A LABORATORY MODEL FOR RESPIRATORY DISEASE INVESTIGATION

AND

ITS APPLICATION TO VARIATIONS IN CLIMATIC ENVIRONMENT

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ABSTRACT

The problems associated with investigation of respiratory disease in calves are examined and the need for a laboratory model study presented.

A germ free mouse colony is described and evaluated in terms of residual disease. Methods are described for exposing germ free mice to aerosols of parainfluenza 1 (Sendai)virus. The distribution of virus within lung tissue was ascertained using immunofluorescent stains. The interaction of Sendai virus with the germ free mouse was measured by titrating virus in lung tissue. The interaction of the germ free mouse with Sendai virus was measured by histological quantitation. A method for comparing the uninfected animal with the infected was developed to produce a numerical count of bronchiolar mucosal cells per millimetre of basement membrane.

The effect of climate on the function of respiratory physiology and pathology is reviewed. The effect of varying environmental temperature and relative humidity on mice infected with Sendai virus is examined. Temperature and relative humidity are both shown to affect the pathogenesis of the disease.

It is concluded that a cooler environment ($10^{\circ}C$) allows a higher virus titre to develop with a resultant increase in cellular destruction, while a lower relative humidity (55%) delays bronchiolar mucosal cell regeneration during the post viral recovery phase.

CHAPTER I

INTRODUCTION

Original premise

This thesis describes the production of a laboratory model to investigate various parameters of respiratory disease. The use of quantitative methods, including those of histological analysis, is emphasized. The thesis consists of seven chapters which define the host animal, the infectious agent, their mode of combination, the measurement of their interaction, and the effect of certain environments upon that interaction.

The study was stimulated by observations made directly and through the scientific literature on respiratory disease in calves. However, experiments with cattle are necessarily expensive and frequently demonstrate a lack of genetic homogeneity in the experimental animal. Basic studies using an inbred laboratory animal were therefore chosen. The experimental studies were designed after careful consideration of the respiratory disease problem in calves.

The respiratory disease problem in the calf

Respiratory disease in calves, as it is recognised in the United Kingdom and certain other countries, appears to be a complex of several diseases which have common features. A discrete aetiological agent has not been incriminated; the wealth of microbial isolates would suggest that many infectious agents can be involved in the disease syndrome which has limited clinical and pathological manifestations. The variety of titles given in the literature illustrates the lack of unity among different research workers e.g. "Pneumonia in calves" (1), "Epizootic bronchitis" (2), "Enzootic

pneumonia" (3, 4), "Virus pneumonia of calves" (5), and "Calf influenza" (7).

Such titles indicate that observation and classification of this disease complex have been based on epidemiological, clinical and pathologic data. In a review of the aetiology and pathology Omar (1) concluded that no one criterion has been clearly evaluated and that microbial classification adds further to the confusion.

Epidemiologically the disease is widespread in housed calves. Omar (1) quotes observations in Europe, North America and South Africa. The disease has also been reported in Australia by Sutherland (8) and in New Zealand by Curtis (9). Beef calves born in the open, naturally suckled and given free range do not appear to suffer in any significant degree from the respiratory complex, except in certain patterns of husbandry under the stress of weaning. The disease is strongly associated with unnatural rearing, including abnormal diet and abnormal social and climatic environments. The age incidence recorded by Barr, McMillan, Jennings and Kelly (3) shows a very wide range - from birth to about six months. A seasonal incidence for the United Kingdon was noted by the same authors (3) lying between late winter and early spring. The figures given by Parker (2), while confirming these observations, show that the condition can occur in summer, though with a lesser frequency.

Clinically the pattern described by Blood and Henderson (6) is narrow. Barr, et al. (3) expand the picture and imply but do not demonstrate that the disease has a progressive character. A range of conditions is described by Curtis (9). Thomas (10) divides this range conveniently and practically into three categories. The first is an acute condition characterized by depression, increased abdominal

respiratory effort and a marked rise in temperature. The second is a mild disease characterized by bouts of coughing and a nasal discharge. The third category is sub-clinical and can only be satisfactorily demonstrated at post mortem as consolidation of the antero-ventral portions of the lung. Interrelationships between the three categories have yet to be defined.

In a limited review of respiratory disease in calves Phillip (11) tabulated bacteria, chlamydia, mycoplasma and viruses as agents isolated from the lungs. Magwood, Barnum and Thomson (12) examined the nasal bacterial flora of healthy as well as pneumonic calves and found similar bacterial populations in both groups. Wilson (13) isolated chlamydia from the faeces of clinically normal cattle, while Davies (14) isolated mycoplasma from healthy calf lungs. On the other hand, Gourlay, Mackenzie and Cooper (15) stated that viruses are rarely, if ever, present in healthy calf lungs. Further, viruses in general are the agents isolated least regularly from pneumonic lungs. Their implication is more frequent by demonstration of a rising antibody titre to viral antigen than by isolation of the actual infectious agent (5). It is relevant to consider that, following experimental inoculation of virus, recovery of infectious virus from either the nasal cavity or the lung can be shown for only one to two weeks (16, 17, 18) and that the majority of natural disease outbreaks are examined many days after the earliest clinical signs were first recorded.

The histopathologic features of calf lungs in respiratory disease frequently overlap. Omar (1) provided a useful classification into exudative and proliferative patterns. The exudative patterns consistently yield bacterial isolates, the type depending on the

character of the exudate. In a different review, on lung pathology, Omar (19) concluded that the proliferative reactions of lung tissues result from many stimuli. Several authors have reported the experimental inoculation of viruses (16, 17, 18); post-mortem examination of damaged lung areas revealed a predominantly proliferative response in all cases. Gourlay and Thomas (20) indicated a similar class of reaction following the experimental inoculation of T-mycoplasma.

These observations have been used as a basis for diagnostic criteria. Where proliferative reactions are found they are suggestive of viral/mycoplasma influence while exudative lesions indicate bacterial involvement. Disturbingly, there has been little attempt to correlate pathologic diagnosis with the clinical picture.

Omar's review (1) of calf respiratory disease in Europe and McKercher's review (21) of the analogous condition of "Shipping fever" in North America both refer to stress factors that are apparent to observers but are usually of unknown quantity and unestablished significance. Such components are transport, exposure, exhaustion, chilling in cold damp conditions and irregular watering and feeding. Although most papers discussing aspects of calf respiratory disease make note of stress factors and conclude that they play a part in the aetiology no correlation between microbiological isolates and stress has been satisfactorily shown. There is, therefore, no record of either a primary aetiological agent or a combination of microbial agents that will produce the clinical disease observed in naturally ocurring outbreaks. When these factors are examined in conjunction with the seasonal variation quoted by Omar (1) and Parker (2) for respiratory disease observed in normally housed, fed and watered

calves, it becomes apparent that climatic conditions may be of paramount importance. In the sheltered conditions of housed calves, temperature and relative humidity assume prominence. There are very few reports of experimental work on respiratory disease related to environmental temperature and humidity: such work has been conducted on laboratory animals of variable and frequently unknown disease status.

Earlier work on the effect of environment on host parasite relationships suggests the need for more precise studies and demonstrates the difficulty of attempting such experiments on farm livestock. A much clearer evaluation is required of the mechanisms involved and the use of defined laboratory animals is indicated.

Use of a defined laboratory animal: the germ free mouse

Environmental control in livestock buildings is technically difficult and very expensive. To provide such facilities in the laboratory is a smaller problem which can be overcome more cheaply. It is likely that the findings of experimental work in mice can be translated into comparative studies of calves by defining the problems more clearly and therein lies particular practical value. Mice can be produced for experiment in statistically significant numbers. Their genetic homogeneity can be well defined as can their disease status.

Experiments designed to measure the interaction of infectious agents and host mammals are exposed to many variables. These variables may be recognised and defined; alternatively they may be recognised but cannot be adequately defined; finally they may not be recognised. It follows that careful experimentation, especially that based upon quantitative methods, must not only take account of all known variables

but aim to control them rigidly or reduce them to small proportions.

The variables of age, diet, management and genetic character are well recognised and can be made uniform for experimental purposes. The macro-environment of experimental animals is frequently uniform but often not directly observed. That the micro-environment of a mouse cage relates to the macro-environment has been shown by Murakami (22). The variables of microbiological association and the related phenomenon of body defence mechanisms cannot be controlled under the more usual laboratory conditions. Bacteriological reference texts (23) state that there is a microbiological flora in the digestive tract, on the skin and in the urinary/reproductive tract of all species; this flora varies constantly both in a quantitative and qualitative manner. The respiratory tract is considered to be sterile in general terms but, because it is exposed to the external environment and connects directly to the pharynx,it is constantly exposed to airborne micro-organisms. Under normal circumstances these are cleared by a variety of defence mechanisms but under pathological conditions bacteria, at least, enhance the body reaction (24, 25).

The widespread use of germ free laboratory animals has shown that the techniques of gnotobiology successfully render animals bacteria free. In this state the humoral and cellular defence mechanisms are stimulated only by food and bedding antigens. The review by Griesemer (26) maintained that defences are functionally competent when the animals are challenged.

The germ free state for experimental animals has been criticised because it is dissimilar in certain respects to the conventional. Wostmann, Bruckner-Kardoss and Knight (27) found caecal enlargement affecting cardiac output and oxygen consumption while Gordon (28)

demonstrated a bioactive substance in the enlarged caecal content. In the review by Gordon and Pesti (29) minor anatomic variations were noted in the skin and respiratory tract; most interest has been stimulated by the morphologic and functional changes in the alimentary tract. These differences have to be weighed against freedom from contaminating bacteria. However, the respiratory tract is not significantly altered, the immune system is not deficient and freedom from bacteria, with resultant freedom from enhanced bronchial inflammation, would be of great advantage.

The choice of respiratory disease agent: Sendai virus

In a review of the established respiratory diseases of mice Brennan, Fritz and Flynn (30) mentioned six aetiologic agents that will constantly produce the same disease upon inoculation into susceptible animals. These are Pneumonia virus of mice, isolated by Horsfall and Hahn (31), Sendai virus, demonstrated by Fukumi, Nishikawa and Kitayama (32), Mouse pneumonitis agent, the chlamydia agent of Nigg and Eaton (33), Newborn mouse pneumonitis agent or K virus described by Kilham and Murphy (34) and the mouse adenovirus of Hartley and Rowe (35).

In selecting from this list of possible agents two factors were important. The mouse pneumonia model was required to compare with calf pneumonia but on the other hand the infectious agent had to be easily handled in vitro. From the above list two organisms are related to agents isolated from calf lung: Sendai virus and the mouse pneumonitis agent.

Sendai virus (parainfluenza 1) was an obvious choice based on the following criteria. It is stable at room temperature for limited

periods. It is easy to propagate, simple to demonstrate, reliably quantitated and produces well defined host lesions. In addition Andrewes and Pereira (36) state that it has a relative lack of pathogenicity for man. Since its original discovery in Japan by Kuroya and Ishida (37) the virus has been extensively studied by many workers (38).

Aerosol clouds for infection

It has been a common practice among experimental workers for many years to infect the respiratory tract by intranasal instillation of infectious fluids. Although there are reports in the 1940's of natural inhalation of virus mists, for example by Wells and Henle (39), the complexity of aerosol apparatus discouraged general acceptance of the method at that time. In 1944 Glover (40), using tubercle bacilli, and Lyons, et al. (41), using radio-active chromic phosphate, demonstrated the superiority of inhalation over nasal instillation 'which provides a rather direct route to the alimentary tract' (41). The work of Bowers, Davies and Hurst (42) showed how aerosol suspensions of influenza virus infected mouse lungs in a uniform and repeatable manner. In providing airborne infection it is assumed that a closer approach to natural infection is achieved. In the apparatus described by Henderson (43) care was taken to expose only the animal's face to the aerosol so that the airborne bacterial spores gained access only to the respiratory passages. In aerosol chambers not only does the animal inhale the aerosol but infectious droplets are deposited on its coat. When Henderson was using anthrax bacilli he found that, subsequent to aerosol exposure, the animal inhaled or ingested an additional dose when cleaning itself. Exposure

of the whole animal to viral aerosols may not therefore completely parallel exposure of only the respiratory system.

Quantitative methods in histology

The interpretation and indeed the preparation of histopathologic sections is empirical and subjective. In comparison with some other biomedical disciplines pathology has lagged in attempting to provide practical quantitative methods. The relatively new science of stereology seeks to redress this inbalance by giving the pathologist a third dimension to his two dimensional tissue section and simple economic methods for accurate measurement.

Stereology is a body of procedures which have the aim of obtaining information about three-dimensional structure from two-dimensional, flat images (44). Morphometric or quantitative descriptive data has been obtained in the past by various means, for example, classical anatomical dissection, wax plate reconstruction and serial sectioning; these methods require great technical skill and are extremely time consuming. When stereological methods are applied to problems the procedures can be efficient, economic and accurate.

The problems met in stereology are, in essence, well known to any morphologist. A section across any organ or tissue cuts randomly across regular structures and yields a variety of shapes for the same structure. Hollow structures often have folded internal surfaces which render appreciation of internal surface area difficult. The relationship of section thickness to structure size usually results in a considerable variation in the two-dimensional image.

The problems can be resolved using geometric probability to derive methods giving numerical data. Such methods pose complex mathematical

problems in their original derivation, but once the method has been established its application is simple and requires only elementary statistical analysis. A requirement of these methods, as in all quantitative methodology, is specific definition of the parameters to be measured. The properties of objects to be measured must be described differentially as must the images of such objects generated by random stereologic probes, in this case tissue sections.

As a further justification of the application of morphometric method to pathologic investigation it is interesting to quote Lord Kelvin who, in 1883 said '...when you can measure what you are speaking about and express it in numbers you know something about it; but when you cannot express it in numbers your knowledge is of a meagre and unsatisfactory kind: it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the state of science' (45).

CHAPTER II

THE HOST ANIMAL

Source of mice

C3H/He/fNMRI/Lac breeding pairs of mice were obtained from the Medical Research Council's Laboratory Animals Centre, Woodmansterne Road, Carshalton, Surrey. They were classified as Category five under the centre's published codes (46) and as normally-born successive generation gnotobiotes (29).

Breeding history

The C3H/He strain of mouse has been inbred since 1941. Following receipt of the original pair, breeding was by brother and sister mating only. The breeding schedule complies with the requirements of Parrott and Festing (47) and is laid out diagramatically in Appendix I, table 9. The mice used for initial experiments to determine the pattern of disease in a normal animal house environment (see Chapter 6) were taken from generations eight, nine and ten. Those used for a hot, wet environment from the ninth and tenth generations: for a cold, wet environment from the tenth and eleventh: for a hot, dry environment from the eleventh.

Phenotypic mutations were observed during the first eight generations but no attempt was made to investigate either the dominance or the penetration of these changes. Breeders for successive generations were selected for lack of the altered character. The mutations observed were two separate changes. The first presented as a shortening and blunting of the tail reducing the tail length by half an inch. The second showed a ring of hair, which lay in different directions from the remainder of the coat. The ring, about half an

inch in diameter, lay over the thoracic spine.

No spontaneous deaths occurred throughout the entire colony during the experimental period. Only fourteen deaths excluding vitamin K deficient animals (see next section) were recorded during the entire history of the colony; these all occurred during the first eighteen months (see Appendix II).

Nutrition and housing

The mice were fed exclusively on Oxoid Pasteurized Rat and Mouse diet *. Following irradiation of the diet (see next section) independent analyses of the dietary constituents, particularly amino acid content, were carried out; these showed that the levels were unaffected by sterilization (see Appendix III). The experience of Ley, Bleby, Coates and Paterson (48) shows that vitamin levels, which were above body requirements in this diet, are not significantly damaged by irradiation. Shortly after the colony was started a number of male mice died from internal haemorrhage. The addition of 0.5 mg/litre of synthetic vitamin K1 (Konakion 1) * to the drinking water prevented any further deaths from the same cause (49).

The mice in the breeding section of the colony were housed in polypropylene mouse boxes *, at a density not exceeding six mice per 50 square inches. The mice under experiment were held in similar boxes at a density not exceeding twelve per 76 square inches. They were provided with wood chips for bedding but no nesting material was supplied. Lighting was entirely artificial; daylight fluorescent tubes were wired to an automatic clock and provided 14 hours light per

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* See Appendix XX

day.

Definition of germ free status

Gordon and Pesti (29) define the germ free animal as one free from all demonstrable associated forms of life including bacteria, viruses, fungi, protozoa and other saprophytic or parasitic forms.

This section indicates firstly the procedures employed to create sterile conditions and secondly the apparatus required to maintain that sterility.

a) Sterilization methods

Three methods of sterilization were used: steam, gamma-irradiation and chemical sprays. Mouse boxes were autoclaved at 121°C for 15 minutes. Drinking water was sterilized in two litre square Pak flasks *, fitted with disposable self sealing caps, by autoclaving at 121°C for 45 minutes.

Gamma-irradiation from a Cobalt 60 source * was used at a dose rate of between 4.0 and 6.5 megarads. All diet and bedding, placed in screw-topped polythene jars, was passed around the radiation source and the dosage checked by perspex markers.

All surface sterilization was achieved with peracetic acid * at a concentration of 2 per cent (volume for volume) in distilled water to which had been added Nacconal 90F *, a wetting agent, at 0.1 per (50). Plastic isolators, isolator ports and covers etc. were either used as received from the manufacturer or washed with detergents and rinsed in tap water prior to spraying with peracetic acid. All equipment that had been either autoclaved or irradiated was surface

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* See Appendix XX

sterilized by wetting with peracetic acid prior to isolator entry.

b) Isolator design

Flexible film polyvinyl chloride isolators based on the original designs of Trexler (51) were supplied by either of two manufacturers *. Modifications to Trexler's original filter design were adopted. Instead of the "candle" filter which uses a wrap of fibre glass filter medium around an expanded metal tube a commerically available respirator filter cartridge * was fitted into a perspex housing using rubber 'O' rings for seals and the outlet filters taped directly into the isolator wall. The advantage of this design was simplicity of assembly and known filtration efficiency. (Appendix IV shows the filter design and tests for efficiency).

Air change rates in the isolators were measured using a method devised by Dr. D. K. Blackmore. Flow rates into the isolator measured with a flow meter were correlated with the internal pressure of the isolator using an inclined manometer. Different flow rates gave different internal pressures. A graph constructed over a wide rate of inflow against internal pressure gave a straight line. The flow rate required in any isolator was regulated by adjusting the main air line pressure manometer readings. The breeding isolators all had an air exchange of 14 changes per hour. The experimental isolators had an air change rate of 10 per hour.

The breeding isolators were maintained in an ambient environment of 21 ± 1°C. The relative humidity of air leaving the outlet filters was within the range 50-70 per cent.

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* See Appendix XX

Sterility testing methods

The quality of microbiological control determines the quality of the germ free animal. There is no positive method of establishing sterility therefore, of necessity, all tests proceed to a negative result. Monitoring for micro-organisms was placed on two levels to reduce the load of technical work. The first was a regular examination for bacteria and fungi carried out twice per month for each isolator. The second, a thorough examination for the presence of mycoplasma and viruses was performed after establishing the colony but before experimental work was started.

a) Bacteria and fungi

There is no single medium which will encompass adequate growth requirements for all common commensal and saprophytic bacteria and fungi as well as the commoner pathogens. The scheme proposed by Wagner (52) attempts to provide a range of media and environments that will locate and identify the more ubiquitous micro-organisms found in the air, on food and on skin surfaces. The tests employed are given in detail in Appendix V. 1. The main variation on Wagner's design is the exclusion of examinations at 56°C. Organisms capable of facultative thermophilia would appear at 37°C but strict thermophiles would not grow in the isolator environment. The use of 56°C would seem to have application only in the food industry.

The media chosen would not support the growth of several well known species of bacteria, for example lactobacilli or mycobacteria but it was not thought likely that such organisms should gain entry as monocontaminants.

Sodium thioglycollate, a widely used in medium for sterility testing (53), has been claimed to inhibit bacterial growth (54). For

this reason two additional broths, Brain Heart Infusion especially for aerobes and Robertson's cooked Meat for anaerobes, were included.

Organisms exposed to gamma irradiation may be damaged by disruption of their nucleic acids (48) but their ability to reproduce may not always be destroyed (55). Subsequent growth in broth media is frequently delayed: broths were incubated for a period of three and one half weeks to cover this possibility.

Isolator contamination was not common. Periods exceeding twelve months would pass without any breakdown. It was rare that the fault could be traced to a specific cause. The number of contaminated isolators that occurred in the course of these studies is shown in Appendix V. 2 together with the nature of the organisms isolated. Following a positive isolation from an isolator sample two further tests were immediately performed. If three consecutive tests had shown an isolator to be contaminated its use was discontinued. No contaminated animals were used for experiment except in two initial aerosol infections where the contamination is recorded.

b) Virus and Mycoplasma

Previously recorded examinations of germ free mice, including the C3H strain, for mycoplasma and viruses have shown that within the limited range of tests devised only carcinogenic viruses (excluding polyoma virus) appear to be transmitted vertically (56, 57, 58, 59, 60, 61, 62). Pollard (58) examined four hundred germ free rats and mice for viral agents using serum antibody detection, cytopathology, skin tests, primary tissue culture preparation from foetuses and kidneys, tissue culture inoculation of faecal material and homogenized viscera, exposure to irradiation and hormone stimulation. His only reported finding was intranuclear inclusion bodies in the lacrimal glands of

male rats over six months of age.

Perhaps the simplest and most sensitive detection of viral contact is the demonstration of specific antibody in the circulation. Examination for the presence of a virus disease in a mouse colony can only be reliably based on isolation or failure to isolate infective virus. However, any negative test does not answer the question " Is the agent present in the colony ? ". This problem is complicated further by the vexed question of latent, dormant or slow growing viruses.

When faced with the problem of detecting polyoma virus in a colony Rowe, Hartley, Estes and Huebner (63) developed a mouse antibody production test (M.A.P.). This test has been expanded for use on several murine viruses (64, 35, 65) and critically evaluated by Lewis and Clayton (66). The M.A.P. sampling technique, as devised by Rowe, is sensitive to successfully established endemic disease. It might be much less sensitive to quiescent virus in a newly established germ free colony where intermouse contact is limited to the small intra-isolator community. The following approach was devised to avoid the heavy technical load undertaken by Pollard (58). It is based on Rowe's M.A.P. test but aims to uncover quiescent virus by serial challenge (58). In order to increase M.A.P. sensitivity blind passage of organs suspected of harbouring virus was initiated using weanling mice whose maternal antibody level, if any, would be low but whose actively acquired immunity was likely to be underdeveloped. Sera from the final pass were distributed to laboratories having the expertise to make routine tests. In addition the organs and the tissues of the final pass were examined histologically.

Lymphocytic chorio-meningitis virus presented a special case

involving immunological tolerance and a different test procedure was carried out (67). No tests were run for either mouse leukaemia or Bittner's mammory tumour agent. Insufficient ex-breeding pairs were kept for their full life span to indicate the presence or incidence of these diseases but the evidence of Pollard and Matsuzawa (60) and Kajima (68) suggest that the former was probably present. Oncogenic viruses were thought unlikely to have a significant effect on the progress of acute respiratory disease in six to nine week old mice.

The list of agents sought, the organs passed, the histological screening examination and the number of animals used are recorded in Appendix VI. 1.

The presence of mycoplasms was sought in the respiratory tract, including the middle ear, but not in the brain nor in the joints. Since mycoplasmal infection may be inapparent, mice were stressed seven days prior to examination by passage of germ free mouse lung homogenate instransally. This test was also repeated using a large intranasal dose of Sendai virus suspended in allantoic fluid. The second test not only stressed the animal severely but also tested the possibility that mycoplasma were present in the original virus pool. The procedures for examination of the respiratory tract are recorded in Appendix VI. 2.

c) Clinical and post mortem observations

During the history of the mouse colony two different lesions were observed (neither occurred during the test procedures described above).

