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Respiratory disease in a colony of rats

II. Isolation of *Mycoplasma pulmonis* from the natural disease, and the experimental disease induced with a cloned culture of this organism

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SUMMARY

Mycoplasma pulmonis was isolated from the pneumonic lung of a rat. Two groups of mycoplasma-free rats were inoculated, one with a culture of the *M. pulmonis* strain which had been cloned four times (group A) and the other with a lung homogenate of the rat from which the strain had been isolated (group B). A third group (C) consisted of uninoculated control animals. Each group was kept in strict isolation and allowed to breed so that the progeny was naturally exposed to any pathogens present in the inoculated animals. After different periods of exposure, rats were autopsied, respiratory tracts and inner ears were cultured for mycoplasmas and bacteria, and sera were tested for complement-fixing antibodies to murine mycoplasmas.

In group-A rats, *M. pulmonis* was consistently isolated from the inner ears or lungs from 50 to 715 days after exposure. Complement-fixing antibody to *M. pulmonis* was detected 20 days after inoculation, but in the naturally exposed progeny antibody took longer than 50 days to develop. Antibodies to the other known mycoplasmas of murine origin, *M. arthritidis* and *M. neurolyticum*, were never found. Purulent otitis interna was consistently found from day 55 onwards, while lung lesions were first observed at 85 days and persisted to 715 days. Pulmonary lesions developed more slowly in inoculated parents than in exposed progeny. Similar results were found in group-B rats, which were examined up to 441 days after inoculation. Uninoculated group-C rats were examined up to 768 days of age, but *M. pulmonis* was not recovered; of the 54 animals whose serum was tested all were negative to the three species of mycoplasmas, except one which had a titre of 16 with *M. pulmonis*. Pneumonia, bronchiectasis or lymphoreticular hyperplasia were not seen in any of these control rats. Bacterial respiratory pathogens were not isolated from rats in any of the groups, nor was antibody to Sendai virus detected.

The results suggest that *M. pulmonis* alone can cause pneumonia and bronchiec-

tasis in rats since mechanical carry-over of another pathogen with the initial cloned inoculum is very unlikely and there was no evidence for the participation of any other rat pathogen. The respiratory disease induced by the cloned culture was comparable with that induced by the lung homogenate, and with the well-known syndrome of chronic respiratory disease and bronchiectasis in the rat.

INTRODUCTION

This paper is concerned with the role of *Mycoplasma pulmonis* in the production of pulmonary disease in the rat. Reviews of the many possible aetiological agents involved in chronic murine pneumonia have been published by Joshi, Blackwood & Dale (1961) and Brennan, Fritz & Flynn (1969). The terminology and wider significance of chronic respiratory disease in rats and its association with *M. pulmonis* has recently been reviewed by Lindsey *et al.* (1971).

Although *M. pulmonis* is the organism that has been isolated most consistently from pneumonia of the rat, and although it is generally accepted that this organism is frequently the cause of infectious catarrh of the upper respiratory tract, there is doubt whether *M. pulmonis* alone causes pneumonia in the rat. Klieneberger & Steabben (1940) demonstrated a close connexion between the presence of the L3 organism (later known as *M. pulmonis*) and the bronchiectatic pneumonia of laboratory rats, but they could not produce experimental lung infection with the organism. Lemcke (1961) found that *M. pulmonis* was established in the nasopharynx of young rats in the first few weeks of life, but they did not develop lung lesions until they were much older, the incidence of pneumonic lesions increasing with age. Klieneberger-Nobel & Cheng (1955) and Klieneberger-Nobel (1962), however, showed that pneumonia and bronchiectasis associated with *M. pulmonis* could be induced in younger rats by bronchial ligation, intubation for anaesthesia or exposure to low temperatures. Nelson (1940, 1967) showed an association between infection with *M. pulmonis* (described in the early paper as 'coccobacilliform bodies') and the development of infectious nasal catarrh, otitis media and possibly pneumonia in rats. However, in an assessment of almost 30 years work, Nelson (1967) came to the conclusion that pulmonary lesions were not caused by *M. pulmonis* alone but that a 'virus' was also involved; this latter agent does not appear to have been characterized sufficiently to enable later workers to test his hypothesis. Gay (1967*a*) provided evidence that *M. pulmonis*, although colonizing the upper respiratory tract of the rat, did not invade the normal lung. He considered that the organism was probably an opportunist following chronic inflammation caused by another agent. Gay also showed that a chronic rat pneumonia could be induced with inocula free from cultivable *M. pulmonis* or other known mycoplasmas; he suggested that his agent might nevertheless be a mycoplasma because of its morphology in electron micrographs, and its antibiotic sensitivity when studied in mice. Vrolijk, Verlinde & Braams (1957) may well have been dealing with the same agent. Bell (1967) also considered *M. pulmonis* to be merely a secondary organism in chronic respiratory disease of the rat. This view was supported by later work (Bell & Elmes, 1969) in which only minor lung changes

were induced in rats by the intranasal inoculation of either *M. pulmonis* alone or *M. pulmonis* in combination with *Streptobacillus moniliformis*. Nevertheless, lung lesions developed in rats inoculated intranasally with lung homogenates from rats with chronic respiratory disease; the responsible pneumonia-inducing agent was not identified.

Kohn & Kirk (1969) induced gross lung lesions in rats inoculated repeatedly with a once-cloned culture of *M. pulmonis*. The disease was more severe in some rats kept in adjacent cages than it was in the inoculated rats. Although Lindsey *et al.* (1971) induced pneumonia with various inocula containing unpurified *M. pulmonis*, their cloned strain (N) did not produce even microscopic lung lesions. A few studies of chronic respiratory disease in rats have included tests for serum antibodies; the plate-agglutination test was used by Kohn & Kirk (1969), who detected antibody to *M. pulmonis* in most of the gnotobiotic rats exposed to this organism but not in unexposed controls. Complement-fixing antibody to *M. pulmonis* was demonstrated in the sera of naturally and experimentally infected rats (Lemcke, 1961); its presence correlated with the demonstration of the mycoplasma by cultural methods. However, Bell & Elmes (1969) were unable to correlate titres of specific complement-fixing antibody with the presence of *M. pulmonis* in conventional rats with chronic respiratory disease or in specific pathogen-free rats inoculated with lung homogenates from rats with chronic respiratory disease. Only one animal with chronic respiratory disease gave a titre as high as 16 and most were less than 4. Similar results had been reported from the same laboratory by Gay (1967*a*).

Thus evidence on the role of *M. pulmonis* in the production of pneumonia in the rat is conflicting. The present work was therefore undertaken to investigate whether a recent isolate of *M. pulmonis* from a case of pneumonia in a naturally occurring outbreak of severe respiratory disease in rats (Lane-Petter, Olds, Hacking & Lane-Petter, 1970) would induce a similar respiratory disease syndrome experimentally, and to study the serological response to experimentally-induced infection.

MATERIALS AND METHODS

Sources of materials for transmission experiments

Seven rats which were affected with respiratory disease in a natural outbreak at Alconbury (Lane-Petter *et al.* 1970) were removed to an isolation cubicle at the School of Veterinary Medicine, Cambridge, where they were held for a few days to allow clearance of the previously administered oxytetracycline before they were killed. The lungs of two of the rats, 3102 and 3103, were quantitatively cultured for mycoplasmas and were examined bacteriologically. A lung suspension from rat 3102 and a mycoplasma isolated from the same lung and subsequently cloned, were used as inocula in the transmission experiments.

Cultivation of mycoplasmas

The media used for isolating and cloning mycoplasmas and for preparing rat inocula were designated A 26. These were the liquid and solid Hartley's broth-based media described by Whittlestone (1969), except that the bicarbonate was

not included. All constituents and containers were prepared and maintained at tissue-culture standard. Constituents were sterilized either by autoclaving or Millipore filtration to avoid Seitz filtration.

Solid medium was poured into 50 mm. plastic Petri dishes with non-sealing lids which were incubated at 37° C. in a humid atmosphere of 5–10% CO_2 in air. Liquid media were sealed and incubated at 37° C.

Titres of mycoplasmas in cultures or tissues

The numbers of mycoplasmas were expressed either as colony-forming units (CFU) on agar medium or colour-changing units (CCU) in liquid medium. The number of CFU were calculated from the colony counts obtained from 0·02 ml. drops of at least three $\log_{10}$ dilutions after about 6 days incubation, after which no new colonies appeared. In liquid medium the titre of CCU was expressed as the reciprocal of the highest dilution at which the phenol-red indicator showed a drop in pH from 7·4 to 6·8 after 14–21 days incubation. In some instances – for example, with the rat inocula – the titrations were made in triplicate to obtain a more accurate estimate of the titre of mycoplasmas.

Purification of cultures

Mycoplasma 3102 was purified by four serial single-colony subcultures on solid medium as follows. An agar block bearing colonies was put into 1 ml. of liquid medium and the colonies emulsified. Tenfold dilutions were made and 0·02 ml. volumes plated on solid medium. After 6 days incubation an agar block bearing a single well-separated colony was cut out and the colony emulsified in 1 ml. of liquid medium. The dilution, inoculation, incubation and cloning procedure was repeated three more times serially before the culture of mycoplasma 3102 was used for preparing the rat inoculum and for serological identification.

Rat inoculations

Group-A rats received the serially cloned mycoplasma derived from lung 3102; the inoculum was a subculture in liquid medium of colonies from the final cloning and contained *ca.* 10^9 CCU/ml.; on agar the inoculum yielded $0{\cdot}5 \times 10^8$ CFU/ml.

