transferences of known volumes of aerosol are desired, as for example in making known dilutions, there is, nevertheless, much to be said in favour of the arrangement recently described by Wells (1940). Here, all operations are conducted with a negative pressure gradient between the system and the outside atmosphere so that air tends to enter, and thus the danger of virus escaping is minimized. However, with either procedure this work calls for complete concentration at every step, if confidence in control is to be retained.

The evidence obtained indicating the superior persistence of infective nuclei carrying influenza virus in dry, as compared with humid, atmospheres is quite definite. Persistence involves two factors: (*a*) the particle size, which determines the rate of settling, and (*b*) the viability of the virus under the prevailing conditions. Experiments have been conducted to determine the relative stabilities of the virus aerosol particles that have settled out on to different kinds of surface. These aerosol particles consist of virus together with protein extracted from the tissue in the preparation of the original suspension and minute variable amounts of water. Where it was desired to minimize the effect of such tissue protein, suspensions of washed influenza virus were made and sprayed. Generally, the virus in the well-dried particle retains its infectivity best, although in the type of experiment instanced the nature of the surfaces on which the virus settles exercises a specific influence as well. These facts are of importance, not only in determining how long an atmosphere may remain infective, but also the period over which the virus settling

out on different types of surface can remain viable and hence constitute a potential source of infection through re-dispersion into the atmosphere as 'dust'. The details of these stability tests have now been reported in a separate paper (Edward, 1941).

The relative efficiencies of sprayed antiseptics and of ultra-violet light as measures for destroying the infectivity of such aerosols of influenza virus have also been investigated by employing the general experimental procedure here described. These results will be communicated later. It is hoped that this account of the methods developed and successfully applied in studies on the infectivity of atmospheres, into which suspensions of influenza virus have been atomized, may prove of interest and help to others who are working in this field. We regret that circumstances beyond our control have prevented an earlier publication of these results which were obtained in 1939 and 1940.

Eaton (1940) has reported that mice placed in the same chamber with mice infected with epidemic influenza virus will, under certain conditions, depending on time of contact and the severity of the disease in the infected mice, contract an infection showing extensive lung consolidation which is seldom fatal. Our colleague, Dr C. H. Andrewes, informs us, however, that in unpublished experiments he failed to duplicate Eaton's results. Only very exceptionally did a normal mouse pick up influenza by contact, though he used a strain of virus kindly sent him by Dr Eaton, the same strain of mice (Swiss) and, so far as could be ascertained, the same environmental conditions.

Andrewes & Glover (1941) have described experiments demonstrating that cross-infection with influenza virus can occur between ferrets infected with influenza A virus and normal ferrets confined in the same room. The animals were separated by about 5 ft., and recipients placed 3 ft. above the donors contracted the disease. Infection was thought to be conveyed either by droplet nuclei or by very fine dust particles.

Still more recently the successful transmission of influenza infection to mice exposed in atmospheres containing atomized suspensions of infected mouse lung tissue has been reported by Wells & Henle (1941), Henle & Zellat (1941) and by Robertson, Loosli, Puck, Bigg & Miller (1941) in their investigations of the value of ultra-violet light and of propylene glycol vapour for disinfecting air infected with influenza virus. The disinfecting power was shown by the protection afforded to mice breathing the treated air.

It is interesting to note that Wells & Henle (1941) observed that the lung lesions produced by the inhalation of air-borne virus were 'decidedly less severe than expected from calculation of the amount of air-borne virus inhaled, as compared with intranasal instillation of similar quantities under anaesthesia'. This accords with our own observations which we have discussed in a foregoing section of this paper.

SUMMARY

1. A method is described for the experimental investigation of aerosol systems formed by the atomization of suspensions of viruses into the atmosphere. Observations on the physical properties of such systems have been made, and an experimental approach found to certain practical problems connected with air-borne virus infections, particularly counter measures involving the use of ultra-violet light and aerosols of chemical antiseptics.

2. Mice, placed in an atmosphere into which the respective virus had been atomized, have contracted the diseases of influenza and infectious ectromelia in a manner closely analogous to naturally occurring air-borne infection.

3. The infection resulting from the inhalation of virus is described. The suggestion is made that this method of infection may be used with advantage (*a*) in studying in detail the histology of lung lesions, and (*b*) in obtaining more uniform infection of large batches of mice.

4. The two methods of experimental infection, (i) intranasal instillation under anaesthesia, and (ii) normal inhalation, have been compared and contrasted. It is deduced from the evidence available that as little as 1 % only of the virus may reach the lungs of mice breathing normally in an atmosphere containing dispersed nuclei of influenza virus.

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