Female breeding mice, particularly at the age of five months and over, frequently developed severe alopecia. The condition did not affect all breeding females but once it had appeared it persisted. Hairlessness occurred all over the body but was seen initially along

the spine. Skin scrapings never revealed ectoparasites. No breeding irregularities were correlated with the condition. Histological examination of the lesions yielded no useful information: the epidermis appeared unaffected. No references were found in the literature to nutritional deficiences inducing alopecia of this type. A genetic mutation was observed by Dickie (69) producing alopecia; he suggested that the character might be semi-dominant.

An isolated occurrence in a ex-breeding male mouse was noted at autopsy. The liver was approximately doubled in size and contained a large circumscribed hepatoma or hyperplastic nodule. The lesion was not observed clinically - the enlarged caecum of older mice makes the abdomen appear distended.

Details of examination of these lesions are contained in Appendix VII.

CHAPTER III

THE INFECTIOUS AGENT

Source of virus

For the work in this thesis a freeze dried ampoule of Sendai virus was obtained from the National Institute for Medical Research, Mill Hill, London from a pool created and described by Sawicki (70). This was received, originally, from Dr. Jensen in the United States of America in 1955 and passaged twelve times in mice, once in rats, twice more in mice and finally 21 times in eggs. On receipt by the author, a pool created from one further passage in eggs was introduced to germ free mice. The lung homogenate from three mice was used to infect a further three mice and passage continued through nine groups of mice. The lungs from the ninth group were homogenized and reinoculated into a large number of eggs by the allantoic route. The allantoic fluid after dilution was stored in 1 ml. aliquots at -70°C.

In summary the passage history since receipt was as follows:-

Original stock received from Dr. Jensen

↓			
Mouse	x	12	
↓			
Rat	x	1	
↓			Passage conducted at Mill Hill
Mouse	x	2	
↓			
Egg	x	21	
↓			
Egg	x	1	
↓			Passage conducted since receipt
Mouse	x	9	
↓			from Mill Hill
Egg	x	1	

Identification of original sample

In the published literature on Sendai virus over the past 20 years Japanese workers have regularly recorded a named strain of virus in their experiments. However other workers merely refer to the 'Sendai strain' of parainfluenza 1 virus. At the moment there is no internationally agreed classification of parainfluenza substrains and therefore comparison between published reports is unreliable. In addition, the passage history of any strain will affect the final interaction of the parasite/host cell systems. It becomes exceedingly difficult, under these circumstances, to do more than examine a given ampoule of virus for the general characteristics attributable to Sendai virus and quote what is known of the original source and its subsequent passage history.

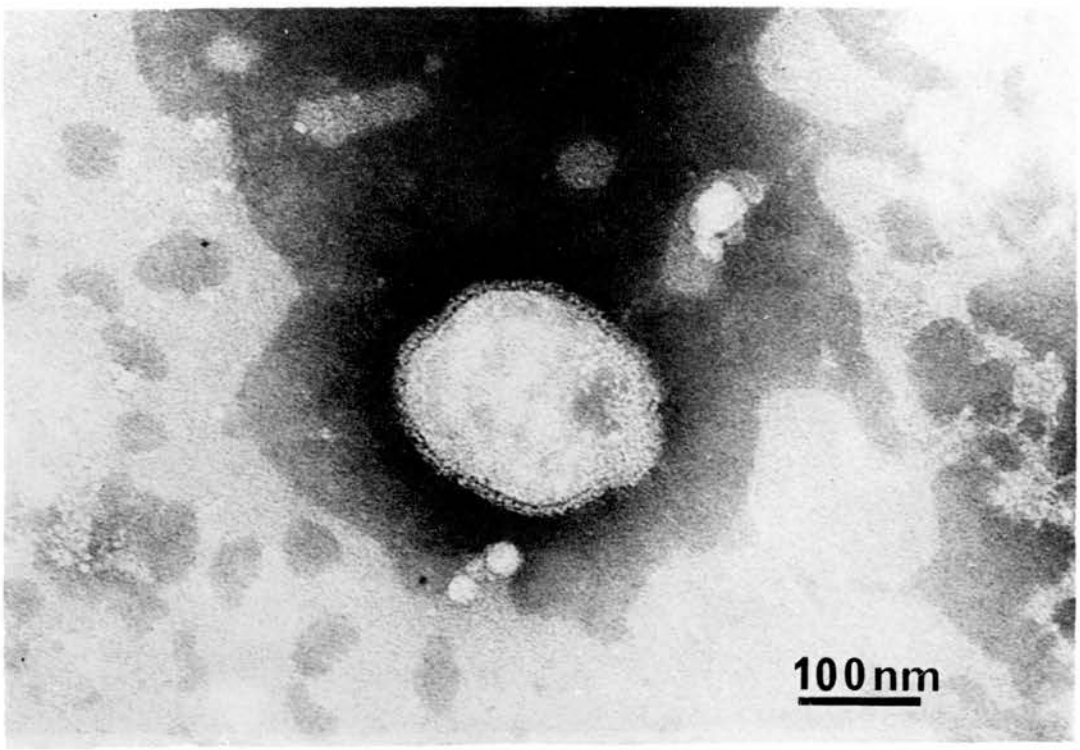
The virus in suspension was characterised by electron microscopy, growth in embryonated eggs, haemagglutination and its inhibition and by infectivity for mice. Detailed results are tabulated in Appendix VIII.

One frozen ampoule from the virus pool was thawed, a drop of the viral suspension placed on a copper grid, washed with distilled water, and negatively stained with phosphotungstic acid. After thorough air drying, the preparation was examined in the Phillips EM 300 electron microscope. Virions varying in size from 100-400 nm. were observed and disrupted virions resembling strands of ribonucleic acid were frequently seen. A detailed description of the virus untrastructure is given in Appendix VIII. 1; electron micrographs of the virus sample are shown in Figs. A, B, C & D.

The virus was inoculated into ten day embryonated hen's eggs by the allantoic route using 0.2 ml. of a 10^{-2} suspension of the frozen

Fig. A : An intact virion of approximately 150 nm diameter

Fig. B : Distorted virions up to 400 nm diameter illustrating the castellated appearance of the surface projections.


100nm

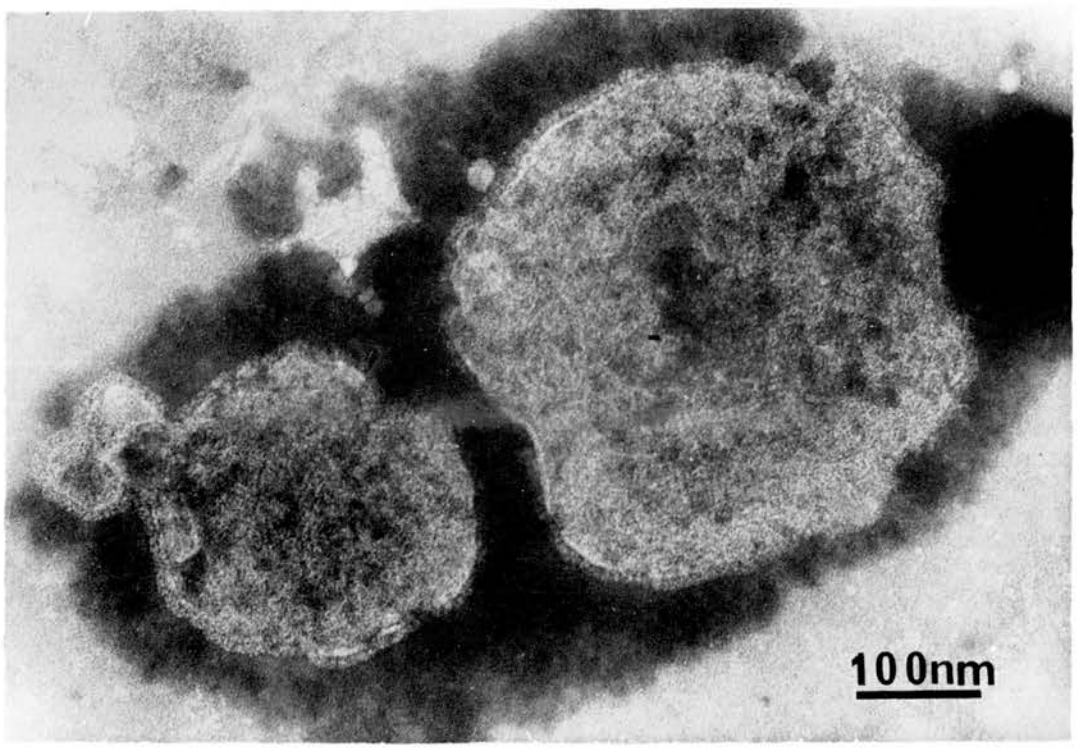
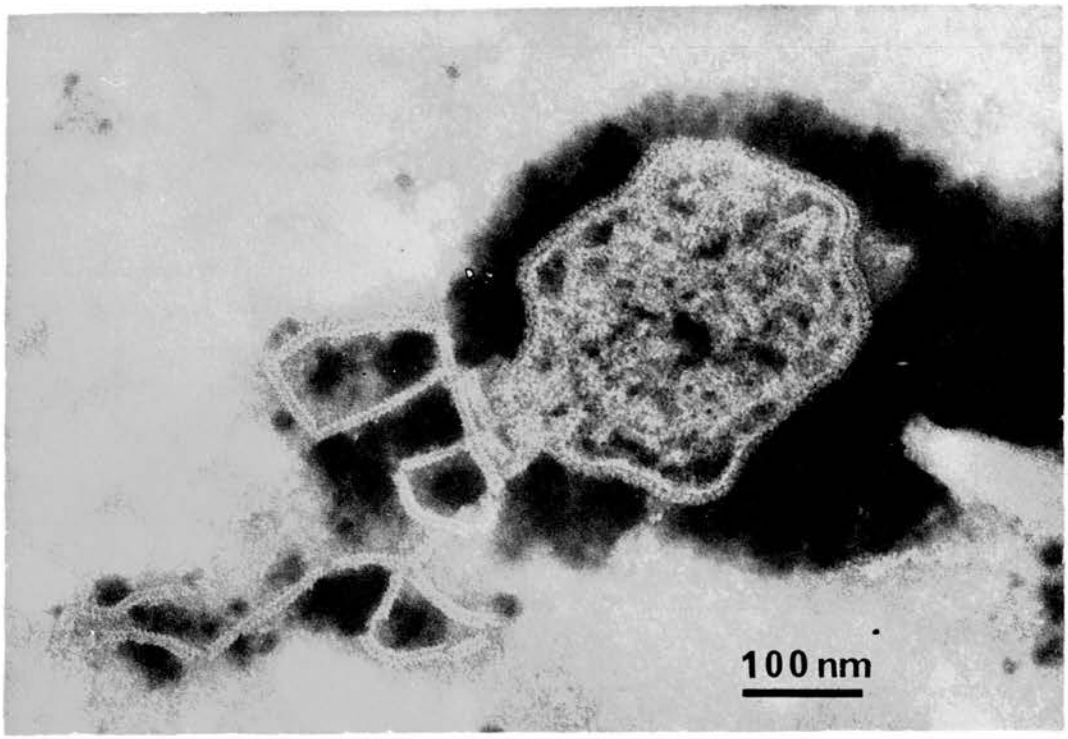
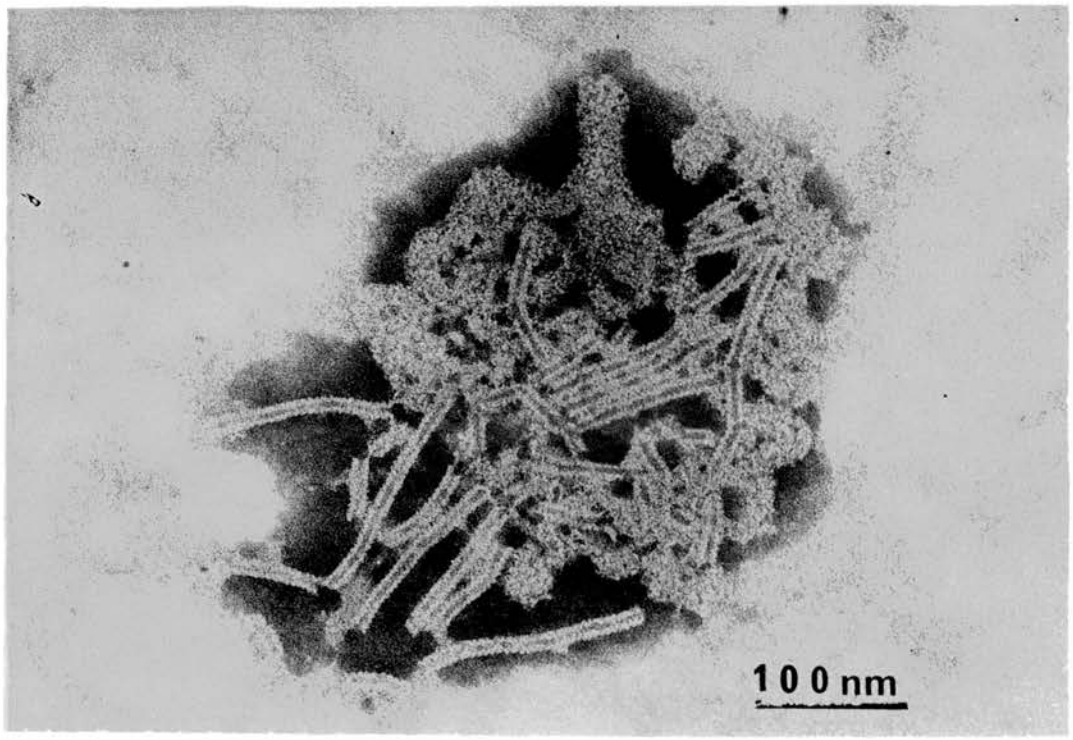

100nm

Fig. C

Both figures show the contents of partially disrupted virions.

Fig. D


100 nm


100 nm

pool. After three days samples of allantoic fluid would agglutinate erythrocytes from chickens, guinea pigs and human type O (33). At 37°C the virus eluted after one hour but at room temperature (18-20°C) agglutination was stable for over 24 hours. Haemagglutinin inhibition was used to demonstrate the specificity of this test. Commercial anti-parainfluenza 1 serum * showed complete specific activity.

The lesions produced by Sendai virus in mice have been studied by several workers (71, 72, 73, 127). The predominant feature is bronchial mucosal necrosis with an associated tissue reaction. That the virus employed in these studies produced typical lesions either when inoculated intra-nasally or after aerosol exposure is shown in detail in Chapter V.

Propagation and checks for purity

Suspensions of Sendai virus can be produced in bulk from harvests of either allantoic fluid of infected eggs or the supernatant from tissue cultures. As will be shown later a higher virus titre can be obtained from egg inoculation. The lung homogenate from the ninth group of infected germ free mice was inoculated at a dilution of 10^{-2} into a large number of eggs. After three days the haemagglutinating titre (HA) of each egg was estimated and those producing an HA at a dilution greater than 10^{-6} were pooled. The allantoic fluid from these eggs was mixed for one hour at 4°C and the mixture filtered through a 3 micron membrane filter *. The filtrate was diluted to contain an estimated 10^5 TCD50/ml. and divided into 1 ml. aliquots. This basic pool of virus suspension was stored at -70°C and a sample

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* See Appendix XX

used for each aerosol exposure.

Since adjacent laboratories had recently used parainfluenza 3 for infectivity experiments a test based on haemagglutination was run to exclude the possibility of related parainfluenza contamination (see Appendix IX).

The possibility of mycoplasma contamination either at source or from egg passage was exluded by attempts at direct growth on sub-culture. This test was combined with the examination of mice for mycoplasma (see Appendix VI. 2).

Demonstration of Virus

a) In fluid suspension

Quantitative demonstration of infective virus from three sources was required: the suspension in the atomiser used for infection, an impinger sample from the aerosol cloud and homogenized mouse lung after exposure to the cloud.

There are various methods of titrating Sendai virus but all are based on the demonstration of viral haemagglutinin. The most popular method described in the literature is egg inoculation via the allantoic route in 10 day old fertile hen's eggs (32, 23). The main virtues in this technique are firstly the marked sensitivity of embryonated eggs to the virus, enabling high titres to develop in a short time from small inocula, and secondly its simplicity. On this basis initial work commenced using egg titration. However, the principal limitations of the method soon became apparent, viz. poor economy and the large number of eggs required per dilution to obtain accurate end points.

For these reasons the technique of Fazekas de St. Groth (74)

using eggshell pieces was attempted. This combines the virtue of allantoic membrane sensitivity with minimal biological variation since one egg supplies sufficient pieces for several titrations. Although Sendai virus grew in the allantoic membrane, titres were always significantly below comparable figures for whole eggs. That method and not technique was imposing the limitation became apparent from independent tests in another laboratory where the technique was established for influenza virus titrations (75).

The third alternative is titration in tissue cultures (71, 76, 77). While the virus produced an easily interpreted cytopathic effect (C.P.E.) on cell sheets the strain used did not form easily read plaques on petri dish cultures, nor were the attempts to show haemadsorption on either plates or tubes successful. The C.P.E. could be read six days after inoculation but the haemagglutinating titre did not reach a maximum until eight days.

The different methods of virus culture are summarized in Appendix X. A choice between the three was made on their relative sensitivities and reliability to reproduce. Egg shell pieces were too insensitive. Whole eggs were sensitive but laborious to prepare for inoculation and large numbers were required for each dilution titrated. Tissue culture was sufficiently sensitive and gave the best economy for repeatable results. Continuous cell lines were insensitive compared to primary cultures which yielded significantly better results. These findings show that the strain of virus used was different in several respects from that reported by van Nunen and van der Veen (77). The reproducability and reliability of the selected test system was studied and the results given in Appendix XI.

b) In complete lung tissue

For the purposes of quantitative histology it was important to demonstrate the distribution of virus in mouse lung after infection. Two methods of locating viruses in tissue were used for light microscopy. After sectioning tissues can be stained either by acridine orange to identify the nucleic acids of the virus or by immunofluorescent techniques. In each case the stains attach to both 'living' and 'dead' virus, but if it is assumed that these two components of the infecting suspension are distributed in a Poissonian fashion, then qualitatively the distribution of 'living' virus is not altered.

The acridine orange method requires large quantities of virus for clear demonstration. Mice exposed to dilute aerosol of virus retain relatively little virus in their lungs. Arya (78) used an ingenious modification to circumvent this difficulty. Using influenza virus he allowed chicken erythrocytes to haemadsorb the infected tissue and then stained it with acridine orange. By identifying the nucleated erythrocytes he was able to locate the virus.

Arya's method was applied to cryostat sections of mouse lung infected with Sendai virus. It was not found feasible under dark ground illumination to distinguish between chicken erythrocyte nuclei and lung cell nuclei.

The immunofluorescent method offers two alternatives: either direct staining with labelled anti-viral sera or indirect staining using a commercially available conjugate. Since the indirect stain usually provides more sensitive detection, especially for small concentrations of virus (79), and since it also avoids the individual preparation of special conjugated sera it was chosen in preference.

It is common practice to prepare tissues for immunofluorescent staining by cryostat sectioning. Sectioning of mouse lung, even at a thickness of 10 microns was found to be difficult. The method of Sainte-Marie (80) with cold alcohol fixation and routine processing to paraffin wax was tried; although successful, the resultant fluorescence was much diminished. A method for cryostat sectioning using gelatin insufflation was developed. This method together with the other technical procedures involved in fluorescent staining are described in Appendix XII.

Stained sections, with appropriate controls, were examined by blue light using a quartz iodine source in a Leitz Ortholux microscope. The exciter filter was a 5mm BG 12 with a 4mm BG 38 to absorb red light; the barrier filter was K510. Where autofluorescence from lung connective tissue was at a high level counterstaining with 1 per cent aqueous Evan's blue solution for five seconds differentiated specific apple green fluorescence very clearly against a red tissue background.

CHAPTER IV

THE METHOD OF COMBINATION

The aerosol method

In Chapter I the work of Bowers, Davies and Hurst (42) was quoted to point to the advantage of infection by aerosol. Although the limited number of studies on comparative infection methods are not convincingly quantitated the results in this thesis complement and extend the work reported by Baskerville (81) and show that greater uniformity and repeatability can be achieved for a given set of experiments.

Experimental aerosols of infectious agents may be static or dynamic. A static aerosol is completely contained within a single chamber and decays primarily by gravitational settling of the particle cloud (82). When animals are exposed to static aerosols their activity disturbs the uniformity of particle dispersion and may produce variations in the infectious dose inhaled. Dynamic aerosols avoid this complication because the aerosol is continuously generated, then led into mixing chamber, before moving through a chamber containing animals. It is sampled at some convenient point, and disposed of by combustion or filtration (83).

Ambient conditions for Sendai virus aerosols

Experimental studies on the infectivity of aerosolized viruses reported in the literature do not include observations on Sendai virus. Parainfluenza 3 was studied by Miller and Artenstein (84) and other myxoviruses notably, influenza and measles, have received attention (85, 86, 87). Summarizing the data from these experiments and others Benbough (88) suggested that 'viruses with structural lipids survived

best in aerosols at low relative humidity, while ether-resistant viruses without structural lipids generally survived best at high humidities'. He pointed out that earlier work by Harper (89) indicated how the composition of the suspending medium in the atomizer was critical and, in his own later experiments, correlated with the relative humidity (90).

In contrast to these carefully controlled experiments with laboratory systems the result of workers in the 1940's, some of whom are quoted by Bowers et al. (42), together with the observations of van der Veen, Poort and Birchfield (91) suggest that animals can be successfully infected at much higher relative humidities. van der Veen et al. (91) offer as a possible explanation that low relative humidity has an adverse effect on nasal mucus secretions. There is no substantiation of this statement and indeed the work of Andersen, Lundqvist and Proctor (92) would appear to contradict it.

In general, therefore, the reported work on aerosolized viruses for animal infectivity experiments contains several contradictions. Relative humidity is probably important and experimentally the suspending fluid composition may be critical. Other external factors such as atmospheric gases, sunlight and temperature are all likely to affect virus infectivity.

In practice systems must be selected empirically taking into account previous experience. A suspending fluid of buffered salts with a low concentration of protein was selected from a series of successful infectivity experiments being performed at the Central Veterinary Laboratory, Weybridge, using Infectious Bronchitis virus with chickens. On the advice of Mr. G. J. Harper a relative humidity of 30 per cent was selected at a convenient room temperature of 21°C.

Infections were conducted in artificial fluorescent light and so avoided natural radiation from the sun. Ordinary room air, after filtration to remove extraneous microorganisms, was used to drive the atomizer and provide a secondary air stream.

A standard infection procedure was adopted for all experiments described in this thesis. These conditions are described in the next section. The composition of the suspending medium is given in Appendix XIII.

Description and operation of apparatus

The aerosol apparatus was designed on the principles laid down by Henderson (43). The actual construction was simplified and the infecting chamber modified (Fig. J). The design is described in detail in Appendix XIV but can be briefly summarised as follows:

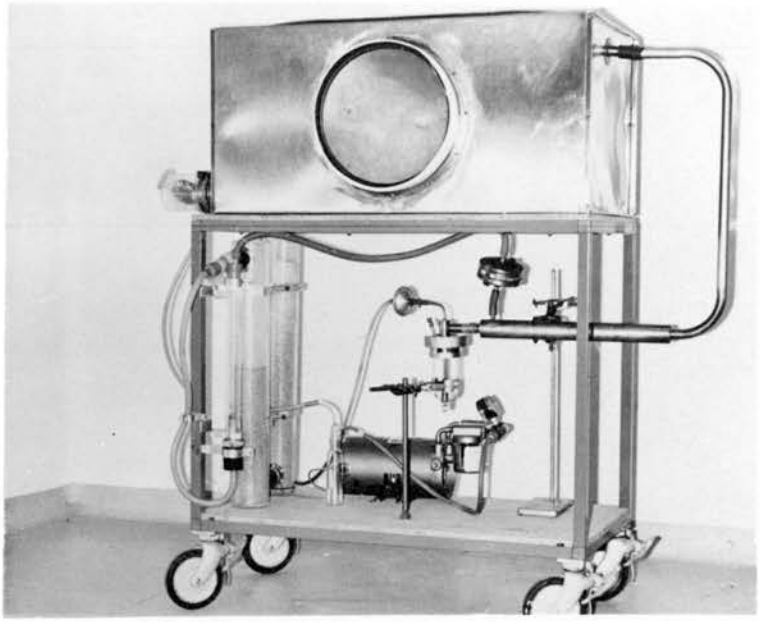
The exposure chamber was a stainless steel cuboid, 30 x 18 x 18", fitted with one inlet and one outlet for the aerosol stream. On the top was a toughened glass window approximately 24" long and 16" wide. One side was fitted with a pair of heavy gauge rubber gauntlets and the opposite side had a 12" diameter entry port fitted with a steel cover. It contained two open mesh cages to hold mice; these were subdivided to prevent the animals from huddling (Fig. K).