Group-B rats were inoculated with a suspension of pneumonic lung from rat 3102. This material, which had been kept frozen at – 60° C. since harvesting, was thawed rapidly and ground with horse serum broth to give a 10^{-1} suspension. A further ten-fold dilution in mycoplasma medium was used as inoculum and contained *ca.* 10^7 CCU/ml. (*ca.* 10^6 CFU/ml.). Rats were anaesthetized with CO_2 gas and their nostrils immersed in about 1 ml. of culture or 0·5 ml. of lung suspension. Each rat inhaled 0·1–0·25 ml. of fluid.

Experimental animals and accommodation

The rats used in the transmission experiments were derived from a small colony of rats established in December 1968; the young foundation stock of four females and two males were primary hysterectomy-derived CFE rats from Carworth,

U.S.A. From this barrier-maintained colony two litters born on adjacent days were divided into separate cubicles as follows: 8 rats which were inoculated with the cloned mycoplasma when 45 days old (group A); 7 which were inoculated with the pneumonic lung suspension when 52 days old (group B); 4 which were retained in the uninoculated control colony (group C). Some limited breeding was allowed in each colony so that the total number of rats in groups A, B and C reached 88, 52 and 109 respectively.

The cubicles in which the three colonies were housed were in a specially designed building provided with filtered air at positive pressure; each cubicle was entered via an ante-room in which protective clothing was put on. Before introducing rats into a new cubicle, all the equipment was either steamed or autoclaved, and the whole cubicle with its equipment then fumigated with formalin. Food was autoclaved and the drinking water acidified (Lane-Petter *et al.* 1970). Rats were kept in plastic boxes with wire-mesh tops and were provided with sawdust bedding and wood shavings.

For the first few months of the experiments different attendants looked after the normal and experimental rats. Later, all the rats were cared for over a period of several months by one attendant, who took a shower after attending infected rats and did not enter another cubicle for at least several hours. The order of attending the groups was C, A, and then B.

Post-mortem examinations

Harvesting tissues and exudates

Rats were anaesthetized with CO_2 gas and bled out for serum from an incision in the axilla. The skin was reflected from the thoracic cavity, the sternum removed, and the lung tissue harvested aseptically.

To collect fluid from the middle ear the external pinna was severed close to the tympanic membrane, which was pierced with a fine Pasteur pipette. The inner-ear samples were collected from the cochlea and vestibule, which were opened ventrally, after exposing the overlying bony bulla and searing it with a hot piece of shaped aluminium foil.

Harvested specimens and cultures were stored at $-60°$ C. or below and sera at $-24°$ C. Rats dying on days when it was impracticable to make a detailed examination were stored at $-24°$ C. in plastic bags and thawed under running cold water before examination.

Histological methods and assessments

For Giemsa-stained touch preparations the method of Whittlestone (1967) was used.

After aseptic collection of lung specimens, the larynx, trachea and lungs were removed, a few blocks were fixed in acetic Zenker and the remainder of the lung usually fixed in 10% neutral formalin before preparing blocks by section at various levels.

Sections were paraffin embedded and stained with haematoxylin and eosin. The degree of bronchiectasis was assessed in the histological preparations, by measuring

the internal diameters of transverse sections of bronchioles of rats from the infected colonies and then subtracting the equivalent figure for rats of approximately the same age from the control colony. The degree of bronchiolar distension thus assessed was recorded numerically in 0·1 mm. units.

The degree of lymphoreticular hyperplasia was assessed similarly and expressed in the same units.

Recording of gross lesions

The extent of pneumonia was recorded as an approximate percentage of the lung surface affected, by using the following scoring system: the right apical, cardiac and intermediate lobes were each allocated 10 points, the right azygos lobe 20 points, and the left lung 50 points if completely pneumonic. The most extensive case of pneumonia in this series thus scored 80 out of a theoretical 100 points for a completely pneumonic lung.

Culture of tissue for mycoplasmas and bacteria

Lung tissue was ground to produce an approximate 10^{-1} suspension in mycoplasma medium or 10% horse serum broth and further ten-fold dilutions were made in appropriate liquid media before incubation or inoculation onto agar. The cultivation of mycoplasmas is described above. For bacterial isolation a drop of lung suspension was sown on pairs of blood-agar, 'chocolate'-agar and MacConkey-agar plates, and on Loeffler's serum slopes. One sample of each pair was incubated anaerobically and the other in air with 5% CO_2. A cooked-meat medium was also sown and incubated in air. Bacteriological media were prepared by the methods of Cruickshank (1965).

The routine cultural examinations of the inner ear, nasal mucosa and trachea were made by preparing approximately 10^{-2} dilutions of the surface exudates in appropriate broth, which were then diluted, inoculated and incubated as described for the lung suspensions. In some instances exudates were inoculated directly on to solid medium.

Serological methods

Reference cultures of mycoplasmas

In all, 18 species were used. The murine mycoplasmas *M. pulmonis* (Kon, M1, MB, and Sabin's type C) *M. arthritidis* (PG27 and Jasmin) and *M. neurolyticum* (KSA) were those described by Lemcke (1961, 1964) and Lemcke, Forshaw & Fallon (1969). Other non-murine mycoplasmas were *M. hominis* (SC4). *M. pneumoniae* (FH), *M. salivarium* (B3), *M. fermentans* (G2), *M. orale* type 1 (837), *M. mycoides* var. *mycoides* (Gladysdale), *M. bovigenitalium* (PG11), *M. gallisepticum* (X95), *M. gallinarum* (Fowl), *M. iners* (M), *M. primatum* (Navel), *M. suipneumoniae* (J), *Acholeplasma laidlawii* (A), avian strain A 36 and Dinter's porcine strain B3 (Lemcke *et al.* 1969; Goodwin, Pomeroy & Whittlestone, 1967).

Media

The basal medium used in the preparation of antigens was that described by Hollingdale & Lemcke (1969), but supplemented with pooled, inactivated human

serum (20 %, v/v) instead of the human plasma. Difco PPLO agar with unheated horse serum (Burrough's Wellcome No. 3) was used for the growth-inhibition tests.

Growth inhibition (GI)

Tests were made by the method of Clyde (1964).

Complement fixation (CF)

Antigens were prepared and the method carried out as described by Hollingdale & Lemcke (1970). Anticomplementary rabbit or rat sera were diluted with normal guinea-pig serum (1/10) and held at 4° C. for 24 hr. before inactivation at 56°C. for 30 min.

Antisera

Antisera to *M. suipneumoniae* and Dinter's porcine mycoplasma B3 were previously described by Goodwin *et al.* (1967). Sera against the other 16 species were those prepared by Lemcke (1964, 1965), Hollingdale & Lemcke (1969) and Lemcke, Forshaw & Fallon (1969), and all inhibited growth of the homologous species.

Antisera against strain 3102 were prepared in two rabbits. Antiserum for growth inhibition, where antibody to foreign serum proteins was unimportant, was prepared in Rabbit 187 by intravenous inoculation of mycoplasma cells from 20 hr. cultures in the human-serum liquid medium referred to above. In all, the rabbit received washed cells from 1 l. of culture; six injections were given at intervals of 3 days, and 3 weeks later a second similar series was given. For CF tests, antiserum was prepared in Rabbit 188 against 3102 grown for 48–72 hr. in liquid medium containing unheated rabbit serum (15–20 %, v/v). The strain was subcultured seven times on agar or in broth containing rabbit serum before the inoculum was prepared. Cultures were harvested when the pH reached 7·0–7·2, washed twice in saline and stored at −30° C. Growth from 250 ml. of culture was emulsified in adjuvant (9 parts of Esso Markol 52 to 1 part of Arlacel) and given in two subcutaneous injections, separated by an interval of 3 weeks. Three weeks after the second inoculation, a saline suspension from another 250 ml. of culture was given in six intravenous injections. Rabbits were bled 1 week after the last injection.

Haemagglutination-inhibition (HI) test for Sendai virus (parainfluenza 1)

For the tests done at Pfizer Laboratories the Sendai virus used was an isolate from Carworth mice and was used at eight haemagglutinating (HA) units. Sera were titrated with and without prior heating at 56° C. for 30 min. Virus-serum mixtures were held at 4° C, for 1 hr., then 1 % chicken red cells were added, followed by a further hour at 4° C. before reading the end-point as the highest dilution showing complete inhibition. As a control, a rabbit antiserum with a titre of 128 was run in parallel.

For the Sendai antibody tests made at the Clinical Research Centre Laboratories the method used was that described by Tyrrell & Coid (1970).

RESULTS

Isolation of mycoplasmas from the natural disease

During the 3–6 days in which the seven rats from the natural outbreak were held in isolation before killing, their clinical condition progressively improved, although they were receiving an antibiotic-free diet.

When killed, five showed extensive pneumonia (scores 18, 20, 30, 35 and 52) with copious catarrhal exudate in the trachea and major bronchi while the other two had small pneumonic lesions. Histologically the group showed purulent or chronic bronchitis and bronchiolitis, peribronchiolar mononuclear cell accumulations, massive lymphoreticular hyperplasia and organization. Perivascular mononuclear cell accumulations were common.

The lungs of two rats, 3102 and 3103, were examined for mycoplasmas; touch preparations showed many small rod-shaped organisms of the mycoplasma type associated with the cilia of the epithelial cells (Pl. 1, fig. 1), while on cultural examination each lung yielded approximately 10^8 mycoplasma colonies/g.