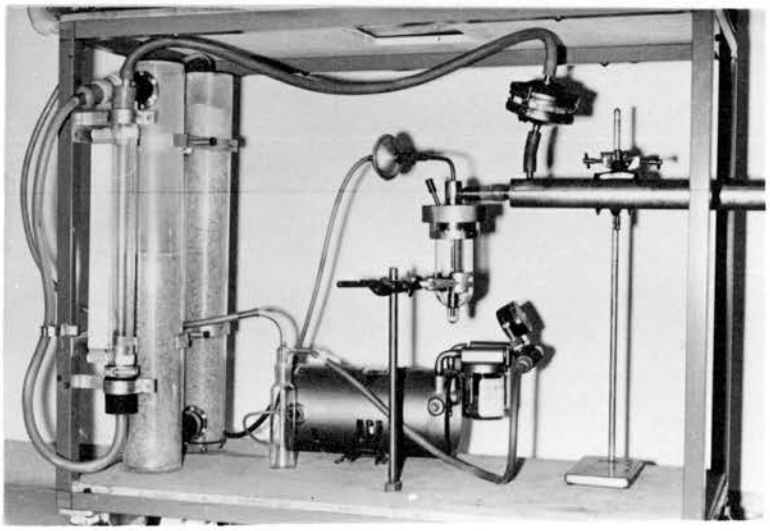
The aerosol was generated in a Collison 2 jet atomiser of the type used by Henderson (43) and passed into a mixing tube. The tube was 16" long, 2" in diameter and connected directly to the exposure chamber. Attached to the mixing tube adjacent to the Collison spray was a filtered inlet for secondary air (Fig. L).

Fig. J : Complete aerosol apparatus and exposure chamber.

Fig. K : The aerosol exposure chamber.

Fig. L : The aerosol generator and sampling impinger.





Compressed air was provided by a rotary compressor *. Exhaust aerosol which was driven by compression not sucked by vacuum pump as in Henderson's original design, passed through a filter cartridge * and after filtration through a thermohygrometer *. The secondary air supply was dried by passing through two towers containing silica gel.

Prior to operation the apparatus was dismantled into three units: Collison spray, mixer tube and exposure chamber. These were sterilized individually, the spray and mixer tube by autoclaving (121°C x 15 minutes), and the chamber by dry heat. When placed in the autoclave the chamber window was sucked inwards from its gasket mounting * during the alternate vacuum and pressure cycles. Hot air sterilization at 100°C for one hour did not damage the rubber fittings and, although this may be theoretically inadequate, never allowed contamination in subsequent experiments. The entry port was protected by a plastic film * and other openings by cotton wool plugs.

The apparatus was assembled under positive pressure air flow starting with the mixer tube and attaching the air supply. The final filter, which was not previously sterilized, always remained downstream from the chamber. The entry port was connected to an animal isolator by a conventional plastic sleeve and the dead space sterilized by peracetic acid spray. When the plastic connecting sleeve was sterile the inner port cover of the animal isolator was removed and excess acid mopped up. The sleeve was left for 24 hours to ventilate.

Immediately prior to use the Melinex film was ruptured and the wire mesh cages passed into the isolator. Mice were distributed in the cages so that no more than three mice occupied one subdivision. When the mice had been placed in the chamber the entry port was shut

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* See Appendix XX

by the steel cover. The secondary air supply was switched on and run until the chamber air had a relative humidity of 30 per cent. At this point exposure commenced. The Collison spray, operated at a pressure of 15 lb/in^2, generated a fine cloud of particles which were dried and so shrunk by the dry secondary air stream (43). The airflow through the Collison was 15.6 litres/minute and the secondary airflow 100 litres/minute. The infective cloud was generated for 30 minutes. At the end of this period the air stream was switched off, the port cover removed and the mice returned to the isolator. The isolator port cover was replaced and the exposure chamber disconnected.

Sampling of aerosol cloud

In their discussion of the Henderson apparatus Dimmick and Hatch (83) quote, among others, the following points as being important: that the temperature and humidity of the air at the point of exposure is known and sampled and that samples are representative of the true aerosol in terms of both physical and biological content. They also require that aerosol output is either constant or changes are measured through the exposure period.

Physical problems arise, since direct readings of humidity in a sterile chamber demand that the measuring device be sterilized. By measuring both temperature and humidity of the outflowing chamber air, after filtration, an accurate record could be kept throughout animal exposure time. For some undetermined reason the humidity within the chamber varied with that on the outflow line but this variation was linear and was plotted prior to infection experiments so that the true chamber readings could be obtained. For each individual experiment the temperature and range of relative humidity are

given.

Samples were taken from the outflow line of the chamber i.e. after contact with the mice but before final filtration. They were collected in a standard Porton all glass impinger * (43) with a limiting orifice that delivered 10.75 litres/minute. The impinger was filled with 10 ml 0.2 per cent gelatin buffer solution containing 0.1 per cent of anti-foam agent *.

Aerosol output was regulated by applying constant pressure to the atomizer and regulating the flow rate of secondary air by a flow meter and valve.

To determine that the chamber contained infective particles small enough to enter mouse lungs, a short series of pilot experiments was performed. Bacillus globigis spores at a concentration of approximately 10^7/ml in 0.2 per cent gelatin buffer solution were sprayed from the Collison atomizer and mixed with a secondary dry air supply. The chamber was filled by the mixed spray and secondary air for 30 minutes and samples collected by a cascade impactor (93) at 5, 10, 15, 20 and 30 minutes. The details of this test are given in Appendix XV. In summary when the chamber had a relative humidity of 30 per cent at a temperature of 21°C particles were produced in the following proportions:

2.5 per cent	20-3 microns
21.5 per cent	7-1 microns
76.0 per cent	3-0.7 microns

Maximum concentration of aerosol was reached after five minutes and remained constant thereafter up to 30 minutes. These results are in

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* See Appendix XX

accord with the findings of Beard and Easterday (94).

It is in the category 3-0.7 microns that particles are claimed to penetrate the upper respiratory passages and reach the airways of the lung (95). No attempt was made to correlate this distribution of bacterial spore with infectious virus particles. If a test was run mixing virus and spores suspensions in the atomiser then the infective particle size, which shrinks in a dry air stream would nevertheless be likely to be much larger than for virus alone. The tests described, therefore, only establish minimal efficiency in the chamber function.

Distribution of virus in mouse lung

Having determined the ability of the aerosol apparatus to produce a heterogeneous range of particles with the largest proportion below 3 microns in diameter, work proceeded directly to discover where virus was deposited in mouse lungs after aerosol exposure. Whatever measurements can be made on particle number, size and infectivity of virus aerosol clouds, the final deposition of infective particles can only be determined directly in the lung. The work of Davis, Griesemer, Shadduck and Farrell (96) using Adenovirus 12 is an excellent example of the disparity between a theoretically calculated dose and the amount of virus actually recoverable from exposed animal lungs.

Groups of mice were exposed to the standard aerosol dose under standard conditions as described earlier. By choosing a 30 minute exposure time it was hoped that a greater uniformity in airway distribution of virus would be achieved and so yield greater efficiency when applying random sampling to histological sections of lung. After exposure the mice were held for three days then sacrificed; the lungs

were removed and frozen.

Blandford and Heath (97) showed that virus was easily seen in the bronchial mucosa by fluorescence three days after infection but virtually no shedding of infective material was detected. At this stage only infective virus that had replicated locally following primary infection would be likely to be seen. Non replicating deposits would be likely to have been removed by phagocytosis. Production of immunofluorescent stained sections from these lungs is described in Appendix XII.

For examination fluorescein stained sections were placed on the microscope stage and, using x10 eyepieces, x10 objective lens and a dark ground condenser, the length and breadth of the sections were measured with the stage micrometer. The centre point of the section was calculated and the section divided arbitrarily into four quarters. Each quarter was examined for specific fluorescence within complete airway cross sections. Under dark ground illumination non-stained airways were not always seen. In order to calculate the proportion of infected airways the sections, after fluorescent counting, were washed free of glycerol mountant and stained with haematoxylin and eosin. Using the same stage micrometer readings the number of complete airway cross sections were counted.

14 mice were examined in 4 experiments. The actual number of airways was compared to the number of airways infected and analysed by the χ^2 test. The details of these experiments and the statistical analysis are contained in Appendix XVI.

In summary it was shown that for any one environmental treatment if two mice were sampled at each time interval then 44 ± 12 per cent of their bronchial cross sections examined histologically would have

received an infective dose of virus from the aerosol.

CHAPTER V

THE MEASUREMENT OF THE REACTION

Range of reaction

Previous accounts of experimental Sendai virus infection in mice indicate variation in the severity of response. van Nunen and van der Veen (77) and Degre and Glasgow (98) reported deaths: the number of fatalities was related to the size of infectious dose. Robinson et al. (71) and Appell, Kovatch, Reddcliff and Gerone (99) stated that Sendai virus infection neither produced overt clinical signs nor caused prominent post-mortem lung lesions, except on rare occasions. Fukumi et al. (100) and Sawicki (70, 101) reported a greater susceptibility in newborn than in adult mice. van Nunen and van der Veen (77) found no significant difference in susceptibility between four and twelve week old mice nor between male and female mice.

The mouse passaged strain of virus defined in Chapter II, when used to infect six to nine week old germ free mice with the standard aerosol dose, produced no clinical signs and no deaths. When large doses of virus were instilled intranasally mice showed clinical signs after seven days: huddling, shivering and ruffled fur were observed, and by nine to ten days all inoculated mice died. These observations are in accord with the published reports quoted above.

Fixation, processing and staining methods

Fixation of lung material poses conflicting problems. How shall fixative be brought into contact with the deeper structures while normal intra-thoracic architecture is maintained? This problem has been considered by many workers but no complete solution has been found (102). Each experimentalist must choose from the variations a

method best suited to his own purpose. In these studies the prime interest was to examine the bronchial mucosa under standard conditions using the light microscope.

A variety of methods was examined. Initially the technique of Forssmann et al. (103) was used; this employs vascular perfusion via the abdominal vena cava. Capillary vasoconstriction is blocked by perfusing 0.1 per cent procaine hydrochloride in physiological Ringer solution and the perfusate then switched to a glutaraldehyde/formaldehyde mixture. Although excellent fixation of lung tissue was achieved using this technique it was not uniform throughout the entire lungs. Since subsequent procedure demanded random sampling such a limitation could not be accepted. Gil (104) discussed the alternative method of fixation from the alveoli. The method of formalin steam used by Weibel and Vidone (105) is generally regarded as unsatisfactory in terms of preventing excessive shrinkage. Tracheal instillation under pressure was reported for rat lungs by Fawell and Davies (106) and Egberts (107). One experiment was performed using this type of fixation. Using a pressure of 10 cm of water lungs were expanded with fixative so that they completely occupied the thoracic cavity. The histological appearance of bronchial mucosa after tracheal instillation is variable and differs from simple immersion fixation. The number of epithelial cells lying on unit length of basement membrane is reduced; many cells are shrunken or deformed in an irregular manner.

The final choice of method was a compromise. After mice were killed by cervical fracture the trachea was exposed and ligated before the thorax was opened. This prevented undue lung shrinkage. The respiratory tract was then dissected out, distal to the larynx, taking

care not to damage the pleural membrane. The ligating thread was tied to a metal weight and the lungs immersed in fixative so that all surfaces were submerged under equal pressure. The lungs were probably inflated below their functional residual capacity but muscular contraction around the airways was insufficient to create folds in the lining mucosa on microscopic examination. The selection of 12 per cent neutral buffered formalin was arbitrary; it produces as little artefact as any other fixative and the author was accustomed to its use.

There is a range of materials and methods available for tissue processing but, in all procedures, the minimum of distortion and shrinkage is to be preferred. Prime concern was given to producing thin sections of complete lungs through their antero-posterior plane. Ideally sections would be about one micron thick. This thickness is not easily cut with a steel knife and cannot be cut repeatably. Thin sections can be cut with a glass knife but the size of section is limited; the cutting face is less than half the width of the embedded mouse lung. Faced with the need to use steel knives the next problem was choice of embedding medium. Ruddell (108, 109) has suggested one solution: tissues may be embedded in hydroxyethyl methacrylate. This plastic is soft enouth to allow sectioning by steel knives and does not significantly affect subsequent tissue staining. The method was used on mouse lungs to produce two micron sections with some success. However, the technical snags such as frequent resharpening of knives and unreliable curing of the monomer were wasteful of time and effort, so it was decided to revert to standard paraffin wax media.

It became apparent that after fixation the greatest shrinkage of tissue occurred during dehydration. It was estimated that the partially inflated lungs shrank by up to 40 per cent after dehydration in graded

alcohols and before clearing or wax impregnation. Unfortunately few methods avoid dehydration without providing greater obstacles at some stage of processing. It was assumed that this shrinkage was common to all the lungs processed. The quantitative results obtained suggest that the range of variation between individual mice was small.

Processing from water soluble fixative to wax embedding is given in Appendix XVII. 1. Blocks were cut on a base sledge microtome * set to cut at four microns. The actual section thickness was estimated to be in the range four to five microns but absolute measurements were not required. Trials with a fibro-wax * gave no better results than standard paraffin wax *. Sections were cut routinely from five levels in the lungs. After trimming the block to about three quarters of its maximum surface area the first section was taken. The remaining four sections were taken at intervals of 400 microns (see Appendix XVII. 2).

When stained, sections had to demonstrate two principal features: basement membranes of branching airways and the nuclei of epithelial cells lining the airways. This was achieved by firstly impregnating the sections with a silver solution as in Gordon and Sweet's method (110) and secondly instead of proceeding to a neutral red counterstain, which gave indifferent cellular differentiation, staining overnight in Giemsa solution. The Giemsa was freshly prepared as a 4 per cent solution in 0.01 per cent acetic acid having a final pH of 3.6. The results of the reaction showed reticulin, collagen and elastic fibres as black staining structures, nuclei as blue to violet and cytoplasm greyish pink. The silver deposits tended to impregnate nuclei,

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* See Appendix XX

nuclear and cytoplasmic membranes and altered the normal Giemsa stain by a greyish discolouration. Nonetheless the epithelial cells could be clearly seen lying on a continuous line of basement membrane (BM). A similar variation on Gordon and Sweets' method was recently published by Lyon and Prentø (111). Details of stain preparation and method are given in Appendix XVII. 3 and 4. Sections were stained in batches of 65 so that sections from one complete experiment (26 lungs with five sections from each) could be stained in only two separate batches; this reduced individual stain variation. After mounting in DePeX the sections were presented for examination in random order.

Normal histology of mouse lung

A brief account of the histology of mouse lung is given by Hummel, Richardson and Fekete (112). Many texts, in describing the general histology of the respiratory system, refer to the mouse for comparison. One of the most complete of these accounts is provided by Krahl (113).

The component of the respiratory tract relevant to the experiments described in this thesis starts where the bronchi enter the lung and ends at the entrance to the alveolar ducts. Hummel et al. (112) described primary, secondary and tertiary bronchi, terminal and respiratory bronchioles. According to the definitions given by Ham (114) all airways in the mouse lung proper are bronchioles. Krahl (121) stated specifically that the mouse has no respiratory bronchioles. In practice, regardless of terminology, there is a graded change in size and quantity of tissue components as the bronchial tree divides. Two helical bands of smooth muscle enclose each airway and this muscle coat reduces progressively down the bronchiolar tree. Bronchioles

are lined by a single epithelial cell layer; these cells may be ciliated or non ciliated. Goblet or mucus-secreting cells are rare; there are no sub-mucosal glands. The epithelial cells are plastic in that they can alter in shape from columnar to cuboidal following muscular contraction and relaxation. When fixed, as described above, the majority of normal cells were cuboidal. At the distal extremities of the branching airway sometimes only one side or a strip of bronchiolar wall opposite to alveolar duct openings may be so lined. Adjacent to the BM are varying numbers of oval nuclei frequently lying parallel to the membrane. These are formative cells which give rise to new ciliated cells; they take up stain less well than established cells. The regular cuboidal epithelium has basal nuclei which stain deep blue-black using the silver and Giemsa method. The nuclear chromatin does not stain with a uniform density.

The epithelial layer rests on a lamina propria which is relatively acellular. This comprises a thin continuous BM underlying which are irregular bundles and strands of elastic tissue. In the germ free mouse very few lymphocytes lie within the lamina propria although accumulations of lymphocytes are sometimes seen near bronchiolar bifurcations.

The turnover rate of these components has not been clearly established. On the basis of work in rats by Bertalanffy and Lau (115) the ciliated epithelial cell of the bronchiolar airway has a life spun of approximately 27 days. Walker (116) has attempted to give an indication of turnover rates for BMs. In rats the replacement of silver impregnated membranes in the colon was within six weeks and in the renal glomerulus not more than every six months.

Pathology of Sendai virus infection

The macroscopic feature of Sendai virus infection were minimal. Lesions, which appeared seven days after infection, did not exceed 1 x 1 mm in size; they had no general pattern of distribution. Their appearance varied from slight depression to slight elevation in relation to adjacent lung surface. Their colour ranged from a dull reddish purple to a dull greyish purple as the lesions aged. No adhesions between adjacent pleural surfaces were seen. Since macroscopic lesions were so slight and, because it was not possible to appreciate changes beneath the pleural surface, no attempt was made to correlate these observations with the disease process.

The histological changes in conventional mice are well described by Robinson et al. (71) and mentioned by Appel et al. (99) and by Degre and Glasgow (98). The changes seen in germ free mice are qualitatively very similar but differ in degree of response and in some lesser details. Furthermore the observations made here followed aerosol infection whereas previous reports (71, 98) refer to intranasal instillation of virus.

The lesions described resulted from active replication of virus in the respiratory epithelium since exposure of control groups of mice to inactivated virus * did not produce any pathological lesions.

For simplicity the events will be examined on a time scale taking individual components of the lung tissue separately. These can be conveniently divided into bronchioles, alveoli and blood vessels (arterial and venous). No study was made of changes in the trachea

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* See Appendix X.6

nor any part of the upper respiratory tract, since the mucosa varies in thickness usually being more than one cell layer thick. Such a pattern poses greater problems than a simple cuboidal epithelium for quantitative analysis.

a) Histological changes in bronchioles

Earliest histological changes were observed two days after infection. These were slight, affecting few cells, but were recognised as a disturbance of the orderly arrangement of epithelial cell nuclei with an occasional cell containing a pyknotic nucleus. Some non-specific cellular debris was found in the bronchiolar lumen accompanied by an occasional polymorphonuclear neutrophil (PMN).

On the third day cellular changes were distinct. Discrete groups of epithelial cells showed partial loss of their staining affinity. The PMN response was marked with cells appearing in the connective tissue (CT) surrounding the airway, migrating through the mucosa and entering the lumen. A smaller number of small mononuclear cells were found among the PMN, in the CT sheath and muscle coat.

By the fifth day the epithelial cells not only failed completely to stain with Giemsa but in many sites appeared to diminish in height. (This tendency to flattening in the mucosa may parallel the events seen in organ culture (117) when cell loss is accompanied by a spreading and flattening of adjacent cells in an attempt to cover the exposed BM.) It was not possible to distinguish between normal basal nuclei of the established mucosal pattern lying parallel to the BM and supposedly flattened cells adjacent to necrobiotic foci. While PMN and limited mononuclear cell infiltration of mucosa and lumen continued, the total numbers of inflammatory cells tended to diminish.

The seventh day following infection revealed areas of mucosa

lined only by cellular debris. A few karyolytic nuclei could be traced in these damaged areas. In contrast with affected areas of mucosa the epithelial cells lying adjacent to lesions stained normally and had no apparent morphological abnormality. The exudation of PMN remained similar to day five but increasing numbers of such cells were pyknotic. The number of mononuclear cells increased both in the CT around the airway and in the lumen.

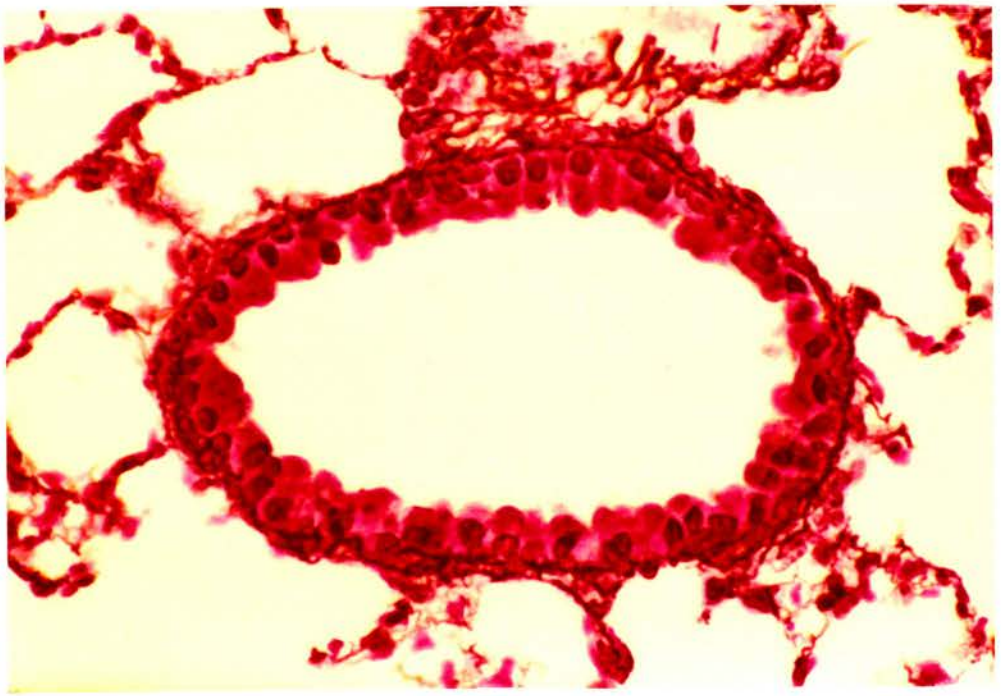
Total desquamation of epithelial cells was clearly seen by the ninth day. In larger bronchioles the reaction was not so positive: ghost outlines of both cytoplasmic and nuclear membranes could be seen (silver impregnation); but in smaller bronchioles the entire mucosa had disappeared leaving a bare BM. Again there was a variable quantity of cellular debris overlying the mucosa of large bronchioles while the smaller airways contained little or no debris. In each case an accumulation of mononuclear cells in the CT sheath was becoming prominent.

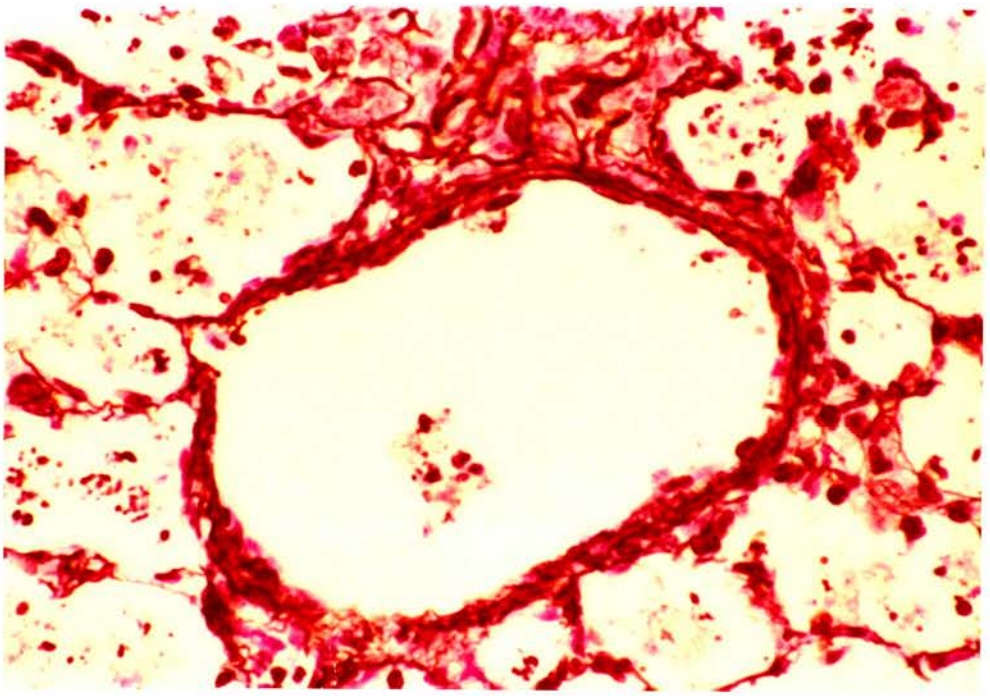
By the eleventh day the destructive effects of the infection began to wane. Widely distributed areas of denuded BM could be found but the PMN response was slight and irregular (see Fig. F). Regeneration of epithelial cells was apparent.

Examination of the epithelial mucosa on alternate days after the eleventh revealed a marked regenerative response. The pattern became less specific on a fixed time scale with a very variable rate of regeneration within the same lung. Characteristically many cells with long thin nuclei appeared grouped on the mucosa. The reaction was not confined to specific foci of previous damage but sometimes affected the entire bronchiolar cross section. Neither nucleus nor cytoplasm took up Giemsa stain well. The nuclear stain reaction was a light blue

Fig. E : Normal bronchiole before exposure to virus: stain Silver and Giemsa. (magnification x 200)

Fig. F : Infected bronchiole ten days after virus exposure: stain Silver and Giemsa. (magnification x 200)





black compared with the deep purple of normal uninfected tissue but considerable variation was found. Among these regenerative cells there was no evidence of infiltrating leucocytes nor was there any appreciable debris in the bronchiolar lumen. From the eleventh day progressively more bronchioles demonstrated this hyperplastic regenerative response. In any infected lung some bronchioles appeared unaffected showing neither evidence of earlier cellular damage nor of regenerative hyperplasia. The distribution of these normal bronchioles did not have any recognisable pattern.

The accumulation of mononuclear leucocytes, predominantly lymphocytes and plasma cell types, increased around some airways but there was no obvious correlation between mononuclear 'cuffing' and the degree of either cell damage or regenerative response.

After the nineteenth day the thin narrow columnar cells became smaller and took up Giemsa stain normally. The appearance was of a densely packed cuboidal cell mucosa with many cells overlying each other and without any visible increase in BM length. The cellular density reduced progressively thereafter to a normal single cell layer.

b) Histological changes in alveoli

The alveolar reaction following Sendai virus infection is not relevant to the stereological model evolved in this thesis but will be described briefly.

Infection of alveolar cells may occur directly by aerosol droplets or subsequent to bronchiolar infection by endobronchial spread. It would appear that in these experiments endobronchial spread was of little significance with respect to alveolar infection. Foci of damage were few and had no evident pattern of distribution. In general, by the fifth day, small areas of alveolar septal thickening were

observed accompanied by partial collapse and emphysema. The thickening of alveolar walls was caused by proliferation of septal cells with a few infiltrating PMN. By the seventh day these lesions were more prominent with foci of alveolar collapse: there was no evidence of consolidation nor of fluid exudate. By the ninth day complete collapse was seen with a slight increase in PMN probably in the alveolar luminal spaces. Such lesions did not appear to re-aerate following total collapse. The cellular density decreased after the eleventh day and PMN were not seen. Small foci of pseudo-epithelialization were sometimes seen among collapsed foci. Consistent with the altered stain reaction in affected bronchioles the distorted architecture of these alveolar areas was also pale staining. By the twenty fourth day these lesions appeared to contain fibrocytes and collagen fibres with a streamed and whorled pattern. Normal architecture was not regained during the limited span of these experiments.

By contrast a mild reaction was occasionally seen. In a wide zone surrounding an affected bronchiole the alveolar lumina filled loosely with PMN and macrophages but there was no apparent septal proliferation. This picture was not seen after the ninth day.

c) Histological changes in and around blood vessels

By the third day following infection many arterial vessels, accompanying affected bronchioles, showed swelling of their endothelial cells together with margination of leucocytes where the intima was alongside the airway.

This reaction increased in intensity by the fifth day and leucocytes were seen lying on the intima and filling the CT sheath around the vessel. The reaction extended to several vessels both

arterial and venous which were not close to any alveolar or bronchial reaction.

By the ninth day distinct "cuffs" of mononuclear leucocytes were seen around several vessels. The cuff could extend around both a bronchiole and bronchial arteriole but developed with equal intensity around venous channels remote from bronchiolar or alveolar reaction sites.

By the nineteenth day the "cuffing" was either clearly established or had resolved leaving only a few mononuclear cells in the CT sheath. At this time the cuffing around blood vessels was much more prominent than the around airways.

d) Comparative observations

A number of interesting comparisons were found between the results just described and those of previous workers. Initially it must be remarked that Degre and Glasgow (98) and Robinson et al. (71) used intra nasal fluid instillation and found a peak in virus recovery at three days. Appel et al. (99) used aerosol infection and produced a peak in virus recovery at six days. The results recorded in this thesis, using aerosol infection, are similar to those of Appel et al. (99). Despite this difference the descriptive histology in all cases is remarkably similar.

In the experiments referred to above, which were performed with conventional mice a marked peribronchiolar oedema was observed by the third day. In the present studies, using germ free mice, no oedema was seen. Likewise in conventional mice the numbers of PMN seen both in the bronchiolar reactions and in the alveoli were greater than in germ free mice. Both these differences, together with absence of metaplasia in germ free mice, may well be explained by the additional

insult due to multiplication of commensal bacteria in the conventional mice. (The numbers of bacteria in the latter were not sufficient for organisms either to be cultured from lung homogenate or seen in Gram stained histological sections. However the observations of Degre and Midtvedt (118) comparing germ free and conventional responses to Sendai virus infection strongly support this contention).

Heath (119) stressed the early and consistent accumulation of mononuclear leucocytes around airways. It is interesting to note that in the experiments reported here a perivascular cuff was the more prominent.

One experiment was performed under the standard experimental conditions described in Chapter IV using mice that had been "conventionalized". These mice were removed from the sterile isolator four to six weeks prior to exposure and fed on unsterilized diet, littered on nonsterile wood chips and given ordinary tap water. They were exposed to direct human contact but were kept apart from any other species or other mice. Following exposure to the viral aerosol virus was recovered from homogenized lung at titres similar to those of germ free mice. However the clinical, post mortem and histological reactions were different from the standard germ free pattern. Three per cent of the total number of mice exposed died two weeks after infection. Clinical signs of sickness - huddling, reduced activity, hyperventilation - were exhibited by about 50 per cent after ten days. The histological appearance of the lungs showed many more PMN infiltrating the airways, an accumulation of mucopurulent material in their lumina and by three weeks some mice had multiple abscessation of one or more lobes. The alveolar reaction was more extensive than in germ free mice.

While the lesions could be classified as typical of bronchopneumonia the most interesting feature of the bacteria-contaminated mice was the marked variation in individual response. 50 per cent only of the mice appeared clinically ill. Of the lungs examined after two weeks approximately one third of the total revealed no greater lesion than a mild, and presumably resolving bronchiolitis. This result extends the findings of Kass, Green and Goldstein (128) who showed that virus inoculation inhibited the clearance of staphylococci from mouse lungs. It also complements the studies of Harford, Leidler and Hara (129) and Gerone, Ward and Chappell (130) who found that influenza virus infection altered the host response to pneumococcal and diplococcal infection.

Both the experiment recorded here and an examination of previous experimental work using mice and Sendai virus amply justify the use of germ free animals for quantitative histological analysis.

Histological quantitation

a) Selection of parameters

The preceeding section describes the range of reaction of mouse lung to Sendai virus infection. For quantitative methods it is essential, as stated earlier, to define the object to be examined accurately. The dynamics of lung response to Sendai virus infection are complex but three features predominate in the histological analysis. These are epithelial cell necrosis and subsequent regeneration, neutrophil infiltration and removal, and the mononuclear leucocyte response.

The principal feature of the response in these experiments was of airway damage without apparent severe alteration to alveolar

function. Infection resulted in bronchiolar epithelial cell death and the inflammatory response occurred in and around the airways. It was only when bacterial contamination was superimposed on virus infection that alveolar structure was significantly altered. Therefore the existing quantitative methods used for lung structure eg. estimation of alveolar surface area, alveolar diameter etc. were of little use in these germ free mouse studies.

Of the three cell types involved only the epithelial cell can be defined and differentiated from infiltrating inflammatory cells at all stages of the reaction. This virtue stems from two factors: the location of the epithelial cell is fixed and its histological stages of cell death do not disguise its original structure. By contrast PMN leucocytes undergo pyknosis rapidly and cannot regularly be differentiated from small macrophages or large lymphocytes.

On the basis of these findings it was decided to quantitate epithelial cell numbers using as a numerical index the number of cells lying on unit length of BM.

b) Theory of quantitation

The first problem to overcome in any detailed study of population is to avoid biased sampling. A routine was devised based on systematic sampling with the first section chosen at random. This was interpreted to mean that all sections were, in effect, selected at random. The routine presented material from dorsal, ventral, anterior and posterior areas of lung in approximately equal proportion. Each section of lung was examined completely and all entire transections of bronchiolar airway were selected for counting.

Having established a practical method of sampling, a technique was evolved to produce an index of epithelial cells per unit of BM.

The estimation of the length of a curved surface in a microscopic section is both a simple technical procedure and a complex theoretical problem. The method derives from a series of historical developments. In 1800 Buffon (120) initiated investigations into geometric probability. Using his results and combining them with the theory of Delesse (121) Tomkeieff (122) applied his methods to biological data. The subsequent refinements of Smith and Guttman (123) were realized in practical biological terms by Weibel and Elias (124), who produced a simple formula for estimating the length of a curved line. The two-dimensional image presented by the section is confronted by a grid of parallel lines of equal spacing. Then

$$L = \frac{\pi}{2} \times D \times N$$

where L is the length of the curved line

D is the spacing between the lines of the grid, and

N is the number of intercepts between the curved line and the lines of the grid.

Hennig (125) has shown that the thickness of the section does not affect such measurements. However, section thickness can clearly affect the cell count. This problem was examined by Haug (126) who showed that the thicker the section in relation to the diameter of cell or nucleus examined the greater the accuracy; he devised certain modifications but these refinements were not found necessary in the methods reported here.

c) Practice of quantitation

After embedding as described each lung block was mounted in the microtome such that sections were cut longitudinally along the lungs from apical to diaphragmatic lobe. After initial trimming a section

was taken from the dorsal region. Thereafter one section was taken at every 400 micron interval until five complete sections had been cut. The final section was always taken from the ventral region of the lung. Complete examination of each section yielded on average of between five and seven complete airway transections thus producing a count of 25-35 airways per lung.

In order to confront the section with a grid of parallel lines an eyepiece graticule * was employed. This contained two sets of parallel lines at right angles to each other, each line being separated by a distance of one millimetre. Airways with a cross-sectional diameter not exceeding 1 mm (in true diameter) were selected because they did not occupy more than two thirds of the microscope field and were not subject to distortion at the perimeter of the field. To minimise error the number of intercepts between each set of parallel lines and their airway BM was counted separately and a mean figure derived. Thus for each airway examined the true length of BM could be calculated using the known graticule measurement and true microscope magnification.

The epithelial cell count was complicated by section thickness. Despite the theoretical abstractions of Haug (126) the thinner the section cut the simpler it became to count a single layer of cells. A compromise solution was found by cutting sections at approximately four microns thick. This is roughly the same diameter as the nuclei of the epithelial cells to be counted. Whenever the nuclear profile was less than normal diameter it was discarded. While this procedure did not produce absolute accuracy it was quite acceptable for a comparative study between different epithelial treatments.

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* See Appendix XX

The final index of cell number per unit length of BM was calculated as follows. The number of BM intercepts for each airway was tabulated against the cell count for each airway. A figure was derived by dividing the intercept number by the cell count. These figures were summed and expressed as a mean figure with standard error. Both number and standard error were multiplied by a factor derived from the equation $L = \frac{\pi}{2} \times D \times N$ and the final figure gave cell count and standard error per mm of BM. This index for normal lung was found to be 154 ± 5 cells per millimetre.

CHAPTER VI

THE EFFECT OF ALTERATION TO ENVIRONMENT

Literature review of climatic influence

a) Physiological response to climatic change

Climate influences biological function through air temperature and humidity, pressure and wind velocity, radiation, photoperiodism and atmospheric pollution. These changes are recognised in mammals by physiological adjustments, primarily as alteration to metabolic and physical activity, respiratory rate and blood circulation. These changes in domestic animals can be observed in body temperature, oxygen consumption, carbon dioxide production, respiratory quotients, heat and moisture losses, blood pictures and endocrine function. These observations are often grouped to produce figures for growth rate, food conversion and meat or milk production. This review concentrates primarily on the measurement of body temperature.

Weihe (131) has provided climatograms for rats and mice. He suggested three ranges of environmental temperature and relative humidity (RH) for differing age groups of animals. His provisions comply with the data collected by Spector (132) who gave 13°C and 31°C as the critical environmental temperatures below or above which the body temperature of a mouse is likely to alter significantly from the normal range. Herrington (133) described the zone of thermal neutrality for a mouse as 30-33°C above which the metabolic rate becomes variable. He measured the rectal temperature of a mouse as 36.5°C ± 0.5°C at an environmental temperature of 26.7°C when the surface temperature of the animal was 31.2°C. Laison, Levine, Bieter and McLimans (134) stated that at an environmental temperature of 21°C the average tissue temperature for a mouse is

38.1°C. Chevillard, Bertin and Cadot (135) indicated that in the range of environmental temperatures 13-31°C the variation in a mouse's rectal temperature would be 0.4°C, but that in any one animal a margin of variation could reach 3.6°C: Nomura, Yamauchi and Takahashi (136) suggested that a mouse is 'physiologically stable' in the environmental range of 25-30°C and that beyond these limits changes in physiological function are a direct result of temperature stress. South (137) pointed out that all measurements so far recorded gave values for restrained isolated animals. The figures derived are not necessarily the same for groups of animals where the individual metabolic rates may be lower.

This brief review of the mouse's body response to a normal environment indicates a relative lack of information rather then conclusive or contradictory evidence for measurements of body function.

When measurements have been made of the response to changes in climate the literature shows a greater divergence and lack of continuity. South (137) defined the differences between acclimation, representing the day to day responses such as vasoconstriction, sweating patterns and panting, and acclimatization representing modification to physiological mechanisms which require intervals of hours or weeks to reach completion. Little attention has been paid to these different classifications when studying the mouse, possibly because the methods of heat loss and conservation by mice have not yet been established. Harrison (138) stated that the mouse has no sweat glands but that the highly vascular tail may be an area of insensible perspiration. Weihe (131) found that the mouse takes six days to acclimatize if the change does not exceed 5°C and 30-40 per cent RH.

Variations in response to heat or cold stress occur not only with

the strain of animal examined but also with its previous history of exposure to environmental temperature. Hart (139) has shown that prior exposure to cold prolongs the life of mice exposed to fatal extremes of cold. Previous experience of heat or cold stress enables an animal to withstand greater extremes of temperature change. Chevillard et al. (135) have briefly reviewed the speed of adaptation for various species and remarked that age is also a factor influencing the power of adaptation.

Very little information is available concerning the response of animals to environmental humidity. Freeman and Lengyel (140) found that in man a high relative humidity inhibited water loss and caused a rise in body temperature. This view was supported by Burton et al. (141) whose subjects 'felt' less cold at higher rather than lower humidities. How far this interpretation of cause and effect relates to different species is not known.

The effect of cold stress on young germ-free mice has been investigated by Sieki, Anderlik, Banos and Radnai (142). When 28 day old germ free mice and conventional mice were exposed to a temperature of $4^{\circ}C$ for four hours 40 per cent of the germ free mice died compared with no deaths among the conventional mice.

b) Alterations in pathological response to climate change.

Several workers have produced numerical evidence showing a broad negative correlation in man between respiratory dysfunction and environmental temperature. Lidwell, Morgan and Williams (143) found that the incidence of common colds in office workers was related to cold spells four days before clinical signs appeared. Holland and Spicer (144) attempted a similar mathematical analysis and found that

lower environmental temperatures were associated with an increase in respiratory disease. Sutton (145) discussed the problems of acute respiratory disease and climate; he suggested that a clear relationship had been established between respiratory mortality or morbidity and low environmental temperatures and humidities. Loudon and Kilpartick (14) noted that a drop of 10°F increased the cough index for participants at a scientific conference. Cassel, Lebowitz and McCarroll (147) critically discussed the relationship between air pollution, weather and symptoms in an urban population; they underlined the dangers of concluding simple cause and effect hypotheses in a complex epidemiologic situation.

In their review of the relationship of calf health to environment Appleman and Owen (7) summarize a common view: 'The cause of calf pneumonia is probably a combination of viral and bacterial infection precipitated by environmental stress.' Parker (2) attempted, by clinical observation, to define this stress more clearly. He observed that the severity of outbreaks of respiratory disease in calves was greatest in conditions of high relative humidity which were created by variations in winter temperatures. Roy, Stobo, Ganderton, Shotton and Ostler (148) discovered, while conducting experiments on calf nutrition in relation to environmental temperature, that pneumonic lung lesions had a higher incidence in conditions of low temperature (14.5°C) and high relative humidity than at higher temperatures (21°C).

Laboratory animal experiments to investigate the effect of temperature on host/parasite relationships present a great diversity in their design. Earlier workers used death or survival as their parameter. McDowell (149), using rats, was among the earliest investigators to show that high humidities combined with extremes of

temperature decreased resistance to pneunmococcal infection. Mills and Schmidt (150) and Mills (151), using mice, confirmed that higher environmental temperatures decrease survival time while Moragues and Pinkerton (152, 153) investigating murine typhus stated that 'By controlling the environmental temperature conditions may be created under which murine typhus will have any desired degree of mortality.' These findings did not translate directly to viral infections. Sarracino and Soule (154) failed to kill mice after a sub lethal infection of influence A virus and subjection to extremes of heat or cold. Later Sulkin (155), while not producing deaths, in a similar system recorded an increase in pulmonary lesions after exposure to 95°F for ten days compared with 60°F. These early results were not explained by any convenient hypothesis although Mills and Schmidt (150) suggested that an impaired phagocytic index might contribute.

Later more precise measurements were performed, some of which investigated the interaction of host ciliary/mucus defence and parasite invasion. Briody, Cassel, Lythe and Fearing (156) inoculated adapted and unadapted strains of influenza virus into three different strains of mice which were held at 19-22°C. Although a similar graded virus growth was recorded in the lungs of each strain greater areas of consolidation were noted in one strain than another. When the experiments were repeated at an environmental temperature of 5°C the lung virus titres were higher in one strain but did not change in another. Boring, Zurhein and Walker (157) and Walker and Boring (158) conducted experiments on mice and observed fatalities after inoculation with coxsackie virus. They observed that at 25°C a small number of mice died whereas at 4°C all mice died but at 36°C virtually all mice survived. Uninfected control mice failed to gain weight so rapidly at

either extreme of temperature compared with 25°C but were clinically healthy. They proceeded to demonstrate that mice acclimatized to 25°C required cold stress for four days only to produce mortality but that this stress was only effective for 4-5 days after inoculation. Six days after inoculation with virus cold stress produced no deaths, nor did it when applied for only two days after inoculation.

Baetjer, Lowry and Bang (159) devised a useful model system by infecting young chickens with Newcastle Disease virus (NDV). They showed that cool wet conditions (72°F and 90% RH) allowed a greater spread of virus in the respiratory tract, but that at a higher temperature (84°F) the extent of virus spread was greater at low humidities compared with high. Later Bang and Foard (161) showed that cold temperatures increased the number of cells initially infected. Because the young chicken is virtually poikilothermic Scholtissek and Rott (162) were able to define a range of 34-41°C body temperature outside which virus replication was inhibited; they suggested that the cause was failure to synthesize viral RNA polymerase.

c) Effect of environment on mucociliary defence

One of the principal defence mechanisms of the respiratory tract is provided by a combination of ciliary activity and mucus flow. Normal patterns of activity have been investigated and the subject was reviewed by Dalham (163). Subsequently Iravani and van As (164) reexamined earlier findings after reporting mucus transport in entire intact tracheobronchial sections of rats. They did not find a 'mucus blanket' as described by earlier workers but observed the movement of droplets, flakes and plaques of mucus in definite patterns. Bronchitic animals exhibited widespread abnormalities and the velocity of

transport was diminished.

There are several reports of the effect of altered temperature and humidity on mucus transport and ciliary activity in normal mucosa. Proetz (165), using isolated pieces of mucosa, showed that frequency of ciliary beat was a function of temperature. Dalham (163) indicated that mucus flow is a more rapid indicator of change than ciliary beat. He noted that mucus changes not only in the quantity secreted but in its viscosity. He also realized that temperature changes might be recognised by a local effect of inspired air or by a systemic effect on whole body temperature. In his experiments he found that an elevated rectal temperature increased mucus flow up to a temperature of 39°C after which the flow decreased. Conversely as rectal temperatures fell below normal, mucus flow and ciliary beat diminished. He varied the relative humidity to which the mucosa was exposed and found that above 70 per cent relative humidity there was no discernable reduction of ciliary activity. Cole (166) investigated the effect of altered environmental temperature on the temperature and humidity of inspired air and found that air in the pharynx remained in the range 34 ± 3°C and almost saturated with water. Anderson, Lundqvist and Proctor (92) exposed humans to air at 30, 50 and 70 per cent relative humidities but failed to demonstrate any change in mucosal function. Both these latter reports examined only the effect of environmental change on the upper respiratory tract but paid no attention to the potential physiological changes in the whole system. Ewert (167) expanded on the work of Dalham and found that if the air temperature alone rose or fell there was no change in cilial beat; however, if the rectal temperature was allowed to rise and fall at the same time as environmental air then ciliary beat was affected. He confirmed

Dalham's conclusion that air above 70 per cent relative humidity has no apparent harmful effect. Taken with the results of Anderson, Lundqvist and Proctor (92) it is difficult to envisage conditions when air reaching the lower respiratory tract will be at less than 70 per cent RH. Nonetheless the NDV and chicken model, subsequently employed by Bang and Bang (160), showed that experimental dehydration of the chicks tended to immobilize ciliary activity.

All these preceding reports involve a greater or lesser degree of interference with normal respiratory function. A different approach to estimating the efficiency of muco-ciliary clearance has been to expose animals to airborne particles and measure their rate of removal. The subject was reviewed by Rylander (168). The effect of various drugs and pollutants has been extensively investigated but relatively little attention has been paid to changes in environmental conditions. Using rabbits Crolley (169) was able to show that extremes of temperature and humidity produced a temporarily depressant effect on the clearance of bacteria; the effect was most marked with sudden changes. Ames and Nungester (170) obtained similar results using cold stress on guinea pigs. Green and Kass (171), with more refined techniques, found that mice exposed to cold stress (13-17°C) suffered similar decreased resistance to bacterial infection using three different species of bacteria. They noted that the effect was more pronounced in male mice. All these findings are complicated by the activity of phagocytic cells which vary in their efficiency.