After storage in the deep freeze, the lung tissue of rat 3102 was cultured for bacteria using a sample of the suspension prepared for inoculation of the group-B rats. No eubacteria were isolated after seeding 0·025 ml. samples on the media already described as well as into Albimi brucella broth.

Cultural characteristics of the isolates from rats 3102 *and* 3103

After 2 days incubation on A 26 solid mycoplasma medium, minute colonies could be detected under the microscope ($\times$ 30 magnification); with dilute inocula the mycoplasma could just be seen by naked-eye examination after 4–5 days incubation. Crowded colonies were usually of the 'fried egg' type whereas in sparse cultures, the colony form was generally convex. There was usually no increase in colony numbers and little increase in colony size after the sixth day of incubation, by which time colonies were over 0·5 mm. in diameter. At this age colonies still could be subcultured readily. Touch preparations of colonies contained pleomorphic elements of the mycoplasma type.

Colony formation was not inhibited by penicillin (200 units/ml.) or thallium acetate (1/80,000), i.e. the concentrations used in the normal growth medium. Both strains (3102 and 3103) were sensitive to the tetracyclines, e.g. in tests with disks containing 5 μg. of tetracycline, the nearest mycoplasma colonies developed 14 mm. away.

Strain 3102 was passaged on solid medium without penicillin or thallium acetate, immediately after primary isolation. It retained its usual colonial appearance during these passes.

In liquid medium both strains produced acid, the pH of the medium changing from 7·5 to 6·8 within 24 hr. if 0·01 ml. of culture was seeded into 1 ml. A 26 medium. Cultures that had just reached a pH of 6·8 contained 10^8 to 10^9 CCU but no turbidity was observed. Where the same material was titrated in parallel in liquid and on solid medium, the liquid media gave a titre of CCU about 1 log higher than the titre of CFU on solid medium.

Table 1. *Reactions of mycoplasma* 3102 *with antisera to mycoplasmas of murine origin*

Antiserum against	Width of growth inhibition zone (mm.) strains: Homologous	Width of growth inhibition zone (mm.) strains: 3102	Reciprocal of CF titres with antigens: Homologous	Reciprocal of CF titres with antigens: 3102
M. pulmonis				
Kon	**5–6**	5–6	**1280**	640–1280
M1	**6**	7	**5120**	2560–5120
MB	**5**	5	**1280–2560**	1280
Type C	**5**	5	**1280–2560**	1280
M. arthritidis				
PG27	**2**	0	**20480**	< 10
Jasmin	NT	NT	**20480**	< 10
M. neurolyticum (KSA)	**4**	0	**10240**	< 10

NT = not tested.
Bold type represents homologous reactions.
Pre-immunization sera corresponding to all sera except M1 and Jasmin were available and gave CF titres of < 10 with both homologous and 3102 antigens.

Serological identification of mycoplasma 3102

Testing of 3102 *against antisera to various mycoplasmas*

In growth-inhibition tests strain 3102 was inhibited by antisera to four strains of *M. pulmonis*, namely Kon, M1, MB and Sabin's type C (Table 1). These antisera were judged to be specific as they did not inhibit any of the other mycoplasmas listed in Materials and Methods. Pre-immunization sera from the rabbits subsequently inoculated with Kon, MB and type C did not inhibit 3102. Pre-immunization serum corresponding to M1 antiserum was not available. Mycoplasma 3102 was not inhibited by antisera to the other 17 species.

With antisera to *M. pulmonis* strains Kon, M1, MB and type C, 3102 gave complement-fixing (CF) titres comparable with those given by the homologous antigens (Table 1). It did not react with antisera to the other murine mycoplasmas *M. arthritidis* or *M. neurolyticum* (Table 1).

Testing of antisera to 3102 *against various mycoplasmas*

Both the antisera prepared against 3102 in rabbits 187 and 188 inhibited the growth on agar of the homologous strain and *M. pulmonis* strains Kon, M1, MB and type C. Zones of inhibition were 5–6 mm. in width with an inoculum of 10^4–10^5 CFU/5 cm. plate. The other mycoplasmas listed above were not inhibited by the antisera.

In CF tests, antiserum prepared in rabbit 188 reacted with Kon, M1, MB and type C as well as with the homologous antigen, but did not react at 1 in 10 with antigens of *M. arthritidis* and *M. neurolyticum* (Table 2).

Mycoplasma 3102 was therefore identified as a strain of *M. pulmonis*. It is closely

Table 2. *CF reactions of antiserum* 188* *with mycoplasmas of murine origin*

Antigen	Reciprocal of CF titres with sera: Homologous antiserum	Pre-inoculation 188	Antiserum 188
3102	**2560**	< 10	**2560**
M. pulmonis			
Kon	**1280**	< 10	2560
M1	**5120**	< 10	1280–2560
MB	**1280–2560**	< 10	320
Type C	**1280–2560**	< 10	2560
M. arthritidis			
PG27	**20480**	< 10	< 10
Jasmin	**20480**	< 10	< 10
M. neurolyticum (KSA)	**10240**	< 10	< 10

* Prepared against Mycoplasma 3102.
Bold type represents homologous reactions.

related by both CF and GI tests to four strains of *M. pulmonis*, but not to *M. arthritidis* or *M. neurolyticum*, the other recognized mycoplasma species of murine origin. No relationship was found by the GI test between mycoplasma 3102 and 17 other species of mycoplasmas or acholeplasmas derived from a wide variety of sources.

Clinical signs in the group-A rats

No clinical abnormalities were detected in the group-A rats for many weeks after they were inoculated with cloned *M. pulmonis* strain 3102; but sneezing was noticed in some of their progeny before they were weaned. It had been noted earlier that the clinical condition of the natural cases of the disease improved when they were moved to a similar isolation cubicle; this suggested that improved environmental conditions could reduce the severity of the disease. Because of the absence of overt signs in the group-A rats, an attempt was made to create conditions in the isolation cubicle that were more likely to favour the development of clinical respiratory disease. From the 79th day after the experiment started, the rat cages were cleaned out only once weekly instead of twice. Following this, an ammoniacal smell was constantly present in the isolation cubicle. The density of rats was also increased by further breeding.

The first case of obvious respiratory disease, which developed when the experiment had been running for 118 days, was in rat 3145 born in the cubicle 78 days previously. This rat had distinct râles when handled, a sign which persisted until it was killed at 85 days of age. Subsequently the number of similarly affected rats increased, so that by about 200 days after the beginning of the experiment nearly all the rats in three-quarters of the cages were affected. The general picture in the colony was one of chronic respiratory infection. However, when the experiment had been running about 450 and 640 days, transient exacerbations occurred.

Pathological findings in the group-A rats

The most consistent finding in the group-A rats was purulent exudate in the bullae of the inner ears. Such exudate was already present in the inoculated rats killed 55 days after infection and in the naturally exposed rat killed at 85 days of age. Thereafter the internal ears of practically every rat were examined and all but two showed copious pus in one or usually both bullae. Very often the bullae were grossly enlarged and the surface bone showed rarefaction so that the creamy-yellow pus could be seen through the bone before the bulla was opened. The older rats (550–715 days) were at least as severely affected as the younger. In one rat (3281) which showed locomotor disturbance for a day before euthanasia, pus had tracked centrally from one bulla through the bone and into the central nervous system.

Table 3 summarizes the pathological findings in the lungs of the group-A rats after different periods of exposure to infection.

The earliest sign of lung involvement in the eight inoculated rats was in one killed at 125 days; this animal showed considerable lymphoreticular hyperplasia with some degree of bronchiolar dilatation. The rat which died at 155 days had quite extensive pneumonia with moderate lymphoreticular hyperplasia and bronchiolar dilatation; the one dying at 573 days had very extensive pneumonia with marked bronchiectasis.

In the rats exposed to natural infection from the inoculated animals, neither gross nor histological pneumonic lesions were detected in the animals killed at 50 days, but at 85 and 108 days quite extensive pneumonia was seen. Thereafter, out of the 40 rats examined up to 715 days of age, 30 showed gross pneumonia. The pneumonic lesions did not appear to become either more or less extensive with increasing age but there were more old animals without pneumonia.

The main histological features seen in the lungs of the group-A rats were associated with the bronchial tree; the earliest cases showed migration of polymorphonuclear neutrophils into the lumina, hyperplasia of the epithelium and an increase in the lymphoid cells surrounding the branches of the bronchial tree.

The later cases showed hyperplasia of the peribronchial glands and accumulation of purulent exudate in the bronchial tree which became grossly distended to form bronchiectatic cavities (Pl. 1, fig. 2). Bronchiectasis was first seen in a naturally exposed rat killed at 85 days and subsequently was obvious during the infection range 291–678 days, but only about half the rats were severely affected. Bronchiolar distension was greatest in animals exposed for 530–570 days, but of 13 rats examined later than this, only three showed any obvious bronchiectasis.