How environmental temperature and humidity affect body response to air borne infectious agents has not been properly established nor are the factors involved well understood. This review condenses findings made in different species and at different sites on the respiratory

mucosa using a variety of techniques. The broad correlation reported by various authors does not necessarily imply that defence function is similar for different areas of mucosa in different species nor that the correlation will exist under pathological conditions. However it appears probable that, among others, the nature of the mucociliary defence is significant. The quality of inspired air affects ciliary rate and force of beat; at the same time mucus secretion rate and viscosity are altered. In an experimental situation several elements may affect the result; among these are the strain of virus and animal selected, the method of inoculation and the sensitivity and therefore accuracy of the measurements made.

Design of Experiments

The preceding review suggests that environmental air temperature has an effect on the host response to air borne infection. The effect would appear to depend on the changes brought about in host body temperature rather than altering the temperature of inspired air reaching the lower respiratory tract. The influence of relative humidity is not clear. It may be that humidity only acts by varying the host ability to thermoregulate, or its influence may be more subtle.

The body temperature of mice is affected by the environmental temperature. The critical air temperature given by Spector (132) suggests that above 13°C or below 31°C the mouse's rectal temperature remains within the normal range but beyond these limits physiological compensation is overstretched and the rectal temperature falls or rises.

In the present experiments environmental temperatures were chosen that would keep the mouse's rectal temperature just within normal

limits. It was hoped that a distinction could be drawn between changes in pathological response due to whole body temperature change and challenge to the respiratory defence system alone.

Humidities were varied within the range experienced under conventional animal housing systems. In effect the infected animals were presented with a cold, damp environment, a hot drier and a hot wetter environment. These were compared with a standard indoor environment of 20°C and an RH of 50-70 per cent.

Virus titres of excised lungs were calculated as a crude measure of virus/host interaction. The histological methods described in Chapter V were regarded as an accurate and sensitive measurement.

Environmental control methods

a) Design and function of the measurement system

Limited observations in a test isolator showed that at an air exchange rate of ten per hour sufficient turbulence could be created to provide a uniform temperature and humidity. The temperature and humidity within the propylene mouse boxes were related to isolator conditions as shown by Murakami (22).

Recordings of temperature and RH within a sterile isolator or in a monocontaminated system demand that the measuring probes are able to withstand some form of sterilization. While this poses no problem in respect of temperature measurement the commonest method available for RH estimation involves the use of a wet bulb thermometer. In such a system the wick moistening the bulb must be kept absolutely clean. It was not considered practical to maintain the required standard of cleanliness within an isolator for a sufficient period.

An alternative method was adopted in which the actual measurements

were made outside the isolator but immediately adjacent to the outlet filter. In a test isolator the correlation between internal and external readings was found to be satisfactory. When changes were made, particularly in RH, the external measurements revealed a time lag of one to two hours, presumably while conditions equilibrated within the isolator.

Measurements were recorded from a thermohygrometer * screwed into a 4" perspex tube which was taped to one of the isolator outlet filters. The assembly is illustrated in fig. K, p.32 attached to the aerosol exposure chamber. Continuous recordings of the isolator outflow were not available but the ingoing air was continuously monitored and a direct correlation established between input and outflow air. The correlation appeared to be independent of the density of mouse population within the isolator.

b) Design and function of control system

The air supply to an individual isolator normally required a flow rate of three cubic feet per minute at a pressure of three inches water gauge. It was required to control this air supply over the range of 10-30°C and the relative humidity within this temperature range from 20-90 per cent. A method was selected for simplicity and economy.

Ambient air was compressed to 15 pounds per square inch *, passed through a commercial refrigerator unit *, and expanded to four inches water gauge. This series of events initially reduced the water content of the air by compressing water vapour to form droplets which were collected in a drain trap. The refrigeration unit passed the air through a condenser operating at 2°C which condensed further water

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* See Appendix XX

vapour and provided saturated air at a low temperature. This cold air was then re-expanded without being able to pick up the condensed water vapour. An air supply was thus continuously created that had a temperature just over 2°C with an RH of 40-50 per cent. The cold air was then passed over a 600 watt heating element capable of lifting the air temperature to 30°C and through a duct containing a water atomiser *. The atomiser drew water from a small self levelling tank and was controlled via a midget solenoid valve * attached to the original compressed air line.

With the compresser and refrigeration unit working continuously it was possible to provide for all extremes of climate from hot dry conditions through to a cold damp climate. Control of the heater and atomiser units was exercised through a recorder controller * that sampled the input air at the isolator entry filter and regulated their function via appropriate electrical relays.

It was found that, whatever temperature settings were selected, the air throughput of the isolator was too small to prevent a large degree of heat loss or gain through the PVC isolator wall. In effect it became necessary to use a commercial air conditioning unit to provide a room temperature close to that required in the isolator. On the other hand the RH selected was completely independent of room conditions, provided that the isolator temperature was not allowed to fluctuate.

The heat and water output of the caged mice exerted a considerable influence on climatic control. Actual calorific values were not measured but within the system described it was possible to compensate

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* See Appendix XX

TABLE 1 : LUNG VIRUS TITRES (-ve $\log_{10}TCD_{50}$/0.1 ml)

No.mice/ day	Temp	Rel. Humidity	Mean Virus Titre Days Post Infection Day 1	Day 3	Day 5	Day 7	Day 9	Day 11
6	10°C	85%-90%	1.6	4.4	4.2	4.2	2.2	0.2
12	20°C	55%-60%	1.4	3.9	4.0	3.5	0.6	0
4	30°C	55%-60%	1.0	4.4	4.4	2.8	0	0
4	30°C	85%-90%	1.4	3.8	3.8	2.9	0.5	0

Standard error of a mean = 0.5

TABLE 2 : IN VITRO VIRUS GROWTH AT TWO TEMPERATURES

Incubation Temperature	Virus titre (-ve $\log_{10}TCD_{50}$/0.1 ml) Expt 1	Expt 2
35°C	4.7 ± 0.43	3.5 ± 0.37
39°C	3.5 ± 0.37	1.9 ± 0.25

the input air to provide the required experimental conditions.

Results of Experiments

a) Lung Virus Titres

A number of experiments were performed in the ambient laboratory conditions to determine the pattern of virus replication within infected mouse lung. The results of these and the subsequent experiments conducted under different environmental conditions are presented in table 1. This summarizes the number of mice examined, the mean virus titre of lung homogenate per observation and the environmental variables.

In vitro replication of virus was examined at the extreme temperatures that might have prevailed in the lungs of environmentally stressed mice. The experiments were performed at each temperature viz 35°C and 39°C showing that at higher temperatures virus replication was reduced. Although the temperature of the epithelial mucosa was not recorded in vivo it appears probable that the changes shown in table 1 between different environmental treatments reflect an alteration in the surface temperature of the mucosa. The results of this test are shown in table 2.

b) Histological Indices

In each experiment the index of mucosal cells per millimetre of BM represents the mean figure for two experiments using one male and one female mouse per sample in each experiment i.e. the mean of four mice. Samples were taken at the same time for both histological analysis and virus titration. An example of data collection and the method for calculating an individual index is shown in Appendix XVIII, table 32.

Mice were sampled on alternate days beginning on day three post

TABLE 3 : EPITHELIAL CELL COUNT expressed as Cells/mm Bronchial mucosa

Days after infection	Temperature °C and Relative Humidity			
	10°/85-90%	20°/55-60%	30°/55-60%	30°/85-90%
0	154	154	154	154
3	154	151	140	143
5	144	149	128	140
7	135	141	116	116
9	67	133	96	108
11	88	109	138	118
13	118	129	148	116
15	115	133	140	137
17	145	148	130	133
19	146	158	139	153
23	148	150	138	156
27	174	132	145	160
31	160	152	116	155
35	152	138	130	154

infection. After nineteen days sampling was at four day intervals. Mice were examined for a total period of five weeks after exposure to the virus aerosol.

The results are presented in table 3 and recorded graphically in fig. M.

An analysis of variance was performed on each experimental treatment to determine whether a significant difference occurred between days of environmental exposure and between male and female mice. The results are tabulated in Appendix XX in tables 4, 5, 6 and 7, and their significance assessed in table 8.

c) Mouse Rectal Temperatures

Rectal temperatures of mice during initial and subsequent exposure to environmental change were recorded. Measurements were performed using a thermocouple inserted 1 cm into the rectum. 128 mice were examined at periods of 2 hours, 5 hours, 24 hours, 3 days and 14 days after exposure to altered temperature and RH. No differences were observed within sexes but a difference of $1^{\circ}C$ was consistently noted between male and female groups. The mean temperatures and the standard errors were

Male mice $38.4^{\circ} \pm 0.02^{\circ}C$

Female mice $39.4^{\circ} \pm 0.01^{\circ}C$

The range of temperature observed for male mice was 37.5° to 39.5° and for female mice 38.4° to 40.1°.

d) Control values

A suspension of virus was inactivated as described in Appendix X.6 and a control group of mice for each experiment were exposed to an aerosol of inactive virus under the standard conditions already described for infective virus exposure. No virus was recovered from

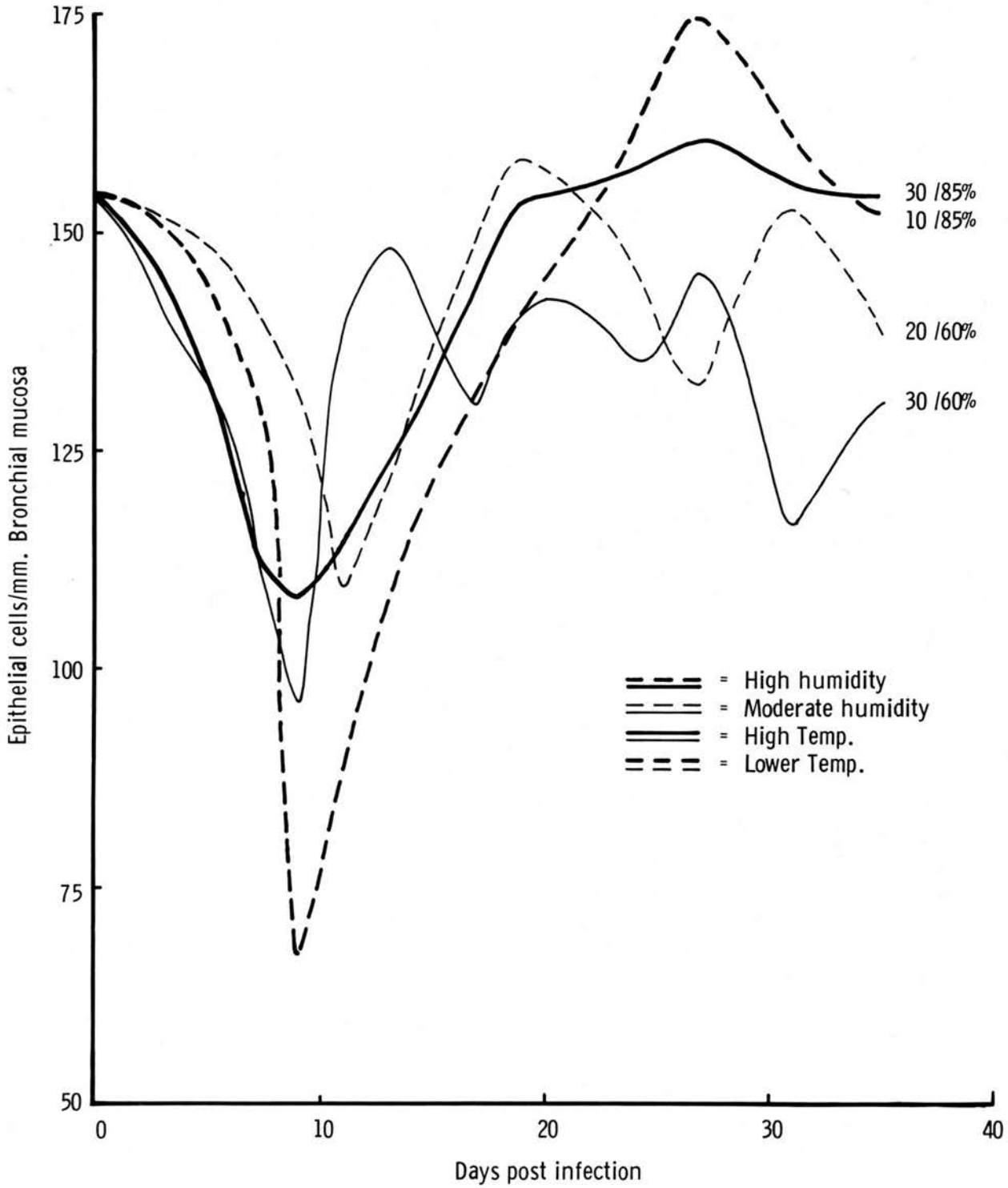


Fig. M : Epithelial cell counts in four different environments.

the lungs of control mice. No lesions were seen either macroscopically or microscopically in the lungs of control mice. Histological cell counts did not vary from the normal value i.e. 154 cells/mm BM than the calculated standard error of 5 cells/mm BM.

Discussion of Results

The effect of different environmental treatments reveals certain similarities and dissimilarities between each set of results. The graph in fig. M illustrates a trend or pattern but the frequency of observations and the limited period of observation do not permit absolute value to be deduced.

It is clear that in each treatment epithelial cell damage was recorded, reaching a maximum value between nine and eleven days after infection. The corresponding pattern of virus replication yielded a maximum titre between days three and five. Although cellular disruption, as observed during viral replication,was presumed to be the cause of initial cell loss the reason that cellular destruction continued is not known. It is possible that virus continued to infect cells but could not be demonstrated by conventional means. Evidence for this hypothesis was presented by Blandford and Heath (97) using Sendai virus in mice when they found specific antibody coating virus particles, thus preventing their attachment to more epithelial cells. The earlier paper of Robinson et al. (71) showing the time sequence of interferon production may also reflect an intracellular component of the interaction although the subsequent paper of Robinson et al. (197) tended to discredit the influence of interferon in this model.

It appears that the colder the environment the greater the damage within the respiratory tissue since the mildest damage was recorded in

the 20-30°C range. This finding corresponds in general with earlier observations and in particular with Bang and Foard (161) who showed that lower temperatures increased the number of cells infected. The milder degree of damage seen at higher temperatures did not evince a marked hyperplastic repair process. The 10°C treatment which produced more severe damage by day nine yielded a correspondingly higher peak in the hyperplastic response.

RH did not have any obvious effect by comparison with temperature. However, when the acute stage of destruction began to resolve, i.e. when epithelial regeneration started, the predominant influence came from RH. Whether the temperature was at either extreme, viz 10°C or 30°C, at high RH cell regeneration rapidly returned the cell count to normal while at the same or different temperatures a lower RH caused fluctuation in the recovery phase. It is probable that the combination of temperature and RH altered the dynamic fluid balance of the mucous membrane lining the airways and either partially or intermittently affected ciliary beat and thus clearance of noxious stimuli, in a similar manner to that shown by Dalham (163) and other earlier workers. The fluctuation shown in both lower humidity treatments was not uniform but when judged from the graph resembles the periodicity generated by a spring expanding and contracting to obey Hooke's Law.

Rectal temperatures did not vary to any significant extent from the normal range during exposure to any treatment. An environmental temperature of 10°C is reported to be outside the physiological range within which rectal temperatures are not altered. It is probable that 13°C is the critical environmental temperature for a restrained isolated mouse as suggested by South (137) and that a group of mice huddled together successfully alter their microclimate. Therefore it is

reasonable to conclude that the variation in response shown by the different groups of mice represented a local variation in respiratory mucosa homeostasis. The changes following a standard dose of virus infection were probably brought about by changes in the balance of surface temperature, secretory pattern, local immune response or other factors created by alterations to the temperature and RH of the inspired air.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

The aim of the work presented in this thesis has been to provide a method to measure the effect of certain aspects of climatic environment upon an established respiratory infection. The complexity of respiratory disease problems in general has, by its nature, demanded specialized techniques in the research methods selected.

The selection of germ free mice for the host animal has been justified. Attempts to make accurate measurements of conventional mice which have a mixed and variable flora were shown not only to be virtually impossible but also to possess a marked variation. The germ free mice were screened for the presence of any parasitic or commensal living form and the negative result implied that homeostasis in each animal should have been very similar. This situation provided for a greater uniformity in the response of each individual animal to the pathological stimulus. It does, however, compare unfavourably with the natural or conventional disease state in which the pattern of events is altered to a marked but variable degree by the indigenous flora of the animal. The effect of a normal flora on the biochemistry and physiology of germ free animals has not been evaluated so that, ignoring the compound effect of lesser pathogens normally commensal with the host, interpretation of the results must make allowance for a considerable difference in host reaction. The genetic component of variability in inbred mice selected from only four generations would be expected to be small. However, genetic selection was based primarily on conformation and not on resistance or susceptibility to Sendai virus.

Measurements of virus titre in these experiments showed that each

aerosol exposure of different groups of mice resulted in a similar quantitative degree of infection. The accuracy of the quantitative measurements was not greater than one logarithmic dilution so that demonstration of differences in degree of viral replication between environmental treatments were only significant on days nine and eleven at 10°C. The demonstration that virus distribution in the lungs of exposed mice was uniform allowed for random histological sampling. Although the concentration of virus three days after infection was greater at the lung hilus than at the pleural extremities there was no distinction between heavier deposition in the large airways and ciliary transport of virus from the periphery to the hilus. It is probable that most of the virus was deposited in the larger airways because by day three relatively little desquamated tissue or viral antigen was seen in the bronchiolar lumen.

The pattern of air streaming within the aerosol infection chamber was not determined and may not have presented a uniform aerosol cloud to the group of 40 mice exposed for each experiment. However, the results as determined by viral replication in exposed animals imply that any variation was less than the technical error in determining virus titre. The aerosol cloud was heterogeneous with respect to particle size. Findeisen (198) stated that ' High mobility of very large and very small particles results in deposition high in the respiratory tract. Middle range particles have a low total deposition rate but a greater net deposition in alveoli'. This early comment has been substantiated by several workers including Beekmans (199). Hyslop (200) defined these ranges as 5-20 microns particles depositing in the nasopharyngeal region and particles between 3-5 microns depositing within the lung. Particles below one micron in diameter may

enter the lung but only a small proportion are deposited, the remainder being exhaled while still in aerosol droplets. The immunofluorescent examinations made in this study suggest that, although the Collison atomiser is claimed to produce many particles below one micron in diameter, the majority of infective virus was deposited on the medium to large airways within the lung. The quantity of virus deposited in the upper respiratory tract was not known and its possible significance as a later source of infectious droplets was not explored. Despite these inherent problems the value of a repeatable morphometric approach to post exposure distribution of virus in lungs should be emphasised.

Methods for the fixation of lung tissue have for many years been the subject of debate. There may have been merit in attempting to instil fixative under pressure into the trachea. The advantage would have been uniformly distended tissue fixed within normal physiological dimensions; the corresponding disadvantage bronchiolar cell loss, caused by the direct action of the fixative in shrinking and rounding off epithelial cells, would be small in proportion to the total number. Difficulties might have arisen in distinguishing between the action of the fixative on normal versus pathological mucosa. The trial of this method mentioned in Chapter V yielded a lower cell count for the normal.

Section thickness both within and between individual lungs was not uniform. In some circumstances the angle of sectioning cut through the periphery of the spherical nucleus and created the false image of a pyknotic nucleus. In others the cytoplasmic section was correspondingly small and a true evaluation could be made. On thick sections the relative number of incompletely cut nuclei was less and thus the cell count could be higher. In addition, by making very small alterations

in the focusing of the microscope the total cell count could be slightly increased. The variance in the count of normal bronchioles was small and the contribution from this source of error was therefore presumed to be insignificant.

There are alternative methods available to quantitate proportional tissue volumes and areas although none were considered superior to the method evolved here. One of the simplest of these, for example, is point counting as originally conceived by Delesse. Such a method would indicate the mass of epithelial tissue overlying the BM and would be quantitatively more precise. However, the information gained would be of less biological interest since it is only the intact cell which protects epithelial integrity and not the mass of tissue lying on the BM.

One of the problems arising when the cells are counted is the necessity to determine when a cell can be classified as functional and entire or as no longer functional. The method used did not distinguish in the mean numerical value obtained between a simple state of cell degeneration compared with a compound state of degeneration combined with hyperplasia at an adjacent site. It is a characteristic of many viral/ host interactions, including Sendai virus and the mouse, that degeneration and regeneration appear simultaneously. A mean figure of or near to 154 cells/mm BM may not indicate a normal mucosa with an even distribution of mucosal cells but a partial recovery phase where the balance between excessive repair and a modest degree of damage happens to yield that value. This situation undoubtedly prevailed in several mice but assessment of the predominant damage or repair state was demonstrated in the mean histological index. Examination of the graph in fig. M shows how this

simultaneous reaction was expressed in the lower humidities. The balance between repair and degeneration fluctuated indicating an overall pattern; at all stages both repair and degeneration were taking place with either one or the other response being dominant.

The concept of numerical assessment of pathological data has great importance. Much of the impetus given to a morphometric approach comes from the inspiration of workers like Weibel (202). The achievement of diagnostic morphometric approach, such as that used in chronic bronchitis by Reid (203), has enabled greater understanding of the pathogenesis of such lesions. The application of similar techniques to experimental pathology provides precise definitions both in experimental design and achievement.

Previous reports of experimental work relating temperature and RH to disease have involved indirect measurements of body function. For example, Lidwell et al. (143) related climate to the incidence of clinical symptoms in man; Parker (2) reported a similar study in calves. It would be reasonable to suggest that an improvement on these observations would identify either environment or body function more precisely. The methods and results reported here have this objective. They provide accuracy in detail of the selected parameters, but may omit the measurement of other parameters of significance. Further work may be suggested using even lower humidities and a longer time scale. How long, for example, does it take for mucosal cells to return to an equilibrium in which cell turnover rates are comparable to normal? Has the variation in temperature of inspired air affected the humoral response? It is reasonable to conjecture that climatic change involves only the mechanical process of mucus secretion or ciliary movement of viral antigen but does not fundamentally interfere

with the local secretion of antibody.

Many previous papers relating RH to respiratory function, particularly mucus/ciliary activity, have reported observations on normal tissue. Such work was rarely extended to pathological states. Andrewes (204) discussed the inadequacy of experiments with common cold viruses. He provided several hypotheses to suggest how chilling of the host might precipitate respiratory disease but favoured variations created in host defence. He suggested that these variations might be brought about by alteration in blood supply to the mucosa or by air currents creating local areas of dessication. If these suggestions are reviewed in conjunction with the normal and bronchitic patterns of mucus clearance in rats described by Iravani and van As (164) then it is reasonable to conclude that areas of dessication and possibly altered blood supply to the mucosa may explain the oscillation between damage and repair at the lower RH treatments reported here. Although Bang and Bang (160) showed that total body dehydration reduced ciliary function the effect of local drying is equally acceptable. Muir (206) emphasized that the most important factor known to interfere with ciliary action is drying of the mucosa.

Andrewes also speculated that upsetting the balance of synchrony between interferon and virus production by climatic stress disturbed a latent infection. Lower temperatures do reduce interferon production and Allen et al. (205) related an outbreak of common colds in man to a fall in environmental temperature despite seventeen weeks of isolated community living. The possibility that Sendai virus might in some fashion assume latency in mouse bronchiolar epithelium should not be overlooked especially with regard to the effect of RH on recovery from acute infection. The nature of epithelial surface presented for

cell counts on low RH treatments had a very similar appearance in the delayed recovery phase to that in the early stages of acute damage. Small areas of cell loss, loss of staining affinity and dwarfing of the cuboidal cells all suggested typical viral damage. Further studies using immunofluorescence and co-cultivation tissue culture techniques might clarify these observations.

The major conclusions of the present studies can be summarized as follows:

Alterations to the climatic temperature affect viral replication rate and thus reflect a short term influence on the pathological changes following primary infection.