The development of bronchiectasis was associated with squamoid metaplasia of the bronchial and bronchiolar epithelium, and in some long-standing cases much of the alveolar tissue was replaced by multiple bronchiectatic cavities with folded walls (Pl. 2, fig. 3). The bronchioles containing exudate often had lost the cilia from the epithelium. Some of the bronchioles which were distended with purulent exudate showed partial or complete loss of epithelium, the purulent

Table 3. *Summary of results from rats (group A) from the colony infected with* M. pulmonis 3102

Rats	Exposure (days)	Extent of pneumonic lesions*	Degree of bronchiolar distension†	Degree of lympho-reticular hyper-plasia‡	*M. pulmonis* in lung	*M. pulmonis* CF titre of serum (reciprocal)
3122	20	0	0	0	–	128–256
3123	20	0	0	0	–	64
3132	55	0	0	0	N	256
3133	55	0	0	0	–	128–256
3143	125	0	0	0	–	128
3144	125	0	2	14	(+)	128
3149	155D	30	6	6	N	N
3335	573D	70	25	0	+	N
3137	50	0	0	0	N	8
3138	50	0	0	0	(+)	8
3145	85	29	2	6	(+)	128
3326	108D	60	80	0	(+)	N
3156	150	33	12	6	(+)	128–256
3157	175	0	0	0	N	64
3158	175	37	6	4	N	64
3321	276D	17	2	0	N	N
3322	291D	47	17	2	+	N
3324	294D	44	5	2	N	N
3319	363D	41	7	35	+	N
3325	372D	70	3	2	N	N
3261	379	35	2	7	+	128
3328	393D	80	25	10	+	N
3258	401	0	N	N	N	N
3329	405D	34	7	3	N	N
3330	452D	4	0	2	N	N
3259	484	38	6	2	N	N
3279	484	65	19	12	(+)	512
3280	488	30	3	16	N	64
3278	496	0	0	0	N	32
3332	500D	30	10	2	–	N
3281	514	0	0	0	N	N
3282	523	0	0	0	(+)	128
3333	535D	25	25	3	+	N
3290	541	80	12	2	–	256
3287	544	12	24	12	N	512
3289	547D	30	24	10	N	N
3293	547	18	21	4	N	256
3292	556	27	8	4	N	256
3294	565D	24	29	5	N	N
3316	597	0	0	1	+	64
3317	600D	7	2	2	N	N
3291	631	75	15	3	(+)	256
3341	678	45	16	2	+	512
3352	706	0	0	0	–	32
3354	707	1	0	2	–	64
3353	711	2	0	0	–	64
3347	712	35	19	2	+	512
3349	713	0	0	0	–	64

Table 3 (*cont.*)

Rats	Exposure (days)	Extent of pneumonic lesions*	Degree of bronchiolar distension†	Degree of lympho-reticular hyper-plasia‡	*M. pulmonis* in lung	*M. pulmonis* CF titre of serum (reciprocal)
3350	713	0	0	0	–	32
3351	713	0	0	0	–	64
3355	714	1	0	2	–	64
3348	715	20	0	2	+	128

The first eight rats were inoculated intranasally with cloned *M. pulmonis* when 45 days old. All other rats were born to these rats or their progeny and thus the length of possible exposure is the same as the age at death.

D = died. N = material not examined. + = isolation of *M. pulmonis*. (+) = isolation of colonies with the morphology of *M. pulmonis*.

* 0 = no pneumonia, 100 = entire surface of lung pneumonic.
† Distension of lumen of bronchioles in 0·1 mm. units
‡ Enlargement of peribronchiolar lymphoid tissue in 0·1 mm. units.

} See Materials and Methods

exudate being contained by connective tissue elements only. There were also frank abscesses within the alveolar areas of the lung.

In many of the rats with severe pneumonia and bronchiectasis there was copious catarrhal or mucopurulent tracheal exudate; such cases usually had shown marked clinical signs.

Microbiology of the group-A rats

The results of the cultural examination of lungs for mycoplasmas are presented in Table 3. It will be noted that the first isolation of mycoplasmas from the inoculated group was from one of the 125-day rats, whereas in the naturally exposed rats one of the lungs was already positive by day 50.

The lungs of 21 rats with pneumonia were examined culturally and 14 yielded colonies of the *M. pulmonis* morphology. Nine of these isolates were checked serologically and all were specifically identified as *M. pulmonis*. This series of examinations included the three oldest animals with substantial areas of pneumonia (3341, 3347 and 3348) which had been exposed to infection for 678, 712 and 715 days; all yielded *M. pulmonis*, the titres being 10^8, 10^6 and 10^4 CCU/g. respectively.

The inner-ear bullae of 26 rats were cultured for mycoplasmas. Of these, 21, including the earliest case (50 days) and the latest case (715 days) so examined, yielded colonies of the *M. pulmonis* morphology. One isolate was checked serologically and identified as *M. pulmonis*.

In addition, various cultures were occasionally prepared from other sites, particularly the nasal and tracheal mucosal surface. Practically all these checks were positive for *M. pulmonis*-type colonies, which were obtained from the nasal cavity up to 379 days and from the trachea up to 631 days after primary exposure.

All the mycoplasmas isolated and judged to be *M. pulmonis* on colonial morphology proved to be this organism when checked serologically.

The cultural examinations made on bacteriological media resulted in the regular

isolation of mycoplasma colonies; these results being comparable with those on mycoplasma media. In contrast, bacterial colonies were obtained infrequently and no species was regularly associated with disease. Thus of 31 lungs cultured bacteriologically, only seven yielded bacteria and from three of these only single bacterial colonies were obtained from 0·02 ml. of a 10^{-1} suspension. The only organisms isolated in appreciable numbers were *Pseudomonas pyocyanea* (three lungs) and *Citrobacter freundii* (one lung), but the last rat died and the citrobacter could well have been a post-mortem invader.

The other bacteria isolated from the lungs, inner ears or nasal mucosae in order of descending frequency were *Proteus mirabilis* (from the lung of one rat and from the inner ears of five), *Staphylococcus albus*, *Enterobacter aerogenes*, *Micrococcus* spp. *Staphylococcus aureus*, *Escherichia coli* and *Proteus morgani*.

It will noted be that no bacteria considered to be respiratory pathogens of the rat were isolated.

Antibody status of the group-A rats

Sera from 33 group-A rats were examined by the CF test. No antibody to *M. arthritidis* or *M. neurolyticum* was detected in any serum sample, even at 1/8, the lowest dilution tested. The CF titres obtained with *M. pulmonis* (3102) antigen are presented in Table 3. The rats inoculated and killed after 20 days already showed antibody and those killed after 125 days showed similar antibody titres. Their progeny, which were exposed naturally to infection, were negative at 1/8 at 50 days but positive after 85 days. Thereafter, every serum sample showed a titre of $\geqslant 32$, the antibody persisting at about the same level (usually 64–512) irrespective of the duration of the infection. The oldest rat killed at 715 days had a titre of 128. Sera from 11 group-A rats were examined for HI antibodies to Sendai virus. All were negative at the lowest dilution tested (1/2 or 1/5). The sera were evenly distributed from the 20th to the 713th day of the experiment.

Comparison of results from group-B rats with those from group A

The group-B rats were included in the experiment to find whether an inoculum of pneumonic tissue would induce disease comparable with that observed at Alconbury. The disease which developed in the group-B rats was similar to that in group A, and in each group the disease was milder for the first few months than in the Alconbury outbreak.

Since the group which received the cloned culture developed the full respiratory disease syndrome, group B was not examined in such detail and the results are therefore summarized only briefly.

The rats inoculated were killed between 13 and 441 days later, and the progeny of these animals were killed between 42 and 401 days of age.

Twenty-eight rats were examined *post mortem*; otitis media was first detected in a rat killed 49 days after inoculation and almost every rat examined later had copious purulent exudate in the bullae of the internal ear. Almost half the rats killed later than 49 days had gross pneumonia, the two most extensive cases scoring 70 and 60 after being exposed to infection for 168 and 401 days respectively.

Catarrhal tracheitis occurred both in pneumonic rats and in some of the rats without gross pneumonia.

The histopathological changes in the pneumonic lungs, including the degree of bronchiectasis and lymphoreticular hyperplasia, were comparable with those occurring in the group-A rats.

Sera from 36 group-B rats were examined for CF antibodies against the three known species of mycoplasmas of murine origin; six of the sera were from rats inoculated with the pneumonic lung suspension, the rest from their offspring. Antibody to *M. pulmonis* was detected as early as 13 days after exposure (titre 32–64) in rats directly inoculated and was still present after 441 days, when the last rat was killed. The naturally infected progeny, like those in group A, took longer to develop a positive titre; antibody was not detectable at 42 days of age, although present at 168 days and thereafter up to 401 days, the age of the oldest rat examined. Titres were of the same order as those in group A, namely 32–256. Antibody to *M. arthritidis* or *M. neurolyticum* was not detected. Sera from six group-B rats were examined for HI antibodies to Sendai virus; all were negative at a dilution of 1/2, the lowest dilution tested.

Absence of diseases in control rats (group C)

The group-C colony remained in good health until completion of the experiment in September 1971; only seven animals died from accident, tumour or old age.

Post-mortem examinations were made on 58 rats, starting 20 days before the group-A and B rats were inoculated. The oldest control rats so examined were more than 700 days old when killed. Apart from the five rats with tumours, virtually no macroscopic abnormalities were detected and only rat 3239 had gross consolidated areas in the lung; these consisted of tumour tissue.

Ten of the 57 lungs examined histologically had tiny discrete areas in which foamy macrophages had accumulated in the subpleural alveolar spaces. The condition was morphologically identical with that described by Beaver, Ashburn, McDaniel & Brown (1963). Otherwise the lungs were histologically normal with no exudates in the bronchial tree; the lymphoid nodules associated with the bronchiolar tree were very small, usually being 0·3–0·4 mm. in diameter, but reaching 0·7 mm. in some rats. These dimensions together with the measured diameters of the bronchiolar lumina were taken as normal in assessing the degree of lymphoreticular hyperplasia and bronchiectasis in the infected rats.

The sera of 54 group-C rats were examined for CF antibody to the three murine strains of mycoplasma. Antibody to *M. arthritidis* or *M. neurolyticum* was never detected. Only one rat had a titre of 16 with *M. pulmonis*; this animal had a mandibular neoplasm.

DISCUSSION

The organism isolated from the pneumonic lung of rat 3102 from the Alconbury outbreak of severe respiratory disease was identified as a mycoplasma on the basis of its morphological and cultural characteristics and its antibiotic sensitivity, and it was identified serologically as *M. pulmonis*. That *M. pulmonis* was estab-

lished in the Alconbury colony (Lane-Petter *et al.* 1970) was confirmed by the regular isolation of mycoplasmas from affected rats – those isolates checked serologically all proved to be *M. pulmonis* – and by the detection of antibody in several other rats (R. J. Olds and R. M. Lemcke, unpublished observations).

All the evidence supports the view that the culture inoculated into the group-A rats contained only *M. pulmonis*. The culture had been purified by four serial single-colony passages on solid medium, and the inoculum yielded only mycoplasma colonies of one morphological type which were identified serologically as *M. pulmonis*. The possibility of mechanical carry-over of a hypothetical virus from the natural disease to the rat inoculum would seem to be remote, since the cumulative dilution of the original pneumonic lesions in the rat inoculum was at least 10^{-25}.

The disease in rats of group A directly inoculated with *M. pulmonis* was characterized first by inner-ear involvement and only later by pathological changes in the lung. Although histological abnormalities were not seen in the lungs of the rats killed at 55 days, CF antibody was already present at 20 days. In the progeny of the inoculated rats, lung involvement and CF antibody were first detected after 85 days exposure, so that lung lesions appeared more rapidly but CF antibody more slowly than in the rats directly inoculated.

The slower development of CF antibody in the exposed progeny may have resulted from their smaller infecting dose, or their younger age at exposure, when they were immunologically less efficient and when they probably had passively acquired antibody. The earlier isolation of the organism from the naturally exposed progeny and the more rapid development of pneumonia in these animals could be related to various factors, such as the organism's adaptation to the natural host, its association with natural exudates, and the size of the inhaled particles. Clearly more work to investigate the possible role of these factors is needed.

An analysis of the results in the naturally infected rats in group A showed that there was a correlation between the extent of pneumonia and the CF titre to *M. pulmonis* at the time the rat was killed. If the rats were divided into two groups on the basis of CF titre, the group with CF titres of 64 and less had an average pneumonic score of 5·0 whereas the group with CF titres of 128 and above had an average pneumonic score of 36·3.

That the long-standing extensive pneumonias consistently yielded *M. pulmonis*, while the lungs with small or no lesions were culturally negative, suggests that there is a close correlation between the continued activity of *M. pulmonis* and both the CF titres and extent of pneumonia.

In other species, pneumonias caused by mycoplasmas resolve slowly; for example, the diseases in man and the pig caused by *M. pneumoniae* and *M. suipneumoniae* eventually resolve. Similarly, in our experiments with *M. pulmonis* there was evidence for eventual resolution of the pneumonia. Although some group-A rats still had pneumonia after 2 years' exposure, the rats killed more than 590 days after exposure more frequently had low pneumonic scores than had rats infected for shorter periods (Table 3).

The fact that both pneumonia and *M. pulmonis* persisted in the presence of high titres of circulating CF antibody suggests that the latter does not play a major part in protection or recovery from the mycoplasma infection. A similar lack of correlation between the presence of circulating antibody and the immune status has been reported in two other pneumonic diseases caused by mycoplasmas – bovine contagious pleuropneumonia (Davies & Hudson, 1968) and enzootic pneumonia of swine (Goodwin, Hodgson, Whittlestone & Woodhams, 1969). CF antibody can nevertheless be a useful specific indicator of infection.

The group-B rats inoculated with a pneumonic lung suspension and their progeny showed a similar pattern of diseases and antibody development to that seen in group A. Group-B rats were no more severely affected than those in group A and there was therefore no indication that the pneumonic lung suspension contained pathogens in addition to *M. pulmonis*.

In the uninoculated group-C rats the absence of histological abnormalities correlated with the consistent failure to isolate *M. pulmonis* and to detect CF antibody to the mycoplasma.

The respiratory disease in the experimental animals of groups A and B was less acute than that observed during the natural outbreak at Alconbury described by Lane-Petter *et al.* (1970), in that it took a long time to become severe and cause deaths. This might indicate that in the Alconbury outbreak an agent in addition to *M. pulmonis* was involved, and that this was not present in the material used to inoculate the experimental rats. On the other hand, if *M. pulmonis* was the prime pathogen in the natural disease, differences between the environments of the experimental and of the Alconbury rats could account for the disease presenting differently. Environmental factors such as degree of crowding, concentration of ammonia in the air and ventilation and humidity are known to influence the establishment of lower respiratory disease in the rat (Giddens, Whitehair & Carter, 1971). Indeed, seven severely affected rats removed from the Alconbury colony to an isolation cubicle similar to those in which the transmission experiments were carried out, improved clinically. The Alconbury workers considered that these rats would probably have died soon if they had been left in the colony.

That more overt respiratory disease developed in groups A and B when the density of the population was allowed to increase, and cages were cleaned out only once a week, also indicates that environmental conditions can affect the course of respiratory disease. If the colony had been of a size comparable to the one at Alconbury a more acute type of infection might well have developed.

M. pulmonis was the only pathogen consistently isolated *post mortem* from rats of groups A and B. All the isolates were of the *M. pulmonis* colony type, which is distinct from that of *M. arthritidis* or *M. neurolyticum*, and all the isolates tested serologically proved to be *M. pulmonis*. Similarly, CF antibody was detected only to *M. pulmonis* but not to *M. arthritidis* or *M. neurolyticum*. That the *M. arthritidis* antigen used to monitor the rat sera in these experiments was satisfactory was shown by its ability to detect specific antibody in another stock of rats from which *M. arthritidis* was isolated (R. M. Lemcke, unpublished observations). The negative results with *M. neurolyticum* are consistent with the fact that this

mycoplasma has never been reported to occur naturally in rats. Although the isolation of urea-metabolizing T-strains was not attempted, these mycoplasmas have never been reported in rodents. There was no evidence, therefore, that mycoplasmas other than *M. pulmonis* were involved in the experimental disease. Similarly, bacterial respiratory pathogens such as *Pasteurella pneumotropica*, *Streptobacillus moniliformis*, *Bordetella bronchiseptica* and *Diplococcus pneumoniae* were not found in the experimental cases.

The possibility of Sendai virus being involved in the experimentally-produced disease was considered since it was present in mice in the Alconbury colony (Lane-Petter *et al.* 1970) shortly before the rat material used in this study was collected. This virus has been shown to cause acute respiratory infection in rats, coupled with high antibody titres within three weeks of inoculation (Tyrrell & Coid, 1970). It is not known how long such antibody persists in rats, but in mice it appears to last for life (Parker & Reynolds, 1968; C. R. McDonald, personal communication). Thus the failure to find Sendai antibody in any of the experimental rat sera is probably significant.

Rat coronavirus induces a fatal pneumonitis in newborn rats (Parker, Cross & Rowe, 1970). It seems unlikely from the clinical picture in our rats that this virus was active, but specific serological checks should be made for it in future studies.

The possible presence of unidentified agents (e.g. the 'virus' of Nelson, 1967; the grey-lung agent, or the similar non-cultivable mycoplasma-like organism described by Gay, 1967*a*, *b*; 1969) cannot be excluded in the absence of definitive tests for such agents. The dilution of the inoculum of the cloned mycoplasma renders the carry-over of any non-cultivable agent unlikely. The possibility arises that the primary barrier-maintained stock used for the experiments were infected with some pneumonia-inducing agent which was exacerbated by intranasal inoculation. All the evidence seems to be against this since the progeny in groups A and B developed respiratory infection merely by being in contact with the inoculated animals. Moreover the histopathology of the uninoculated control animals from the same stock over a period of 2 years (group C) did not suggest the presence of an infective agent.

The progressive development of pulmonary lesions in this series of experiments was similar to that described by Lindsey *et al.* (1971). It should be noted, however, that in their work frank pulmonary lesions resulted only from the inoculation of pneumonic lung or of inocula containing unpurified cultures of *M. pulmonis*. Their only cloned strain (N) did not produce otitis media, tracheitis or even microscopic lung lesions; nor were the tracheal cultures of rats inoculated with this strain positive for *M. pulmonis*. This would suggest either that their cloned strain had lost its pathogenicity or that their experiments were not of sufficient duration for the organism's pathogenicity to manifest itself. If we had killed all of our rats by 60 days (as did Lindsey *et al.* with their cloned *M. pulmonis* group) we would have seen virtually no evidence that our cloned strain was pathogenic for the lower respiratory tract.

Although considerable lymphoreticular hyperplasia developed in a proportion of the group-A rats, this feature did not progress as the duration of infection in-

creased, and it did not reach the proportions often seen in rat colonies naturally affected with chronic respiratory disease. This suggests that factors in addition to *M. pulmonis* might be involved in progressive lymphoreticular hyperplasia. On the other hand it is also possible that under different circumstances *M. pulmonis* alone might induce this change.