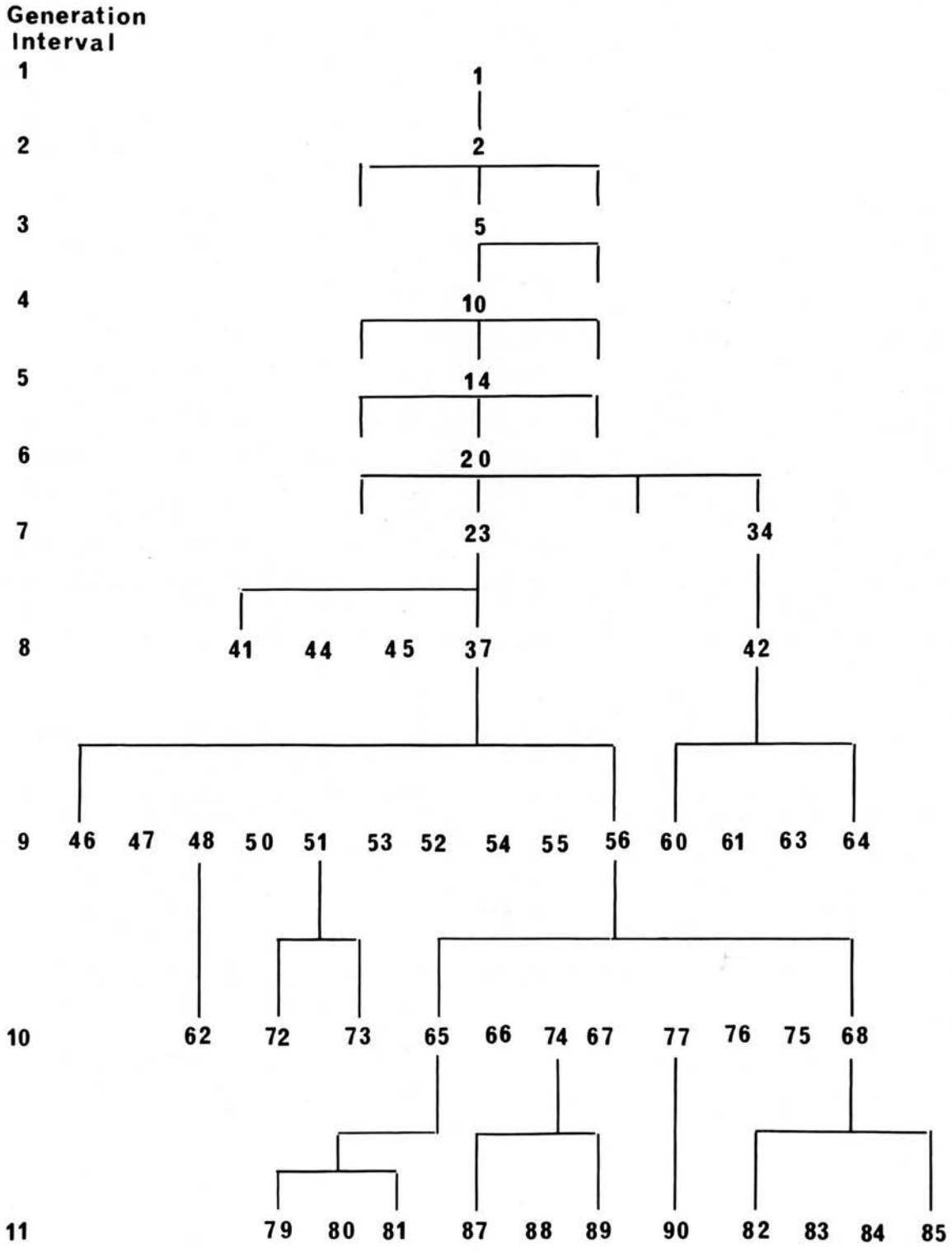
Alterations to the relative humidity of the environment affect the density of the bronchiolar mucosa and reflect a longer term influence on the recovery phase following infection.

The development of this experimental model provides a new tool for a variety of studies in the important field of research in respiratory disease.

APPENDIX I

TABLE 9

MOUSE BREEDING SCHEDULE



Breeding pairs were numbered sequentially. The offspring of those numbers shown in generations 8 - 11 inclusive were used for experiment as described in Chapter 2.

APPENDIX II

SPONTANEOUS DEATHS IN THE MOUSE COLONY

All mice dying spontaneously were examined histologically apart from the first death which underwent severe autolysis. The only significant observation made histologically was to confirm internal haemorrhage in suspected vitamin K deficiency. In all cases specimens from the dead mice were examined for bacteria and fungi according to the schedule laid down in Appendix V. 1 : no microbial isolates were made. The spontaneous deaths are tabulated in table 10.

TABLE 10

Observation	Number of Mice	Sex	Age span at death
Vitamin K deficiency	21	male	64-221 days
Torsion of caecum	2	female	200-243 days
Signs of CNS disorder	1	female	14 days
Liver lesions *	1	female	120 days
Unknown cause	4	female	42-150 days
Unknown cause	1	male	

* Centri lobular necrosis without inflammatory response

It will be seen that death from vitamin K deficiency only occurred in male mice and that caecal torsion was only found in females: the latter was probably caused by careless handling.

APPENDIX III

TABLE 11

PER CENT ANALYSIS OF DIETARY CONSTITUENTS BEFORE AND AFTER STERILIZATION

Constituent	Before irradiation	After irradiation
Crude protein	21.29	21.58
Crude fibre	3.30	3.25
Ether extract	3.55	3.55
Ash	6.59	6.52
Moisture	11.90	12.10
Soluble carbohydrate	53.37	53.00
Calcium	0.95	1.11
Phosphorus	0.42	0.47
Magnesium	0.10	0.12
Potassium	0.76	0.78
Sodium	0.61	0.59

The analysis in table 11 was performed by the Nutrition Laboratory of the Royal (Dick) School of Veterinary Studies, Edinburgh. Their report states that any differences shown are within the limits of error implicit in the estimation techniques used. These results, therefore, indicate no real difference between samples and it may be concluded that on a proximate basis irradiation with Cobalt 60 did not alter the constituents of the feed.

An analysis of amino acids in the diet was carried out at the

TABLE 12

PER CENT ANALYSIS OF DIETARY AMINO ACIDS

	Edinburgh		High Wycombe	
Amino Acid	Before	After	Before	After
Lysine	0.91	1.08	1.45	1.18
Methionine	0.34	0.32	0.47	0.37
Cystine	-	-	0.23	0.20
Valine	0.76	0.99	0.84	0.79
Leucine	1.23	1.45	1.18	1.35
Isoleucine	0.60	0.71	0.68	0.60
Threonine	-	-	0.58	0.51
Phenylaniline	0.71	0.84	0.92	0.80
Tyrosine	0.60	0.63	0.68	0.57
Histidine	0.40	0.47	0.44	0.41
Arginine	1.11	1.20	1.02	1.09
Glutamic acid	3.92	5.13	4.90	4.77
Glycine	0.82	1.14	1.14	1.00
Serine	-	-	1.26	0.93
Aspartic acid	1.37	1.90	1.83	1.71
Alanine	0.83	1.06	1.22	0.99
Ornithine	0.20	0.12	-	-
Proline	-	-	1.89	1.26

same laboratory in Edinburgh and also at the Lord Rank Research Centre in High Wycombe, Buckinghamshire. The results are recorded in table 12.

These figures indicate that technical error in analysis was greater than any difference due to gamma irradiation treatment. It may reasonably be concluded that amino acid levels in the diet were not significantly affected by the sterilization treatment.

Fig. G

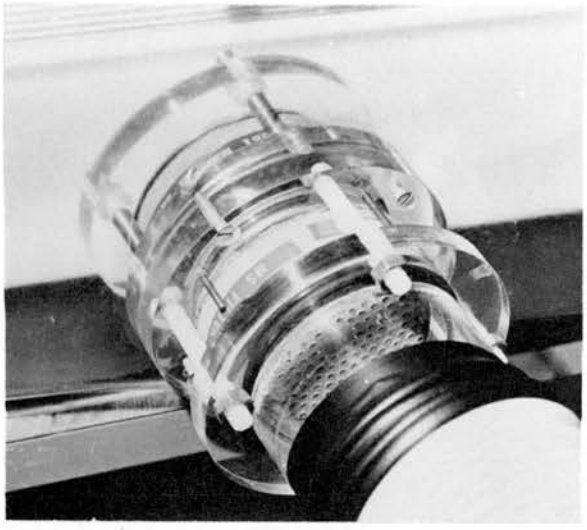


Fig. H

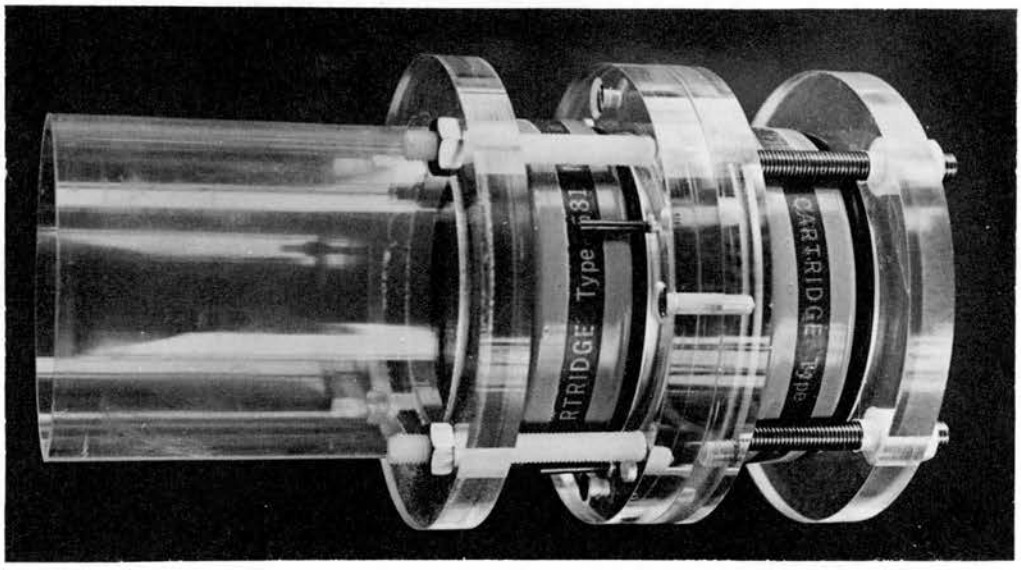
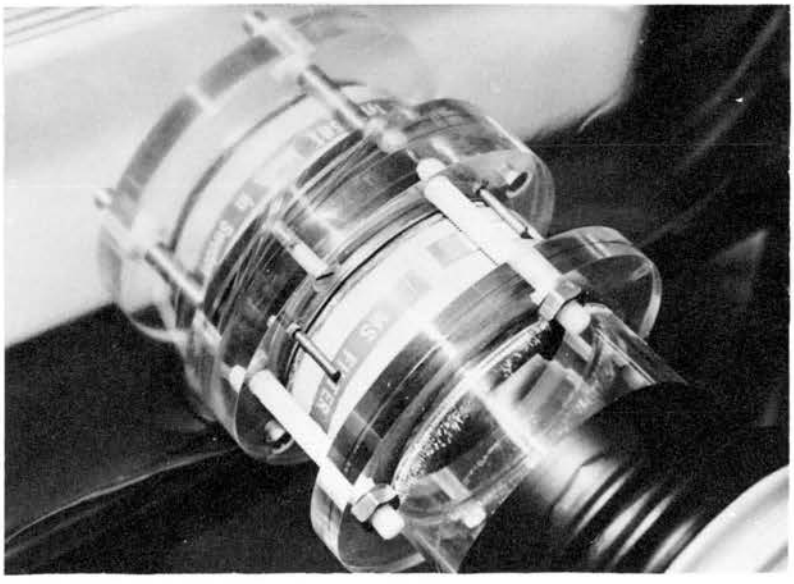


Fig. I



APPENDIX IV

DESIGN OF ISOLATOR FILTERS AND TESTS FOR THEIR EFFICIENCY

The conventional 'candle' filter designed by Trexler (51) has two main disadvantages. The filter media wrap is easily damaged during isolator assembly but the damage may not be obvious; secondly the assembled filter cannot be practically tested for filtration efficiency. These two factors combined with the bulk and clumsiness of the original design prompted a search for improvements.

A respirator filter cartridge, manufactured originally for the Swedish Army, is commercially available in the United Kingdom. It is robustly constructed with a strong aluminium casing. The filter medium is a grade of glass fibre paper which is claimed to retain 99.998 per cent of 0.3 micron particles at a linear air speed of 14.2 cm/sec *.

This cartridge was assembled in a housing of perspex flanges. The filter was sealed into the housing with rubber 'O' rings and the perpex flanges were screwed across the isolator wall to provide an air-tight seal. Figures G, H and I demonstrate the construction and use of the cartridge and filter housing.

Each filter cartridge is individually tested after manufacture and supplied already sterilized by 2.5 Mrad. radiation. A sample of eight cartridges were tested at the Chemical Defence Establishment, Porton using the methods described by the British Standards Institution (172). At flow rates ranging between 120 and 150 litres/minute the penetration of each cartridge was less than 0.0002 per cent.

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*See Appendix XX

APPENDIX V

1. TEST PROCEDURES FOR THE GERM FREE STATE: BACTERIA AND FUNGI

Material sampled

Two types of sample were obtained for examination. At fortnightly intervals freshly extruded faecal pellets were taken from at least four different mice in at least two different boxes in each isolator. These were suspended in approximately 10 ml of drinking water from a used water bottle. The concentration of faeces in water varied. The suspension, in a universal screw topped container, was passed out of the isolator and transferred to the laboratory bench for examination. At irregular intervals, depending on colony production, but not less frequently than once every three months a complete mouse was sacrificed. The animal was killed and dissected inside the isolator and the following organs or tissues sampled: liver, kidney, stomach, small and large intestines including the caecum, nasopharynx, lung, heart, uterus or gonads and axillary skin. The sample bottle, again containing a sample of drinking water, was then removed from the isolator.

Method of examination

Wet mounts and direct air dried smears of the suspensions were not routinely examined. Experience indicated that contaminations could be recognised without this step.

All samples were inoculated into broth media. For the purposes of uniformity all media were reconstituted from Oxoid * tablets or granules. All media were incubated at 30°C for 24 hours prior to use

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* See Appendix XX

Inoculated media were kept at two different temperatures and grouped for primary inoculation as shown in table 13

TABLE 13

25°C	37°C
Brain heart infusion broth	Brain heart infusion broth
Thioglycollate medium USP	Thioglycollate medium USP
Sabouraud's liquid medium	Cooked meat medium

The functions of these media overlap. All media at 37°C will grow aerobes and anaerobes. However, cooked meat medium requires a larger inoculum but grows a greater range of organisms than brain heart infusion broth which requires a very small inoculum. Thioglycollate medium may prove inhibitory but most organisms would eventually appear in either of the other media. At 25°C yeasts and moulds will grow in brain heart infusion but grow out more readily and rapidly in Sabouraud's medium.

All media were inoculated with about 0.5 ml of test suspension and incubated. They were examined daily for turbidity or other signs of growth. To avoid the risk of laboratory contamination sub-cultures were made only once, after three weeks. Broths were sub-cultured to five per cent ox blood agar plates. One set of plates was held aerobically; the other set were incubated anaerobically in modified McIntosh and Fildes jars. The anaerobic environment was checked by including a plate inoculated with Clostridium tetani: this organism has strict anaerobic growth requirements. The sample, and thus the

TABLE 14

Date	Number of isolators	Organisms	Identification										
			Sugar					Other tests					
			Glu	Lac	Sac	Sal	HF	Gel	Ls	RCM	Nit	Ind	Mot
10/69	1	Clostridium	+	+	+	-	F	L	NL	P	+	-	-
4/70	2	Clostridium	+	+	+	-	F	L	NL	P	+	-	-
			-	-	-	-	NA	-	NL	P		+	-
7/70	3	Clostridium	+	+	+	-	NA	L	NL	P	+	-	-
			+	+	+	-	NA	L	NL	P	+	-	-
			+	-	+	+	O	-	NL	P		-	-
4/71	1	Bacillus subtilis											
5/71	1	Bacillus subtilis											
7/71	1	Bacillus subtilis											
6/72	2	Clostridium	+	+	+	+	F	L					
	1	Bacillus	white colonies										
		Bacillus	orange colonies										
		Micrococcus											

isolator at the time of sampling, was regarded as sterile if neither the fluid media nor the agar plates indicated microbial growth.

These tests in effect include and exceed the recommendations of the Committee on Standards of the American Institute of Laboratory Animal Resources (173).

2. ISOLATOR CONTAMINATIONS IN BREEDING COLONY

The colony was started in July 1968, held at the Royal (Dick) School of Veterinary Studies, Edinburgh until December 1970 then transferred to the Institute for Research on Animal Diseases, Compton, Berks. Table 14 gives details of the analysis of isolator contaminations. The Key to the table is given below:-

Glu	glucose	Nit	Nitrate
Lac	lactose	Ind	Indole
Sac	saccharose	Mot	Motility
Sal	salicin	F	Fermentation
HF	Hew and Leifson medium	NA	No action
Gel	Gelatin	L	Liquified
RCM	Robertson's cooked meat medium	NL	Not liquified
Ls	Loeffler's slope	P	Pink

APPENDIX VI

1. TEST PROCEDURES FOR THE GERM FREE STATE: VIRUSES

Viral agents sought in the test procedure

1. Pneumonia virus of mice (PVM) described by Horsfall and Hahn (31).
2. Sendai virus described by Fukumi et al. (32).
3. Newborn mouse pneumonitis or K virus described by Kilham and Murphy (34).
4. Mouse adenovirus described by Hartley and Rowe (35).
5. Polyoma virus described by Gross (174).
6. Ectromelia described by Marchal (175).
7. The hepatitis virus (es) (MHV) first described by Gledhill and Andrewes (176).
8. Reoviruses (Reo) of the types described by Rosen, Hovis, Mastrota, Bell and Huebner (177).
9. Theiler's mouse encephalomyelitis viruses (strain GDVII), (178).
10. Agents of the encephalomyocarditis group originally described by Helwig and Schmidt (179).
11. The thymic agent of Rowe (180).
12. Salivary gland virus described by Smith (181).
13. Epidemic diarrhea and lethal intestinal viruses of infant mice described by Kraft (182).
14. Minute virus of mice (MVM) described by Crawford (183).
15. Lymphocytic choriomeningitis (LCM) described by Traub (184).

The following diseases were included in this category although the first two are of unknown aetiology and the third is caused by a chlamydium.

16. Enzootic bronchiectasis described by Nelson (185).
17. Grey lung disease described by Andrewes and Glover (33).

18. Nigg's pneumonitis described by Nigg and Eaton (30).

Viruses of the leukaemia complex, Bittner's mammary tumour agent and Riley's lactic dehydrogenase viruses were not sought.

Details of test procedure

The passage experiments were divided into three groups:

1. Intra-peritoneal inoculation of liver and spleen homogenates.
2. Intra-nasal instillation of lung homogenates.
3. Intra-cerebral inoculation of brain and spinal cord homogenates.

In each case groups of mice between three and four weeks of age were used. Selected mice were removed from the isolators in sterile containers and transferred to a sterile hood in the laboratory. They were killed by cervical dislocation, dissected with sterile instruments and the various tissues homogenized in phosphate buffered saline to produce an approximate 10 per cent volume suspension. All tissues were ground up using a 'teflon' pestle homogenizer *. The liver and spleen homogenate was centrifuged for five minutes at 1,000g, the supernatant placed in a sterile bijou bottle (other homogenates were not centrifuged) and reintroduced to the isolator for reinoculation. Intra-nasal and intra-cerebral inoculations were carried out under 'halothane' anaesthesia induced by the method of Cook and Dorman (186). The scheme of passage is given diagramatically in table 15.

Mice from the final passage of each group were exsanguinated, their sera pooled, heat treated at 56°C for 30 minutes and tested for viral antibody using either a haemagglutination inhibition test (H.I.T.) or a complement fixation test (C.F.T.). Sera for testing were distributed as follows:-

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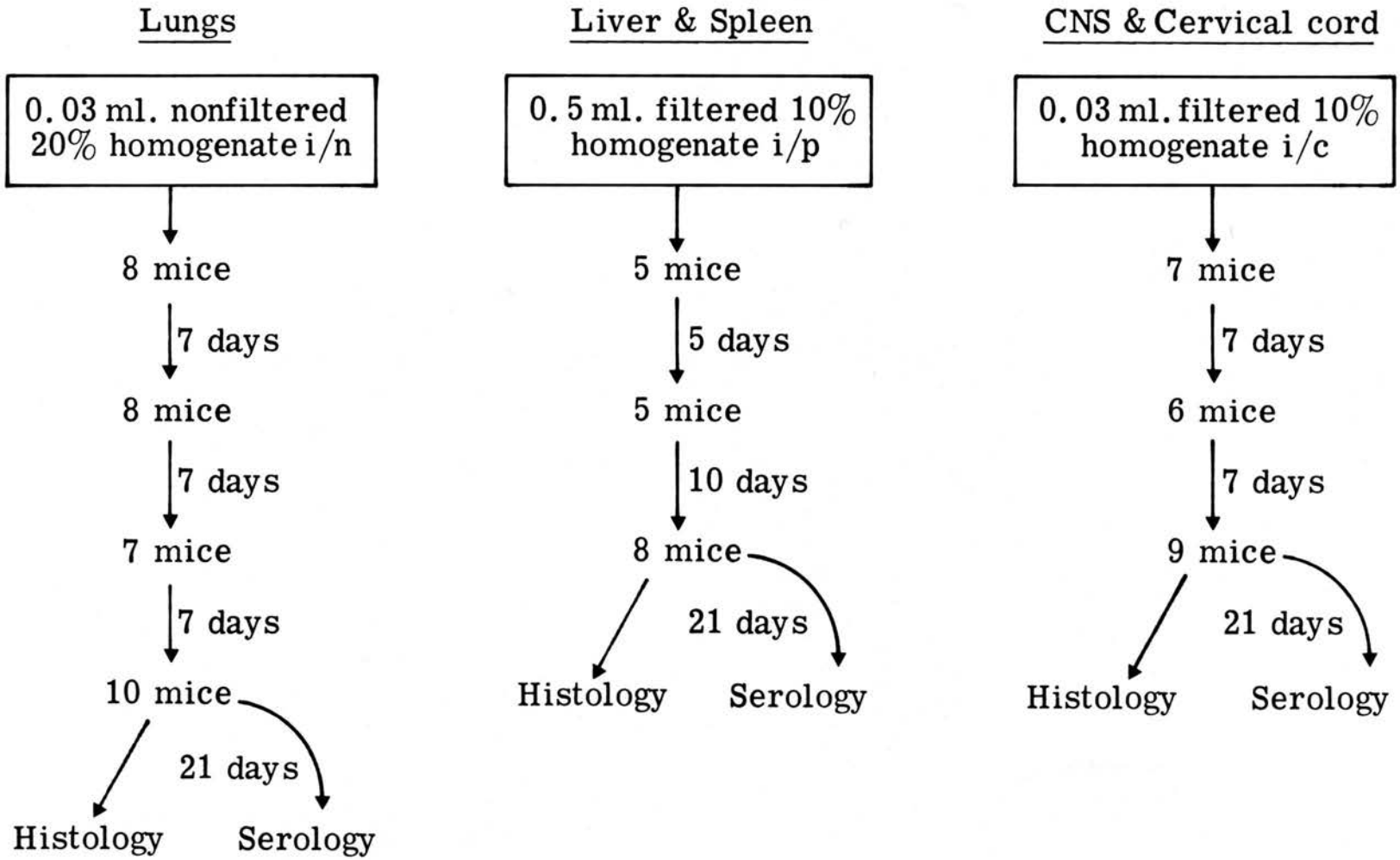
* See Appendix XX

TABLE 15

PASSAGE HISTORIES



All mice were used at 22 - 32 days old

Sexes were balanced within each group

Laboratory	Test performed
Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2	H.I.T. for Polyoma
Clinical Research Centre, Northwick Park, Harrow, Middlesex	H.I.T. for Sendai
M.R.C. Common Cold Unit, Harvard Hospital, Salisbury, Wilts.	C.F.T. for MHV3
Microbiological Associates Inc. Bethesda, Maryland, USA.	H.I.T. for PVM, Reo 3, GDVIII Polyoma, Sendai, MVM, K virus C.F.T. for adenovirus and MHV

All tests with one exception were negative for antibodies to the viral antigens listed. The exception was an H.I.T. to Theiler's encephalomyelitis virus strain GDVII where agglutination was inhibited at an initial dilution of 1 : 20 in the sera from the liver/spleen and the lung passage. Illogically the CNS pass sera did not inhibit agglutination. This isolated positive result did not provide convincing evidence of encephalomyelitis virus in the colony but nor can the findings be discounted.