We noted that, in addition to the otitis media frequently associated with *M. pulmonis* infection, this organism also invaded the cochlea and vestibule of the inner ear and caused a persistent purulent otitis interna.

Thus, our experiments showed that pneumonia and bronchiectasis as well as inner-ear infection can develop in a barrier-maintained rat stock infected with a serially cloned culture of *M. pulmonis* in the absence of any other demonstrable pathogen. Only Kohn & Kirk (1969) have obtained similar results in gnotobiotic rats with their once-cloned *M. pulmonis*. The cloned strain of Lindsey *et al.* (1971) appeared not to be pathogenic by the intranasal route. Bell & Elmes (1969) did not produce chronic respiratory disease or CF antibody response using intranasal infection of rats; they called their organism *M. pulmonis*, but did not provide evidence of its identity. The unidentified mycoplasmas inoculated into rats by Pankevicius, Wilson & Farber (1957) and Joshi, Dale & Blackwood (1965) also failed to produce pathological changes in the lungs.

These apparently conflicting results might be explained by differences in the experimental procedures. For example, in most experiments, a single inoculum of the mycoplasma was used, whereas the rats of Kohn & Kirk (1969) were repeatedly inoculated or naturally exposed. Similarly, in our experiments, the group-A progeny in which pneumonia was most severe were also repeatedly exposed to infection by being in direct contact with the rats which had been inoculated. Further, pneumonia developed only slowly in our rats, and some of the experiments described by others may not have been run long enough to allow the development of lung lesions. The environmental conditions under which the rats were kept in the various experiments probably affected the rapidity with which pulmonary infection developed; it is likely that different strains of rats vary in their susceptibility to *M. pulmonis* infection. This obtains in the development of both pneumonia (Brennan & Feinstein, 1969) and arthritis (Hannan, 1971) following the inoculation of *M. pulmonis* into different strains of mice. Moreover the strains of *M. pulmonis* used by different workers probably differed in their pathogenicity. Virulence may be lost on passage in artificial media as it is with *M. pneumoniae* (Couch, Cate & Chanock, 1964) and the type of medium upon which the organism is passed may influence the rate of loss of virulence. The medium used in our experiments for the isolation, cloning and propagation of *M. pulmonis* 3102 was that developed for the isolation of *M. suipneumoniae* and, as it differs in several respects from the formulae generally used for mycoplasmas, it may favour the maintenance of pathogenicity.

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made by Mr L. G. Benfield of Pfizer Ltd., Sandwich, Kent; other sera were similarly tested at the Medical Research Council's Clinical Research Centre, Harrow, Middlesex, through the courtesy of Dr D. A. J. Tyrrell.

ADDENDUM

The culture of *Mycoplasma pulmonis* (3102) purified by four serial single-colony subcultures has been deposited at the Mycoplasma Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W.9

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Fig. 1

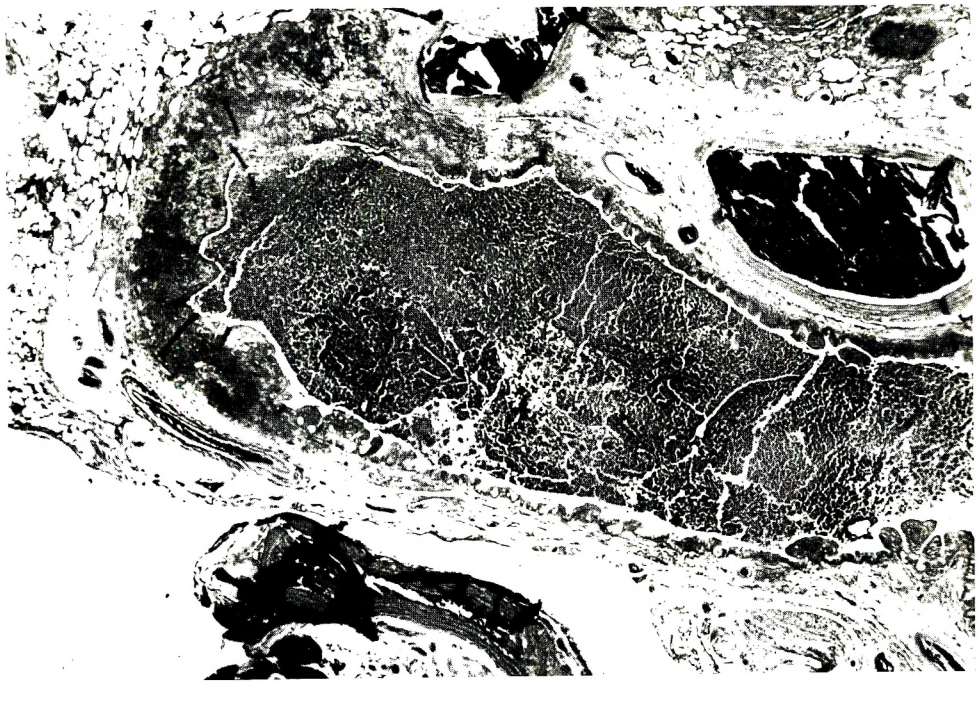

Fig. 2

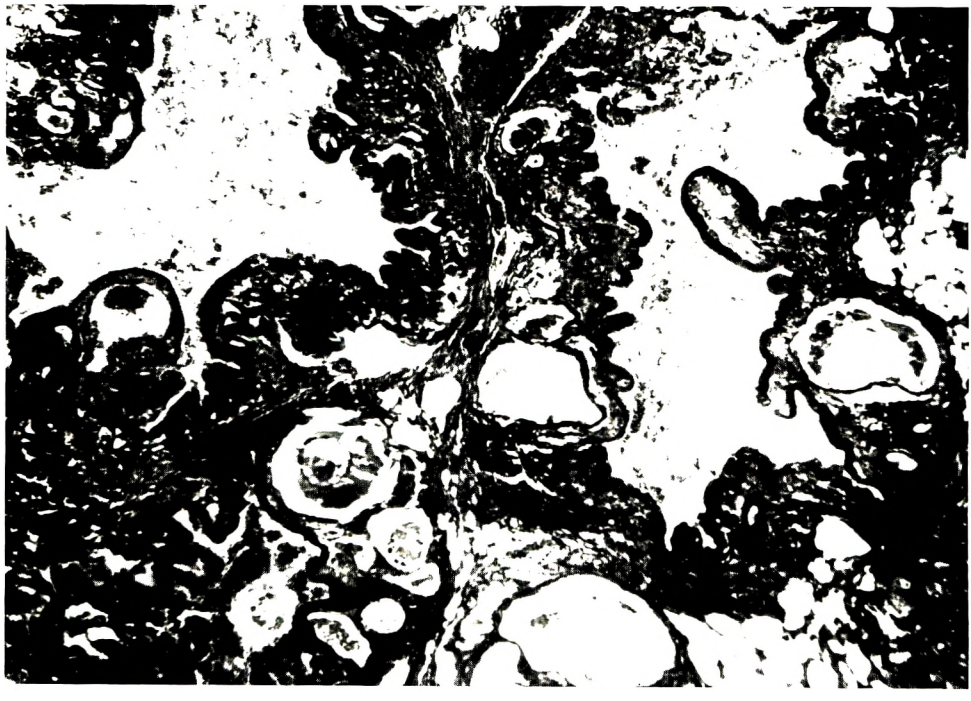

Fig. 3

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Touch preparation of pneumonic lung of rat 3102 showing large numbers of rod-shaped and coccal organisms of the mycoplasma type in the cilial area of bronchiolar epithelial cells. Giemsa, × 1600.

Fig. 2. Section of lung of rat 3341 exposed to cloned *Mycoplasma pulmonis* for 678 days. Marked distension of the bronchial tree due to accumulation of purulent exudate. H & E, × 40.

PLATE 2

Fig. 3. Section of lung of rat 3291 exposed to cloned *Mycoplasma pulmonis* for 631 days. The alveolar tissue is replaced by multiple bronchiectatic cavities with folded walls. H & E, × 40.

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A method for predicting proportions of affected herds from proportions of affected animals

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SUMMARY

The frequency of herds affected with 13 different diseases is shown to bear a simple relationship to the frequency of affected animals. The relationship seems to be useful for predicting proportions of affected herds.

From time to time an estimate is needed of the proportion of herds, in a population of farms, likely to contain animals affected by some disease. Given the proportion of affected animals in the population, an estimate could be obtained from the distribution of herd sizes if the relationship between population disease frequency, herd size, and proportion of affected herds were known.

We have studied published data on 14 different categories of disease in cattle and show that from a simple mathematical relationship between the three factors, the proportion of affected herds can be predicted with useful accuracy.

The data, summarized in Table 1, are taken from reports of national surveys of diseases in cattle on random samples of the farms of Britain (Leech, Davis, Macrae & Withers, 1960; Leech, Vessey & Macrae, 1964; Leech, Macrae & Menzies, 1968). The published tables show percentages of affected herds within from 4 to 8 herd-size groups. The percentages usually increase with increasing disease frequency and with increasing herd size.

For random, independent events (which diseases are not), the binomial distribution predicts that the proportion, Q, of unaffected groups of size n is q^n, where q is the proportion of unaffected individuals. Because $\log Q = n \log q$, trends relating $\log Q$ to group size are straight lines through the origin with slope equal to $\log q$. A plot of $\log Q$ against n using the data of Table 1 showed that relationships fitting the data would be curved and might miss the origin. Other transformations of Q and n were tried, but none rectified the curvature.

A plot of logit P (or logit Q) against $\log n$ showed much better promise of obtaining a reasonable fit (logit $P = \log (P/Q)$; we imply natural logarithms in both 'log' and 'logit'; note that some tables of logits use $\frac{1}{2} \log (P/Q)$). This plot suggested that either parallel or radiating straight lines might fit the points. Any one such line has the formula

$$\text{logit } P = a + b \log n$$

where a, the logit of the proportion of affected herds of one animal, might be expected to be related to logit p (p being the proportion of affected animals).

Table 1. *Data, taken from reports of national surveys, showing percentages of herds containing affected animals and population percentages of animals affected*

Disease condition	Herd size							
Calf mortality	1–5	6–10	11–20	21–30	31–40	41–60	61–80	81+
No. of herds	100	209	551	331	173	144	53	46
Percentage affected	8·0	24·9	33·1	47·7	56·6	68·8	77·4	89·1

Percentage animals affected = 3·74

	Herd size				Percentage animals affected
	⩽ 19	20–39	40–59	60+	
No. of herds	475	482	129	72	
	Percentage affected				
Johne's disease	5·1	7·1	9·3	11·1	0·35
Summer mastitis	2·1	12·0	19·4	29·2	0·50
Grass tetany	5·1	8·1	20·2	34·7	0·57
Dystokia	21·1	29·0	28·7	44·4	1·55
Stillbirth	18·1	34·0	42·6	62·5	1·82
Acetonaemia	18·1	25·9	47·3	56·9	2·01
Abortion	24·0	39·0	47·3	63·9	2·15
Foul-in-the-foot	21·1	32·0	54·3	66·7	2·79
Acute mastitis	30·1	47·9	59·7	81·9	3·53
Milk fever	33·1	47·9	69·0	81·9	3·65
Retained placenta	32·0	57·1	71·3	79·2	4·24
Mild mastitis	44·0	58·9	66·7	81·9	6·78

Udder brucellosis	Herd size				
	⩽ 19	20–29	30–39	40–49	50+
No. of herds	599	623	390	236	414
Percentage affected	6·3	14·0	15·9	16·9	19·3

Percentage animals affected = 1·09.

These considerations suggested that the general relationship

$$\text{logit } P_{ij} = a + b \text{ logit } p_j + (c + d \text{ logit } p_j) \log n_{ij} \qquad (1)$$

(where i represents a size-group and j a disease) should be tried, and some of the parameters fixed or eliminated to test the relative value of simpler relationships. Equation (1) implies a set of straight lines relating logit P_{ij} to $\log n_{ij}$, radiating from a point with coordinates $\log n_{ij} = -b/d$, logit $P_{ij} = a - bc/d$, and with slopes $c + d$ logit p_j.

Various parameter values were tried, using a computer program that searched for the minimum of the log-likelihood ratio by the simplex method of Nelder & Mead (1965). The log-likelihood ratio (L) was calculated from observed proportions of affected herds (P) and predicted proportions ($\hat{P}$) using the relationship

$$L = \Sigma \{R \log (P/\hat{P}) + (N - R) \log (Q/\hat{Q})\}$$

where N is the number of herds in a size-group of which R were affected; $Q = 1 - P$. This relationship implies an assumption of binomially distributed residual errors, which is contradicted by the analysis of χ^2 shown in Table 3. However, the use of

this likelihood ratio will still be justified if the errors have variances proportional, rather than equal, to binomial errors.

A series of trials with equation (1) showed:

(1) that there was no gain (in terms of the value of the log likelihood ratio per degree of freedom) from fitting more than one parameter;

(2) when b was fitted and the other parameters fixed at $a = 0$, $c = 1$, $d = 0$, the log likelihood ratio was only trivially smaller than when a was fitted and the others fixed at $b = 1$, $c = 1$, $d = 0$, which gave the equation

$$\text{logit } P_{ij} = -0{\cdot}1227 + \text{logit } p_{ij} + \log n_{ij}, \tag{2}$$

using natural logarithms, from which

$$P = \frac{0{\cdot}885\, np}{q + 0{\cdot}885\, np}. \tag{3}$$

From equation (3) P can be estimated by simple arithmetic; this seems a worthwhile advantage over the equation with b as the fitted constant, which requires logarithms for its solution.

The nature of the relationship represented by equation (3) is seen at its simplest by looking at the odds (P/Q) on a herd of size n being affected. These odds are $n(0{\cdot}885\, p/q)$. Setting s equal to the proportion within brackets, we see that the odds, ns can be represented by straight lines, with slope s, passing through the origin of a graph whose axes are P/Q, and n. However, such graphs give a misleading impression of discrepancies between observed and fitted odds that are associated with large values of P, because the statistical error in P/Q increases without limit as P approaches 100 per cent. A more realistic impression is given in Fig. 1, which shows a selection of observed points and corresponding prediction curves derived from equation (3). This figure shows, for example, that the predicted trend is less steep than the observed trend of calf deaths, but more steep than the observed trend in Johne's disease.

For other diseases, equation (3) gave predictions that also departed more or less systematically from the observed trends. We examined these discrepancies in detail (Tables 2 and 3) and conclude that they are not large enough to detract from the general usefulness of equation (3).

In Table 2, the errors are calculated as proportions of the predicted numbers of affected and unaffected herds pooled over all herd sizes. The error for udder brucellosis is shown separately; the brucellosis data were excluded from the fitting because the survey report (Leech *et al.* 1964, p. 19) comments that the examination of milk samples from individual cows was incomplete in one category of herds. The observed proportions of herds with udder brucellosis in the survey were therefore almost certainly less than the actual proportions. The other errors in Table 2 are all below 15%, which seems adequate accuracy for the purposes for which prediction might be required.

Estimates of χ^2 (Table 3), which combines the errors for affected and unaffected herds, were calculated for the total discrepancy and for discrepancies from the individual observations of Table 1. Large values of χ^2 (per degree of freedom) in

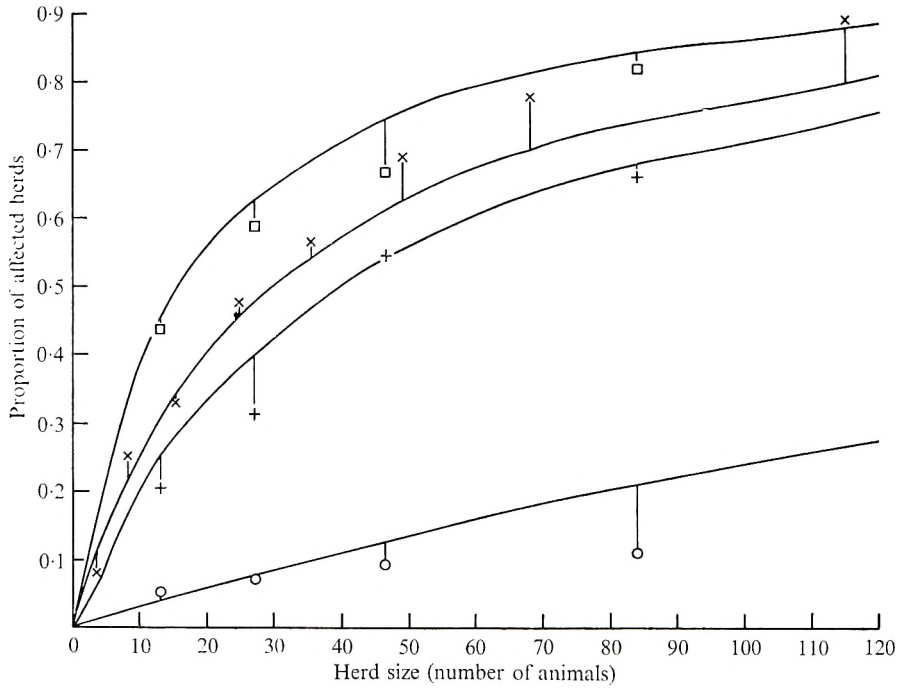

Fig. 1. Lines showing the predicted proportion of herds affected by four diseases, with the associated actual proportions. × calf deaths, ○ Johne's disease, + foul-in-the-foot, □ mild mastitis.

Table 2. *Error in estimating percentage of affected herds from* $P = 0{\cdot}885\,np/(q+0{\cdot}885\,np)$

	Discrepancy as a percentage of predicted number of herds	
	Affected	Unaffected
Calf deaths	3·4	−2·4
Johne's disease	−6·7	0·5
Summer mastitis	−4·3	0·5
Grass tetany	−14·6	1·9
Dystokia	4·7	−1·6
Stillbirth	6·6	−2·6
Acetonaemia	−10·9	4·8
Abortion	10·8	−5·1
Foul-in-the-foot	−14·0	8·3
Acute mastitis	3·0	−2·2
Milk fever	6·3	−4·9
Retained placenta	4·9	−4·4
Mild mastitis	−6·1	8·6
Udder brucella	−41·9	12·8

Table 3. *Error* (χ^2) *in estimating percentage of affected herds from* $P = 0{\cdot}885\,np/(q + 0{\cdot}885\,np)$

Disease	For the overall discrepancy (1 d.f.)	Summed over discrepancies of herd-size groups	
		d.f.	χ^2
Calf deaths	1·29	8	7·06
Johne's disease	0·42	4	8·73
Summer mastitis	0·24	4	11·84
Grass tetany	3·22	4	8·96
Dystokia	0·87	4	19·26
Stillbirth	1·99	4	4·33
Acetonaemia	6·02	4	11·08
Abortion	6·33	4	8·72
Foul-in-the-foot	13·46	4	18·57
Acute mastitis	0·77	4	3·33
Milk fever	3·53	4	7·73
Retained placenta	2·51	4	11·04
Mild mastitis	6·07	4	9·65
Total	46·72 (13 d.f.)		130·30 (56 d.f.)
Udder brucella	121·04 (1 d.f.)		144·31 (5 d.f.)

the second column of Table 3 associated with small values in the first column, show where the shape of the observed curve differed considerably from that of the predicted curve.