Gledhill (187) demonstrated that ectromelia virus was harboured in the intestinal tract of apparently healthy mice and that contaminated faecal pellets represented the source of infection for susceptible mice. Since the passage method may not have revealed latent ectromelia infection, the large intestinal washings of six mice aged three weeks were inoculated intra-cranially into eight new born mice. No clinical signs were observed over a period of seven days. The mice were killed, the whole carcase fixed in 10 per cent formal saline, sectioned and stained with haematoxylin and eosin. No pathological lesions were recognised in any tissues.

Tissues from the final passage mice, after exsanguination, were fixed in 10 per cent formal saline and examined microscopically, as

haematoxylin and eosin stained sections. The following tissues were examined in each animal: CNS, myocardium, lung, thymus, liver, spleen, stomach, duodenum and ileum, caecum, colon, pancreas, kidney, adrenal and testis or ovary. Only one lesion was noted: it appeared as multiple, discrete, small patches of myocardial calcification. They stained positively by von Kossa's method. Similar lesions, specifically in the C3H strain of mouse, are described by Russell and Meier (188) who quote three further references to similar observations. In all cases the lesions appear to have arisen spontaneously but their cause and significance has not been established.

Tests for LCM virus were conducted separately at the Animal Virus Research Institute, Pirbright, Surrey (189). Three mice of the original breeding stock from MRC Carshalton, when aged 42 weeks, were challenged by the method of Hotchin and Benson (190) and a positive response was observed on the seventh to ninth day. They could therefore be said to be LCM-free. The female grandparent of the colony and her seventh litter were tested separately for tolerant and non-tolerant infection. When the litter was six days old it was mixed with a tolerantly infected litter from another female. Contact with infected young lasted three weeks at which time three of the four young mice were clinically ill and had an LCM viraemia. The mother at this point was immune to challenge. It may be reasonably assumed therefore that there was no LCM infection in the colony.

2. TEST PROCEDURES FOR THE GERM FREE STATE: MYCOPLASMA

Incidence of infection in conventional colonies

The actual incidence of chronic respiratory disease in best known for rats in which rates ranging from 50-100 per cent of adults have been reported. Meaningful data on prevalence in contemporary stocks of rats and mice simply are not available (191).

Material sampled

Three male and eight female mice (aged 18-28 weeks) were inoculated intra-nasally with an estimated 10^2 EID_{50} Sendai virus suspension in 0.05 ml. After one week the mice were killed. The nasopharynx and lung were cultured by swabbing and the middle ear was sampled by lavage with sterile saline.

Media

The swabs and fluid were transferred to liquid and solid media. The liquid medium was made up from:-

70 ml DIFCO PPLO broth
1 ml penicillin G, 100,000 units
2 ml thallium acetate 2.5 per cent
10 ml yeast extract 25 per cent
20 ml horse serum, Wellcome Number 6
2 ml phenol red 0.1 per cent

The solid medium differed from the above by the inclusion of 1 g OXOID Ionagar No. 2 and the exclusion of the phenol red indicator.

Result

No mycoplasma were isolated from any of the mice tested.

APPENDIX VII

PATHOLOGICAL LESIONS OBSERVED IN THE MOUSE BREEDING COLONY

Alopecia in breeding females

Haematoxylin and eosin stained sections of normal and hairless skin were examined from six ex-breeding female mice.

The epidermis revealed patchy thinning of the stratum corneum associated with a reduced quantity of keratin. At the level of the stratum granulosum it was common to find heavy granular deposits of basic staining material. This lesion was not seen at the periphery of the bald patches where the hair was growing normally. The material resembled that seen on an eroded epidermis where evaporation of exudates creates a thin crust of cell and fluid debris.

The dermis appeared normal. There was no evidence of cellular infiltration, no alteration in the ratio of glandular to follicular to CT tissue. Broken hairs and the associated trauma were not seen.

Liver lesions in an ex-breeding male

Mouse aged 657 days at autopsy. Macroscopic examination revealed a white cauliflower-like mass in the right lobe visible from the diaphragmatic surface. The mass measured approximately 2 x 1.5 x 1 cm. It had a solid consistency, was white in colour, and on cut surface revealed an irregular lobular pattern of sub-division. Small cystic cavities were present on cut surface. The entire mass appeared to be enclosed by a thin fibrous capsule.

Microscopically sections were stained with haematoxylin and eosin, and by Masson's trichrome method, Gordon and Sweet's method and the periodic acid Schiff reaction (PAS). The parenchyma of the liver

contained multiple discrete foci of coagulative necrosis varying in size from 0.1 - 1.0 mm in diameter. The principal lesion was demarcated by a rim of compressed hepatocytes at its periphery but no fibrous capsule was apparent. The lesion was poorly lobulated with portal triads not well defined. Where bile ducts were present they were frequently dilated. Mononuclear cell accumulations were occasionally seen around both central and portal vessels. The 'cystic' cavities seen macroscopically appeared as grossly dilated sinusoids. The density of the reticulin framework was reduced in the lesion by comparison with adjacent normal parenchyma. Similarly the quantity of PAS positive material in the normal parenchyma was greater than in the abnormal tissue. Within the lesion were many individual pyknotic cells; approximately one mitotic figure was seen per medium power field.

APPENDIX VIII

IDENTIFICATION OF SENDAI VIRUS

1. Electron microscopy

The morphological characteristics of the paramyxoviruses have been concisely summarized by Andrewes and Pereira (192). Using comprehensive references to original investigations they gave the following basic structure: 'Virions consist of a helical nucleocapsid surrounded by an approximately spherical envelope 100 to 200 nm in diameter, but easily deformed giving rise to aberrant forms of variable size which may reach 600 nm. The envelope is covered with projections 12 to 15 nm long and 2 to 4 nm wide. Partially disrupted virions are frequently seen, revealing an internal nucleocapsid with helical symmetry, about 18 nm in diameter, with a central hole about 5 nm across.' These characteristics are revealed in figs A,B,C and D (pages 22 and 23).

2. Production of a haemagglutinin in culture

The original virus sample received from Mill Hill was inoculated into 10 day embryonated hen's eggs and the allantoic fluid harvested after four days. The haemagglutinating titre of this fluid suspension was 1/2560. Using an 0.2 ml inoculum of the allantoic fluid diluted 100 fold, four eggs were inoculated by the allantoic route and samples tested for haemagglutination at the intervals shown in table 16.

3. Specificity of haemagglutinin

A sample of the deep frozen virus pool was diluted tenfold in tissue culture maintenance fluid to an estimated 10^7 TCD_{50}/ml. 0.1 ml of the suspension was inoculated into each of five tubes of primary calf kidney cells. Six days after inoculation supernatant fluid from

TABLE 16

Hours after inoculation	H. A. titre								
	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2560
24	+	+	±	-	-	-	-	-	-
36	+	+	+	+	+	-	-	-	-
48	+	+	+	+	+	+	+	-	-
72	+	+	+	+	+	+	+	+	-
96	+	+	+	+	+	+	+	+	-

TABLE 17

	Serum dilutions						
P.I.1	1/10	1/20	1/40	1/80	1/160	1/320	1/640
	-	-	-	-	-	±	+
P.I.3	1/100	1/200	1/400	1/800	1/1600	1/3200	
	+	+	+	+	+	+	

the tubes was harvested and pooled. The haemagglutinin titre was established and four haemagglutinating doses (4 HA) were estimated to be in 0.2 ml of the original 10^7 TCD_{50}/ml suspension. Serial two-fold dilutions of parainfluenza 3 and parainfluenza 1 antisera * were added to 4 HA units of virus. The results in table 17 indicate that haemagglutination was inhibited only by parainfluenza 1 antiserum.

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* See Appendix XX

APPENDIX IX

EXAMINATION OF THE VIRAL SUSPENSION FOR PURITY

A viral suspension was prepared to contain an estimated 4HA units of parainfluenza 3 virus (P13). These were tested against commercial antisera * as shown in table 18.

TABLE 18

Test	Serum dilution					
	1/20	1/40	1/80	1/160	1/320	1/640
4 HA units Sendai v. Equine anti-Sendai serum	-	-	-	+	+	+
4 HA units Sendai v. Guinea pig anti-Sendai serum	-	-	-	-	-	+
4 HA units P13 v. Equine anti-Sendai serum	-	-	+	+	+	+
4 HA units P13 v. Guinea pig anti-Sendai serum	-	-	-	-	-	-

The tests using guinea pig serum show complete specificity and identify the test suspension as containing Sendai haemagglutinins but no P13 contamination. The equine sera show some non-specific cross reactions. The guinea pig sera were obtained from intra-nasal challenge; their purity should be of a higher order than the equine samples obtained from intra-venous inoculation.

* See Appendix XX

APPENDIX X

VIRUS DEMONSTRATION METHODS

1. Egg inoculation titrations

Virus titrations were performed initially in eggs. 10-day embryonated hen's eggs were found to be the most sensitive medium for virus replication but, as the following result shows, they exhibited a larger variation in their response to inoculation than other media tested.

Virus was diluted by logarithmic steps in nutrient broth. 0.2 ml of each dilution was inoculated into each egg by the allantoic route. Three days after inoculation 0.5 ml samples of allantoic fluid were mixed with an equal volume of 1 per cent washed guinea pig erythrocytes in a WHO plate. After one hour at room temperature plates were examined for haemagglutination (+) or sedimentation (-) of the erythrocytes.

TABLE 19

TITRATION OF VIRUS POOL USING EGGS

Virus dilution	Egg number									
	1	2	3	4	5	6	7	8	9	10
10^{-4}	+	+	+	+	+	+	+	+	+	+
10^{-5}	+	+	+	+	+	+	+	+	+	+
10^{-6}	+	-	+	D	±	+	-	+	+	+
10^{-7}	-	-	-	+	+	+	+	-	-	+
10^{-8}	-	-	-	-	-	-	-	-	-	-

D indicates death of embryo

2. Egg-shell piece (chorio-allantoic membrane) titrations

Using the method described by Fazekas de St. Groth (74) a 10-day embryonated egg was dissected to produce 90 pieces of egg shell to which was still attached the chorio-allantoic membrane. Uniformity of size was maintained by using a modified paper punch to cut the shell. Three experiments were performed the first being examined for haemagglutinin production at 48 hours and the second and third at 72 hours. In each case the same virus dilutions were inoculated into four complete 10 day embryonated eggs.

TABLE 20

TITRATION OF VIRUS POOL USING EGG SHELL PIECES

Virus dilution of inoculum	Haemagglutination (a) in whole eggs (b) in egg shell pieces					
	Experiment 1		Experiment 2		Experiment 3	
	(a)	(b)	(a)	(b)	(a)	(b)
10^{-1}	-	10/10	-	10/10	-	10/10
10^{-2}	4/4	10/10	4/4	10/10	-	10/10
10^{-3}	4/4	0/10	4/4	0/10	-	0/10
10^{-4}	4/4	0/10	4/4	0/10	-	0/10
10^{-5}	4/4	0/10	4/4	0/10	4/4	0/10
10^{-6}	0/4	0/10	4/4	0/10	4/4	0/10
10^{-7}	0/4	0/10	4/4	0/10	4/4	0/10
10^{-8}	0/4	0/10	4/4	0/10	4/4	0/10

3. Tissue culture preparation and titration

Primary cultures of calf kidney (CK) cells were prepared by the method of Luther (193) modifying the technique to suit calf kidney tissue. The only significant change was in the growth medium where lamb serum was replaced by foetal calf serum. This was reduced to two per cent in the maintenance medium. Cells were cultured in tubes without coverslips by inoculating approximately 10^5 cells per tube. After seven days confluent sheets of cells were established. Logarithmic dilutions of virus suspension were made in Earle's lactalbumen salt solution and 0.1 ml of each dilution inoculated into each of five tubes. The virus was absorbed for one hour at 35°C, maintenance medium added, and the tubes incubated at 35°C for six days. After six days virus growth was demonstrated by a cytopathic effect (CPE). This was clearly seen without staining as patchy areas of cell necrosis or complete lysis of groups of cells. Eight days after inoculation the haemagglutinin production reached a maximum titre and samples of the supernatant fluid were tested against one per cent guinea pig erythrocytes.

Confirmation that the CPE was produced only from viral replication and was not a product of other components of the inoculum was demonstrated. Pooled end points (the last dilution completely positive for HA) of the test titration were diluted in logarithmic steps and inoculated as described above into five tubes per dilution. After six days the CPE was examined and after eight days the HA activity tested. The results of four separate tests are shown in table 21.

TABLE 21

Virus dilution	Numbers of positive CPE + HA per number of tubes inoculated			
	Test Numbers			
	1	2	3	4
10^{-3}	5/5	5/5	5/5	5/5
10^{-4}	5/5	5/5	5/5	5/5
10^{-5}	5/5	5/5	5/5	5/5
10^{-6}	5/5	5/5	5/5	5/5
10^{-7}	0/5	5/5	5/5	5/5

This test is interpreted to mean that, since the end point of a titration has a maximal dilution of virus and any other toxic product, eight days after reinoculation into further tubes a positive CPE at high dilutions indicated that the virus has replicated to produce the CPE but that any toxic product could not reproduce and create a CPE at the higher dilutions.

4. Comparison of different titration methods

The data provided in sections 1 and 2 of this appendix indicate that whole eggs are the most sensitive indicator of viral titre in a test suspension. Figures are given in table 22 of titrations carried out at different times but using the same stock from the frozen virus pool. They indicate the relative sensitivity and within group variation for each system.

TABLE 22

Virus dilution	System		
	Eggs	CK	MDBK
10^{-1}	-	-	5/5
10^{-2}	-	-	5/5
10^{-3}	-	-	1/5
10^{-4}	10/10	5/5	0/5
10^{-5}	10/10	5/5	0/5
10^{-6}	7/9	0/5	0/5
10^{-7}	5/10	0/5	0/5
10^{-8}	0/10	0/5	0/5

Eggs : 10 day embryonated eggs
CK : primary cultures of calf kidney cells
MDBK : continuous cell line originating in bovine kidney

5. Preparation of lung homogenate

Sample mice were removed from the isolator and carried to the laboratory in open containers. They were killed by cervical dislocation and dissected on the open bench using sterile instruments and techniques. Complete lung tissue was stored for short periods, not exceeding ten days, in glass bottles at -20°C. The entire lung, from which had been removed lymph nodes and trachea, was minced with scissors and homogenized in 1.3 ml nutrient broth using a teflon pestle homogenizer *. This produced an approximate 20 per cent weight for volume suspension of

* See Appendix XX

lung. The suspension was centrifuged at approximately 1,000 g for fifteen minutes. Containers used for preparation were held in an ice bath for all stages except centrifugation. Supernatant from the final treatment was distributed into two aliquots which were stored at -20°C prior to titration. Storage at -20°C never exceeded three weeks. Antibiotics were not included in suspending media except during the preparation of dilutions for tissue culture inoculation.

6. Inactivation of virus suspensions

5 ml volumes of Sendai virus in buffered gelatin were exposed to ultra-violet irradiation. In a period of 30 minutes the total intensity of radiation was 1.282×10^{-20} ergs/cm^2.

Inoculation of samples of the suspension, after irradiation, into fertile hen's eggs yielded no viral growth when estimated by haemagglutinin production.

APPENDIX XI

REPRODUCIBILITY OF VIRUS TITRATIONS

The testing procedures for any quantitative titration are established in basic virological technology. The following tests were attempted and the results presented in tables 23, 24 and 25.

a) Using a sample of the deep frozen pool serial logarithmic dilutions were made from 10^{-2} to 10^{-7}. Samples of these dilutions were inoculated into three sets of tubes, using five tubes per dilution:-

TABLE 23

Virus dilution	Test A	Test B	Test C
10^{-2}	5/5	5/5	5/5
10^{-3}	5/5	5/5	5/5
10^{-4}	5/5	5/5	5/5
10^{-5}	5/5	5/5	5/5
10^{-6}	5/5	2/5	4/5
10^{-7}	0/5	0/5	0/5
Log TCD_{50}/0.1 ml	5.5	4.9	5.3
s.e.	0.4	0.4	0.5

End points were calculated by the Spearman-Karber method and the standard errors (s.e.) using the 'smoothing' procedure of Irwin and Cheezeman (194).

b) Using a sample of the deep frozen pool five sets of serial logarithmic dilutions were made from 10^{-2} to 10^{-7}. Samples of each dilution were inoculated into five tubes per dilution.

TABLE 24

Virus dilution	Test A	Test B	Test C	Test D	Test E
10^{-2}	5/5	5/5	5/5	5/5	5/5
10^{-3}	5/5	5/5	5/5	5/5	5/5
10^{-4}	5/5	5/5	5/5	5/5	5/5
10^{-5}	4/5	2/5	5/5	2/5	1/5
10^{-6}	0/5	1/5	2/5	0/5	0/5
10^{-7}	0/5	0/5	0/5	0/5	0/5
Log TCD_{50}/0.1 ml	5.3	5.1	5.9	4.9	4.7
s.e.	0.5	0.5	0.5	0.4	0.4

c) The stability of deep frozen aliquots and the variation between samples were examined by titrating individual samples at different times.

TABLE 25

Virus titre	25.IV.72	16.V.72	10.X.72	7.II.73
10^{-2}	5/5	5/5	5/5	5/5
10^{-3}	5/5	5/5	5/5	5/5
10^{-4}	5/5	5/5	5/5	5/5
10^{-5}	5/5	5/5	5/5	5/5
10^{-6}	4/5	0/5	2/5	5/5
10^{-7}	0/5	0/5	0/5	0/5
Log TCD_{50}/0.1 ml	6.3	5.0	5.9	6.5
s.e.	0.5	0.5	0.4	0.4

d) The stability of virus suspensions on the laboratory bench was examined over a period of four hours. The virus infectivity did not appear to be affected under these conditions by the suspending medium.

TABLE 26

DEMONSTRATION OF VIABILITY OF STORED VIRUS

Virus dilution	Storage at 20°C for 4 hours		Storage at 4°C for 4 hours	
	Suspension in gelatin buffer	TC medium	Suspension in gelatin buffer	TC medium
10^{-3}	5/5	5/5	5/5	5/5
10^{-4}	5/5	5/5	5/5	5/5
10^{-5}	0/5	3/5	5/5	5/5
10^{-6}	0/5	0/5	0/5	0/5
Log TCD_{50}/0.1 ml	4.5	5.1	5.5	5.5
s.e.	0.3	0.4	0.4	0.4

APPENDIX XII

DEMONSTRATION OF VIRUS IN LUNG SECTIONS

1. Cryostat sectioning

If mouse lungs are excised and allowed to partially collapse, frozen with liquid nitrogen, and sectioned at -20°C on a cryostat microtome, sections cut at less than fifteen microns thick tend to fragment readily. If lungs, after removal from the mouse, are artificially collapsed in a vacuum chamber then sections may be cut at four to five microns thickness. However, when such sections are examined microscopically under dark ground illumination it is not possible to discern normal lung architecture. To resolve this difficulty the following method was successfully applied.

Lungs were excised from the open thorax, placed on a petri dish and held in a vacuum chamber at 25" Hg for a few seconds. The totally collapsed lungs were then reinflated with a 12 per cent solution of gelatin in distilled water via a syringe and plastic canula. Gelatin solution was warmed to about 40°C and introduced via a canula which was pushed into the trachea to produce an air-tight fit. By gently squeezing the plunger of the syringe the lungs were expanded to approximately their normal maximum inspiratory volume. The trachea was ligated with a cotton thread, the canula removed, the heart dissected away from the preparation and the remainder of the tissue mounted on a cold microtome chuck. A drop of gelatin solution held the preparation in place while the surrounding air space was filled with gelatin solution in a preformed mould. The complete chuck was lowered into a mixture of liquid nitrogen and iso-pentane and frozen. After allowing it to warm to -20°C the block could be trimmed of surplus gelatin and mounted in the microtome. With this preparation it was

possible to cut sections at six to eight microns thickness.

2. Stain preparation

The indirect staining method requires two stains. The preparation of guinea pig anti-Sendai serum is described below; fluorescein isothiocyanate conjugated swine anti guinea pig serum was purchased commercially *. Eight guinea pigs 8-10 weeks old were bled by cardiac puncture and their sera tested by haemagglutination inhibition for anti-Sendai antibodies. No antibodies were detected. The guinea pigs were inoculated intramuscularly with 1 ml Sendai virus suspended in allantoic fluid; the inoculation was repeated twice at two weekly intervals. Six weeks after initial inoculation they were bled by cardiac puncture and yielded sera with haemagglutination titres ranging from 1/320 to 1/640.

The conjugated swine serum and the guinea pig serum were both absorbed against mouse lung powder as follows. Lungs from 150 mice were mixed with acetone and homogenized in a Silverson L3 mixer emulsifier *. The product was filtered through coarse grade filter paper, the deposit washed several times in acetone, and dried overnight at room temperature. The dried tissue was ground to a powder in a mortar and 100 mg powder added to each ml of serum. The mixture was stirred for 2 hours, centrifuged and the supernatant sterilized by Millipore filtration *.

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* See Appendix XX

3. Immunofluorescent staining

Cryostat sections were mounted on glass slides at room temperature, allowed to dry, and fixed for one minute in Analar acetone at 4°C. It was found that the guinea pig anti-Sendai serum could be diluted using phosphate buffered saline (P.B.S.) so that its H.A. titre was 1/160. The conjugated serum was diluted to 1/8 of its concentration as purchased. Sections were stained as follows:

a) 30 minutes with guinea pig anti-Sendai serum

b) 20 minutes washing with P.B.S. at pH of 7.3

c) 30 minutes with swine anti guinea pig conjugate

d) 20 minutes washing as in stage (b)

e) 5 seconds with 1 per cent aqueous Evan's blue

f) Wash briefly with P.B.S.

g) Mount under coverslips using a mixture of 90 per cent Analar glycerol and 10 per cent P.B.S.

Using a known positive preparation control stains were prepared for each fresh batch of stain. To show that only virus infected tissue fluoresced normal guinea pig serum was substituted for anti-Sendai serum. To show that the conjugate was specific for guinea pig antibodies control sections were stained with conjugate alone. In neither case was any specific fluorescence seen.

APPENDIX XIII

SUSPENDING MEDIUM FOR VIRUS AEROSOLS

A 0.2 per cent solution of gelatin in 0.008 molar McIlvaine's buffer was prepared using highly purified, acid free gelatin *. The buffer was prepared from 0.1 M citric acid and 0.2 M disodium hydrogen phosphate. A solution was prepared as follows:

5.22 ml	0.1 M citric acid
34.78 ml	0.2 M disodium hydrogen phosphate
2.0 g	dehydrated gelatin

This was distributed into 20 ml aliquots, sterilized by autoclaving, and held at room temperature until required.

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* See Appendix XX

APPENDIX XIV

DESIGN OF THE AEROSOL INFECTION APPARATUS

The exposure chamber was designed to hold sufficient mice for one exposure per experiment and to attach to an isolator using the standard transfer sleeve. It was convenient to mount the chamber on a trolley for mobility and to assemble the aerosol generation apparatus on a lower shelf. The general layout is shown in Fig. J (page 32).

The exposure chamber was fitted with a 12" entry port in which was a stainless steel removable door. The door had a rubber gasket around its perimeter for making a good but not necessarily absolute seal. A pair of rubber gauntlets was fitted to enable the mouse containers to be passed through the entry port and along the transfer sleeve to the isolator entry port. The inlet pipe for the aerosol stream was fitted at the top of one end of the chamber and the outlet at the bottom of the opposite end. A sterilizing filter was attached to the outlet and a thermohygrometer in a perspex tube attached to the filter. The perspex tube had a flap cover to prevent recording of extraneous ambient air.

Compressed air at 15 lb/in^2 was supplied to the Collison atomizer after passing through an efficient air filter *. Air from the same source was passed through a double bank of silica gel columns to dry it and a metered quantity, measured in a flow meter, passed through another air filter. The aerosol stream and the dry air stream were passed into a mixing tube, then through a connecting tube to the chamber above. The dry inlet to the mixing tube was angled to promote maximum turbulence. The actual humidity and temperature of

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* See Appendix XX

the aerosol stream was not known: recording was made after contact with the mice whose respiration and urination added moisture to the cloud. The flow rate of the aerosol stream was known from measuring the secondary air stream and adding to the fixed flow rate from the atomizer.

Samples of the aerosol cloud were taken from the outflow pipe just before the sterilizing filter. Suction was applied by a rotary vacuum pump *, creating a vacuum of 40-50 cm Hg. Samples of the cloud were collected in a Porton all-glass impinger with a critical orifice to control flow rate.

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* See Appendix XX

APPENDIX XV

EXPOSURE CHAMBER FUNCTION TEST USING BACILLUS GLOBIGIS SPORES

A washed suspension of Bacillus globigis spores in phosphate buffered saline (PBS) was obtained from the Microbiological Research Establishment at Porton. This suspension was titrated by plating out 0.2 ml of serial logarithmic dilutions onto nutrient agar plates using the method of Miles and Misra (195). The suspension contained 2.4×10^9 spores per ml.

An estimated suspension of 10^7 spores/ml was prepared in 0.2 per cent phosphate buffered gelatin * and atomised into the exposure chamber under standard conditions †. Serial samples of the aerosol were collected in the cascade impactor for one minute. A critical orifice governed airflow through the impactor so that each sample contained airborne particles in 17.5 litres of air. Spores were collected on 3" x 1" microscope slides previously coated with a 4 per cent gelatin/30 per cent glycerol mixture in water. After collection the slides were washed in a measured volume of warm nutrient broth for one minute, logarithmic dilutions prepared immediately, and titrated as described above. Two experiments were performed using these methods: the means and standard errors of per cent proportions spores collected at each impactor stage are shown in table 28.

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* See Appendix XIII

† See Chapter III

TABLE 28

Time after starting aerosolization	Percentage of spores for each Impactor stages 2	3	4
5 minutes	2.7 ± 0.7	18.0 ± 3.0	79.4 ± 9.8
10 minutes	2.7 ± 0.4	19.3 ± 4.4	78.1 ±10.4
15 minutes	2.0 ± 0.3	26.9 ± 4.0	71.1 ± 8.6
20 minutes	1.9 ± 0.2	20.8 ± 5.6	77.3 ± 7.3
30 minutes	2.9 ± 0.3	25.8 ± 3.7	71.3 ± 7.5
Variance between times (1)	0.21	15.9	15.5
Variance within times (2)	0.22	17.9	17.5
F Value	< 1	< 1	< 1

(1) calculated using the ordinary variance formula on five means

(2) calculated as average of squares of S.E.S.

e.g. for stage 2 $\frac{1}{5}$ $(0.7^2 + 0.4^2 + 0.3^2 + 0.2^2 + 0.3^2)$

The analysis of variance showed that there was no significant difference between different samples for any given collection stage. This result is similar to that of Rylander (196) who, using a similar apparatus, stated that a stable number of bacteria were produced in the chamber after 0.9 minutes. The impactor stages have a sharp 'cut-off' for particles of different sizes but the stages overlap. So, on average, 2.5 ± 0.7 per cent particles were between 3 and 20 microns in diameter; 21.5 ± 8.5 per cent particles were between one and seven microns in diameter and 75.9 ± 18.2 per cent particles were

between 0.7 and 3 microns in diameter.

APPENDIX XVI

ESTIMATION OF VIRUS DISTRIBUTION IN MOUSE LUNG

Four experiments were performed to determine the distribution of virus in mouse lung three days after exposure to virus aerosol. Table 29 shows the number of fluorescence positive airways per total number of airways observed for each mouse. On the basis of the results in the first experiment it was decided that five sections from each of three mice per experiment would be a sufficient number for statistical analysis.

The average number of infected airway cross sections was found to be 44 per cent. A X^2 test was applied to these figures and the results subjected to an analysis of variance in table 30.

TABLE 30

Source	d.f.	S.S.	M.S.	F value	
Experiments	3	3.865	1.288	2.55	NS
Mice	10	5.062	0.506	1.23	NS
Sections	62	25.405	0.410	0.65	NS
Remainder	156	44.827	0.287		

It will be seen that there is no significant difference between each component. Therefore lungs could be sampled randomly for histological analysis without any bias resulting from uneven distribution of the initial inhaled dose.

The estimate of lung infection given as 44 per cent of airway

Table 29.

Expt. No.	Section No.	Lung Quarters																			
		Mouse 1				Mouse 2				Mouse 3				Mouse 4				Mouse 5			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	1	0/3	0/0	0/1	1/2	0/0	2/4	0/0	0/1	0/2	0/6	0/1	0/5	0/3	3/12	3/5	4/7	0/0	1/2	1/1	1/1
	2	1/1	1/6	2/4	2/4	0/2	1/2	0/0	1/2	0/4	5/6	0/3	1/1	0/2	3/6	2/2	2/8	1/1	3/5	3/5	0/1
	3	2/2	2/4	1/2	1/3	1/1	0/1	0/1	1/7	0/2	1/5	0/4	3/7	5/10	1/1	6/7	0/1	2/2	1/5	1/3	2/5
	4	3/4	2/2	1/3	1/3	1/2	3/4	4/6	1/2	1/3	1/4	3/4	3/7	0/2	2/5	2/4	0/0	0/4	1/1	0/3	0/2
	5	3/3	2/5	5/6	3/7	4/4	3/7	2/3	0/2	2/2	3/6	2/3	1/2	1/2	4/6	0/1	0/0	0/1	0/0	0/0	0/0
	6	3/4	2/2	3/7	2/4	0/0	2/1	0/0	1/1	0/1	3/6	2/4	1/2								
	7	3/8	2/6	0/8	3/10	2/4	5/6	3/4	2/2												
	8					1/2	1/3	1/2	1/2												
2	1	0/1	0/2	0/0	0/2	1/1	3/5	1/5	0/0	2/3	1/2	0/0	1/2								
	2	0/2	2/3	0/1	0/1	0/1	2/2	0/0	0/3	2/4	1/1	0/0	1/4								
	3	0/0	0/0	2/2	1/3	1/1	3/3	1/2	2/4	2/4	0/0	1/1	3/4								
	4	0/0	1/2	2/2	1/4	1/1	0/1	2/2	4/4	3/4	1/2	0/5	2/3								
	5	0/1	2/3	2/4	0/0	1/6	3/4	2/5	1/3	1/4	2/3	0/1	3/4								
3	1	0/0	0/0	1/4	0/8	0/1	0/1	0/4	0/10	1/3	2/4	2/5	2/9								
	2	2/3	0/1	1/3	0/2	0/0	2/2	4/8	1/6	1/3	3/4	2/5	2/6								
	3	1/3	0/5	0/0	1/4	----------				1/1	1/6	1/1	3/7								
	4	1/3	1/3	1/1	1/8	0/2	2/7	0/1	1/5	3/2	5/8	0/3	1/8								
	5	0/2	0/2	2/4	1/5	1/1	6/7	0/0	0/5	2/2	0/3	2/3	0/4								
4	1	1/4	3/5	3/7	2/5	0/0	2/1	0/0	1/4	3/4	0/4	1/1	2/5								
	2	3/5	1/3	0/0	3/6	0/0	5/6	1/2	0/4	3/4	0/3	0/1	2/5								
	3	4/7	3/4	1/1	2/3	0/2	4/5	1/1	1/2	3/5	0/3	1/3	0/1								
	4	0/4	0/3	0/0	2/4	----------				3/4	1/8	2/2	2/2								
	5	2/3	1/4	0/0	0/3	0/2	2/3	2/2	1/1	6/7	1/2	1/3	2/6								

cross sections has a variance which was calculated as follows:

TABLE 31

Source	d.f.		X^2	M.S.	
Experiments	3		8.031	2.677	2.93 ($p<.05$)
Mice/expt.	10		8.313	0.831	
Sections/mice	60	259	61.518	1.025	0.913
Remainder	189		166.673	0.882	

A significant difference is revealed in the amount of lung tissue infected between different experiments. Insufficient experiments were performed to validate such an analysis. However, the variance between the other components is very similar and no significant differences are shown. The total variance within any one experiment is given as the average value for these readings.

$$\text{Total variance} = 2 \times \left(\frac{0.913}{259}\right)^{\frac{1}{2}}$$

$$= 0.1187$$

Using the principles of Delesse expounded in Chapter V relating areas to volumes, it is possible to say that after exposure to the standard aerosol 43.8 ± 11.9 per cent of the lung airway was infected.

APPENDIX XVII

HISTOLOGICAL PREPARATION

1. Processing schedule for mouse lungs

The total thoracic contents were fixed intact for a minimum of 24 hours. The tissues were then further dissected to remove the heart, oesophagus, aorta and vena cava from the lungs. The trachea was cut off just above its bifurcation. The lungs were then processed according to the following programme, each stage lasting for 30 minutes:

80 per cent alcohol	x 1
95 per cent alcohol	x 2
Absolute alcohol	x 3
Toluene	x 2
Paraffin wax	x 2
Paraffin wax (under vacuum)	x 1

2. Section sampling from lung blocks

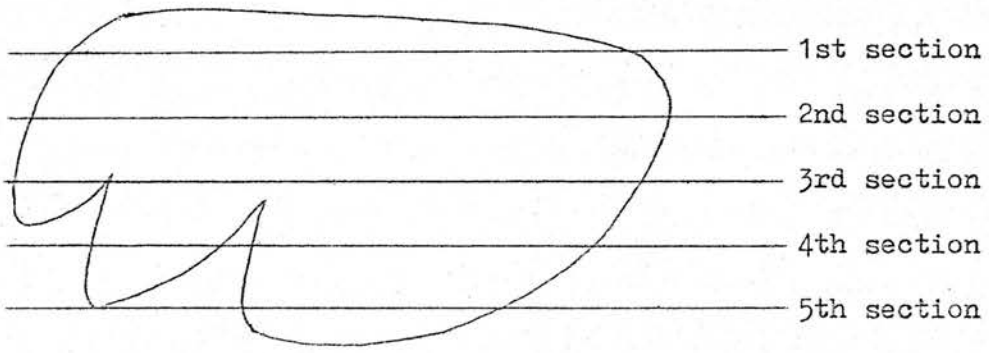

Fig. M : Diagrammatic view of fixed lungs

The first section was taken after initial trimming of the block. The cut surface was cooled by ice cubes for ten minutes before cutting each section.

3. Stains preparation

Acidified Potassium Permanganate

Potassium Permanganate	0.5 g.
3% aqueous Sulphuric Acid	5 ml.
Distilled Water	95 ml.

Oxalic Acid

Oxalic Acid crystals	1 g.
Distilled Water	100 ml.

Iron Alum

Iron Alum (Ammonium Ferric Sulphate)	2.5 g.
Distilled Water	100 ml.

Silver Solution

10% aqueous Silver Nitrate	5 ml.

Add strong ammonia solution drop by drop until the resultant precipitate is just redissolved. Then add:-

3% aqueous Sodium Hydroxide	5 ml.

Add strong ammonia solution drop by drop until the precipitate is just redissolved (the solution should be faintly opaque). Make the volume up to 50 ml. with distilled water.

Formalin solution

Formaldehyde (40%)	10 ml.
Distilled Water	90 ml.

Gold Chloride

Gold Chloride (Yellow)	0.2 g.
Distilled Water	100 ml.

"Hypo"

Sodium Thiosulphate	5 g.
Distilled Water	100 ml.

Add a crystal of Thymol to prevent the growth of moulds.

Giemsa Solution

Giemsa stain	2 ml.	10 ml.
1% aqueous Acetic Acid	5 ml.	25 ml.
Distilled Water	45 ml.	225 ml.

4. Staining method

1. Remove wax from sections and hydrate as usual.
2. Oxidise in Potassium Permanganate for 1-5 minutes.
3. Wash in distilled water.
4. Bleach in Oxalic Acid for 3-5 minutes.
5. Wash in distilled water, then tap water, then in 2 or 3 changes of distilled water.
6. Mordant in Iron Alum for 10 minutes to 2 hours (10 minutes will usually suffice).
7. Wash in several changes of distilled water.
8. Treat with freshly prepared, filtered Silver solution for 30 seconds.
9. Wash in several changes of distilled water.
10. Reduce in formalin solution (section goes black).
11. Wash in tap water, then in distilled water.
12. Tone in Gold Chloride for 10-15 minutes.
13. Wash in distilled water.
14. Fix in "Hypo" for 5 minutes.
15. Wash well in tap water, then in distilled water.
16. Stain over night in Giemsa Solution at room temperature.
17. Wash in distilled water.
18. Dehydrate through 2 changes of acetone; 10 seconds in the first, and about 1 minute in the second change.
19. Transfer to a mixture of equal parts of acetone and xylene.
20. Clear in 2 changes of xylene. Mount in DePex containing 1 per cent Butylated Hydroxytoluene as antioxidant.

Stain Characteristics

Reticulin fibres	Black
Nuclei	Blue to Violet
RBC's	Grey to Pink
Collagen and Muscle	Pinkish
Bacteria	Blue

APPENDIX XVIII

TABLE 32

EXAMPLES OF DATA COLLECTION FROM LUNG SECTIONS

Control mouse after 27 days at 10°C/85-90% RH		Infected mouse after 9 days at 10°C/85-90% RH	
No. BM Intersections	Cell Count	No. BM Intersections	Cell Count
50	88	25	5
40	67	28	8
50	87	50	47
41	61	36	29
59	86	27	19
53	73	24	12
62	100	49	59
52	76	37	40
54	70	48	23
47	69	41	52
50	73	44	60
46	68	45	64
51	70	36	26
42	54	36	28
56	92	48	21
37	53	33	0
38	51	53	71
36	58	25	10
39	60	51	30
51	82	39	54
45	65	45	7
43	69	41	0
43	75	21	4
47	73	43	65
33	52	56	36
40	67	35	53
45	77	41	15
		36	20
		43	64
		31	9
27	n		30
1. 53	Mean $\frac{\text{Cell Count}}{\text{BM intersects}}$		0.754
0.019	Variance		0.242
0.138	Std. dev.		0.492
0.027	Std. error		0.090

A correction factor was applied to the mean figure of cell count / BM intercepts. This factor takes into account the dimensions of the eyepiece graticule, the magnification factor of the microscope and the formula derived by Weibel and Elias (see Chapter V : Theory of quantitation).

Thus the length of the BM, L, is given by

$$L = \frac{\tilde{n}}{2} \times D \times I_1$$

where D is the grid space interval in mm

and I_1 is the intercept factor.

$$L = \frac{22}{7} \times \frac{12}{1900} \times I_1$$

$$= 100.76\ I_1$$

Applying this factor to the two examples given on the previous page

Cells/mm. BM = 1. 53 x 100.76 = 154

and = 0.754 x 100.76 = 76

The standard error is corrected in a similar manner.

APPENDIX XIX

TABLE 4:ANALYSIS OF VARIANCE ON 2 EXPERIMENTS AT 10°C/85-90% RH

Day	Total ♂	♂	Total ♂	Total ♀	♀	Total ♀	Grand Total	Mean
3	156	156	312	153	150	303	615	153.75
5	133	147	280	151	143	294	574	143.50
7	131	131	262	134	144	278	540	135.00
9	79	76	155	66	47	113	268	67.00
11	51	112	163	121	67	188	351	87.75
13	117	110	227	122	123	245	472	118.0
15	115	135	250	116	92	208	458	114.5
17	157	119	276	158	145	303	579	144.75
19	147	156	303	139	140	279	582	145.5
23	142	153	295	158	138	296	591	147.75
27	160	178	338	160	198	358	696	174.0
31	172	162	334	174	133	307	641	160.25
35	148	158	306	156	146	302	608	152.0
	1708	1793	3501	1808	1666	3474	6975	

Standard error of a mean = 8.7

Total S.S. = 987819.0

S.S. between sexes = 935602.96

S.S. between days = 978770.25

S.S. for days x sexes = 980635.50

Correction factor = 935588.94

TABLE 5:ANALYSIS OF VARIANCE ON 2 EXPERIMENTS AT 20°C/55-60% RH

Day	Total			Total			Grand Total	Mean
	♂	♂	♂	♀	♀	♀		
3	150	152	302	151	149	300	602	151
5	147	151	298	148	150	298	596	149
7	140	145	285	133	144	277	562	140.5
9	125	133	258	136	140	276	534	133.5
11	99	107	206	114	110	224	430	107.5
13	129	125	254	127	133	260	514	128.5
15	140	148	288	121	123	244	532	133
17	150	150	300	140	156	296	596	149
19	162	166	328	163	149	312	640	160
23	139	141	280	176	172	348	628	156.5
27	129	131	260	136	132	268	528	132
31	145	145	290	156	160	316	606	151.5
35	143	149	282	133	135	268	550	137.5
Total	1798	1843	3631	1834	1853	3687	7318	

Standard error of a mean = 2.3

Total S.S. = 1045124

S.S. between sexes = 1032725

S.S. between days = 1042390

S.S. for days x Sexes = 1044615

Correction factor = 1032684.3

TABLE 6:ANALYSIS OF VARIANCE ON 2 EXPERIMENTS AT 30°C/55-60% RH

Day	Total			Total			Grand Total	Mean
	♂	♂	♂	♀	♀	♀		
3	155	137	292	141	126	267	559	139.75
5	129	128	257	120	134	254	511	127.75
7	115	114	229	125	119	234	463	115.75
9	84	53	137	143	105	248	385	96.25
11	121	135	256	137	157	294	550	137.5
13	151	145	296	150	144	294	590	147.5
15	121	132	253	153	154	307	560	140.0
17	135	126	261	124	133	257	518	129.5
19	159	136	295	128	134	262	557	139.25
23	150	141	291	127	135	262	553	138.25
27	142	142	284	148	148	296	580	145
31	115	117	232	117	117	234	466	116.3
35	133	128	261	127	131	258	519	129.75
	1710	1634	3344	1740	1737	3467	6811	

Standard error of a mean = 5.0

Total S. S. = 911623

S.S. between sexes = 895071.73

S.S. between days = 904263.75

S.S. for days x sexes = 909175.5

Correction factor = 894731.55

TABLE 7:ANALYSIS OF VARIANCE ON 2 EXPERIMENTS AT 30°C/85-90% RH

Day	Total					Total	Grand Total	Mean
	♂	♂	♂	♀	♀	♀		
3	145	145	290	139	143	282	572	143
5	137	152	289	141	128	269	558	139.5
7	100	121	221	108	136	244	465	116.25
9	121	108	229	76	126	202	431	107.75
11	133	110	243	122	105	227	470	117.5
13	88	117	205	136	124	260	465	116.25
15	140	132	272	139	137	276	548	137
17	116	118	234	149	143	292	526	132.5
19	152	140	292	159	159	318	610	152.5
23	150	150	300	171	152	325	623	155.75
27	164	160	324	158	158	316	640	160
31	172	138	310	160	149	309	619	154.75
36				146 155	158 156			153.75
	1618	1591	3209	1658	1660	3318	6527	

Standard error of a mean = 6.7

Total S.S. = 908327

S. S. between sexes = 887783.54

Day S. S. = 901907.25

S. S. for day x sexes = 904320.5

Correction factor = 887536.02

TABLE 8 : ANALYSIS OF VARIANCE ON EACH TREATMENT

$10^{\circ}C/85-90\%$ RH

	df	SS	MS	F value
Days	12	43,181.31	3,598.44	12.02 ($p < .001$)
Sexes	1	14.02	14.02	0.04 NS
Days x sexes	12	1,851.23	154.25	0.51 NS
Error	24	7,183.50	299.31	
Total	49	52,230.06	1,065.91	

$20^{\circ}C/55-60\%$ RH

Days	12	9,705.7	808.8	38.1 ($p < .001$)
Sexes	1	40.7	40.7	1.9 NS
Days x sexes	12	2,184.3	182.03	8.6 ($p < .001$)
Error	24	509.0	21.21	
Total	49	12,439.7	253.9	

$30^{\circ}C/55-60\%$ RH

Days	12	9,532.2	794.4	12.0 ($p < .001$)
Sexes	1	340.18	340.2	3.3 NS
Days x Sexes	12	4,571.57	381.0	3.7 ($p < .001$)
Error	24	2,447.5	102.0	
Total	49	16,891.45	344.7	

$30^{\circ}C/85-90\%$ RH

Days	11	14,371.23	1,306.48	7.2 ($p < .001$)
Sexes	1	247.52	247.52	1.4 NS
Days x sexes	11	2,165.73	196.88	1.1 NS
Error	22	4,006.5	182.11	
Total	45	20,790.98	462.02	

APPENDIX XX

SUPPLIERS OF COMMERCIAL PRODUCTS

Oxoid Pasteurised Rat and Mouse Diet.

Oxoid Ltd., London, SE1

Synthetic Vitamin K1 (Konakion).

Roche Products Ltd., Welwyn Garden City, Herts.

Polypropylene mouse boxes.

North Kent Plastic Cages Ltd., Dartford, Kent.

Square Pak flasks (ASPF).

Medisco Equipment Ltd., Windsor, Berks.

Gamma irradiation.

Irradiated Products Ltd., Wantage, Berks.

Peracetic Acid.

Laporte Chemicals Ltd., Luton, Beds.

Nacconal 90F.

Kingsley & Keith Ltd., London, SW1.

Flexible film isolators.

Plysu Industrial Ltd., Woburn Sands, Bucks.

Vickers Medical Ltd., Basingstoke, Hants.

Filter cartridges Type MS 681.

Stora Kopparberg, Sweden.

Parainfluenza antisera.

Flow Laboratories Ltd., Irvine, Scotland.

Membrane filters.

Millipore Ltd., Wembley, Middlesex.

Rotary compressor Model 1550.

Gast Manufacturing Ltd., High Wycombe, Bucks.

Thermohygrometers.

Pastorelli and Rapkin Ltd., London, N13 4XS

Rubber gaskets : Claytonrite.

North British Rubber Co. Ltd., London, SW1

Plastic film (autoclavable) : Melinex polyester sheet.

I.C.I. Chemicals Ltd., Macclesfield, Cheshire.

Porton all glass impingers.

A. W. Dixon & Co. Ltd., London, SE20

Anti foam agent : Polyglycol P2000.

Dow Chemical Co. Ltd., Wimslow, Cheshire.

Base sledge microtome.

E. Leitz Ltd., London, W1

Fibro wax embedding medium.

R. A. Lamb Ltd., Wembley, Middlesex.

Paraffin wax embedding medium M.p. 57°C.

G. T. Gurr Ltd., High Wycombe, Bucks.

Eyepiece graticule.

Graticules Ltd., Tonbridge, Kent.

Tissue grinders.

Arthur H. Thomas & Co., Philadelphia, U.S.A.

Fluorescein conjugated antisera.

Nordic Pharmaceuticals and Diagnostics,

Tilburg, The Netherlands.

Tissue emulsifier.

Silverson Machines Ltd., London, SE1

Fine Leaf gelatin.

Societe de Produits Chimiques Coignet.

Brussels, Belgium.

Air filter for atomiser air supply.

Microflow, Fleet, Hants.

Air refrigeration Unit : Deltech Refrigerated Dryers Model RFA-04C.

Oxy Fluid Control, Woking, Surrey.

Pressure regulating valves : Fairchild Regulators.

Efco Ltd., Woking, Surrey.

Water atomiser : Mistaire Spray Nozzles.

Delavan-Watson Ltd., Widnes, Lancs.

Midget Solenoid Valve.

A. Schrader Son, Cannock, Staffs.

Recorder controller : Clearspan P105 Potentiometric Recorder/Controller.

Foster Cambridge, London, N12.

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