Although the discrepancies for calf deaths in Fig. 1 look systematic, the values of χ^2 in Table 3 show that they were of the size that would be associated with binomial error; it therefore seems more reasonable to attribute them to sampling error than to systematic departure from the model. The χ^2 for Johne's disease dicrepancies would be exceeded only in about 6 % of random samples. The survey showed that this disease was much more frequent in Channel Island than in other breeds and that the herds of Channel Island cattle were relatively small. This observation provides a sensible explanation of the systematic departure from the model. It suggests also that if data classified into Channel Island versus other breeds existed, equation (3) would give good individual predictions for the two breed groups. Breed differences could also account for the discrepancies from the predicted curve for foul-in-the-foot. In general, when the frequency of disease per animal depends greatly on factors associated with considerable differences in herd size, systematic discrepancies from a curve calculated from the average frequency per animal are to be expected. Grass tetany was about four times more frequent (per animal) in herds in the north of Scotland (averaging 41 cows) than in herds in the south-west of England (averaging 24 cows). The national average frequency of grass tetany therefore tends to overestimate the proportion of affected small herds and to underestimate the proportion of affected large herds. In such conditions, separate predictions for different geographical areas, using the regional proportions of affected animals, should be used when accurate estimates are required.

DISCUSSION

For our purposes, an empirical model seems better than a theoretical model such as the negative binomial, partly because the empirical model is simpler and partly because no single theoretical assumption about the associations between occurrences seems appropriate when such a range of diseases is being considered.

The relationship (equation (3)) has been fitted only to data for diseases in herds of cattle in Britain. Data giving both the frequency per animal and the frequency of affected herds in the same population are uncommon. We have used all the data we could find. If the same relationship is found adequate for describing the situation in other species and other countries, its general usefulness will be enhanced.

Field observations determine a proportion of affected animals much more precisely than a proportion of affected herds. This is partly because the number of herds per size group is necessarily small relative to the numbers from which the average frequency per animal is calculated. Because the frequency of affected herds in a group covers a range of herd sizes, it may be a slightly biased estimate of the frequency for the mean herd size of the group. Furthermore, mean herd size could not always be calculated precisely from the published data, and the use of some approximations may have introduced extra divergences from the relationships in the original observations.

The prediction errors in applying equation (3) to our data are presented as values of χ^2. It is clear from these that we cannot assume binomial errors for the proportion of herds infected. At least in part, this is because some factors closely associated with variation in the frequency of some diseases were unequally distributed among herd size groups.

A relationship such as equation (3) will probably be useful when the predicted proportion of affected herds is not close to zero or 100%. The useful range is affected by herd size and by disease frequency. Very large herds are expected to have at least one animal affected by any disease that is of economic importance in the population to which it belongs. Very common diseases are expected to occur in almost all herds.

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The effect of two methods of cooking and cooling on *Clostridium welchii* and other bacteria in meat

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SUMMARY

A comparison was made of beef cooked in conventional and moist air (Rapidaire) ovens. In both large (*ca.* 4·5 kg.) and small (*ca.* 2·7 kg.) joints, spores of *Clostridium welchii* survived after cooking but vegetative cells, *Escherichia coli*, and *Staphylococcus aureus*, did not, regardless of the type of oven used.

Cooling at room temperature after cooking permitted growth of *Cl. welchii*. Although some multiplication also occurred in the centre of large roasts cooled under refrigeration, the viable counts were considered too low to constitute a potential health risk.

INTRODUCTION

The ability of bacteria to survive cooking and to multiply within a particular food during cooling and subsequent storage is an important factor in the epidemiology of food poisoning outbreaks. Adequate cooking and rapid cooling of foods, particularly meats and poultry, is frequently advocated as a means of reducing the incidence of food poisoning within the population. Unfortunately, there is only a limited amount of published quantitative information available on the temperature changes that occur within meat during cooking and cooling, and the effect of these temperature changes on the survival and multiplication of food-poisoning bacteria. Sylvester & Green (1961) recorded a temperature of 79° C. in the centre of a 4–5 lb. roast after cooking for 2¼ hr. in an oven heated to approximately 215° C. Woodburn & Kim (1966) studied the survival of *Clostridium welchii* in turkey stuffing during roasting at 94, 163 and 232° C. They found that *Cl. welchii* spores could survive these cooking temperatures, although vegetative cells were generally killed. These workers found that after cooking in an oven at 94° C for 10 hr. the temperature of the stuffing was 62° C. in lightweight turkeys (12–16 lb.) and 41° C. in heavyweight birds (20–24 lb.). Final cooking temperatures were not given for turkeys cooked at 163° and 232° C.

The following work was carried out to compare temperature changes in portions of beef of different sizes cooked in a traditional hot air oven (213° C.) and in a Rapidaire moist air oven (82° C.).

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The rate of cooling of meat samples was investigated at room temperature (15° C.), in a refrigerator (4–5° C.) and in a blast freezer (−30° C.). The effect of different cooking and cooling procedures on the survival and multiplication of food poisoning organisms inoculated into the meats before cooking was also investigated. The work was carried out in the kitchen of a large mental hospital.

MATERIALS AND METHODS

Equipment for cooking and cooling meat

Electronic thermometer. This was type 166C, manufactured by Comark Electronics Limited, Littlehampton, Sussex, used with chrome alumel thermocouples 4–7 ft. long. The accuracy of the system was ± 1° C.

Moist air oven. The Rapidaire oven was manufactured by Eureka Engineering Company, Pratts Bottom, Kent. Meat was cooked in this oven at a temperature of 82° C. and at an RH of 95 %. The cooking process therefore differs greatly from that of steam cookers

Hot air oven. The large coke-fired hot air oven was used routinely in the hospital kitchen; it was fitted with an external recording thermometer. Meat was cooked at 213° C.

Blast freezer. The commercial 'Prestcold' blast freezer was operated at −30° C. with an air flow rate of 12,000 ft.^{3}/min.

Cold room. The room was commercially designed and operated at 4–5° C.

Meat

Lean beef from which surface fat had been removed was used as follows:

(*a*) Bulky cuts of irregularly cubical shape, weighing approximately 4·5–5 kg.

(*b*) Long slim cuts, approximately 5–6·5 cm × 9 cm. in section, weighing about 2·7–3·2 kg. When cooked in the Rapidaire oven these smaller cuts were placed in polythene Visicase bags, which was the routine practice of the chef. Thermocouple leads were inserted through the neck of the bag before sealing.

Media

Veal cooked-meat medium. A modification of Robertson's meat medium (Cruickshank, 1965) was used, made with boneless veal instead of bullocks' hearts. A similar meat medium with the addition of 10 % NaCl was used for *Staphylococcus aureus* enrichment.

Neomycin blood agar (Sutton & Hobbs, 1968). A modified 5 % horse blood agar was used.

MacConkey broth and MacConkey agar. These media were prepared from basic materials.

Phenolphthalein phosphate agar (Barber & Kuper, 1951) was modified according to Hobbs, Kendall & Gilbert (1968) and Gilbert, Kendall & Hobbs (1969).

Organisms

Clostridium welchii

Spores of F2063/67 (Type 17, heat-resistant) and F3795/67 (type xii, heat-sensitive), together with vegetative cells of F9191/66 (type iv, heat-sensitive) and F6849/67 (type 18, heat-resistant) were used. The term 'heat-sensitive' when applied to spores of *Cl. welchii* has led to confusion. Although these spores (F3795/67) are less resistant at high temperatures (90–100° C.) than the so-called heat-resistant food poisoning strains, they are, nevertheless, able to survive temperatures as high as 80° C. for relatively long periods of time, particularly if heated in a meat medium (Sutton, 1969). They are, therefore, much more resistant to heat than vegetative cells of *Cl. welchii* or other food-poisoning organisms.

Specific antisera were available for each of the strains. A selection of colonies isolated from the meat samples after cooling was tested against the specific antisera using a slide agglutination technique.

Preparation of spore suspensions. Spores of *Cl. welchii* present on meat are usually derived, either directly or indirectly, from the faeces of animal or man. For this reason, and because of the difficulty in obtaining large numbers of spores of *Cl. welchii* in laboratory media, and of the probable reduction in heat resistance of laboratory prepared spores, the spores used in this work were obtained from the faeces of food-poisoning patients.

Faecal samples known to contain large numbers of *Cl. welchii* spores (F. 2063/67 and F. 3795/67) were emulsified in water to give approximately 1/10 suspensions, and filtered through gauze. The filtrates were centrifuged at 3500 rev./min. for 30 min. and the centrifuged deposits washed twice with water before making 1/100 suspensions in water. The final suspensions contained approximately 10^5 spores/ml. and were relatively free of faecal matter.

Staphylococcus aureus

NCTC 4163; phage type 6+/42E/47/54/42D.

Escherichia coli

NCTC 7152; O 25, H 12, K 19.

Inoculum

The same inoculum was used for each sample, consisting of:

Cl. welchii	Spores	$1{\cdot}5 \times 10^5$ type 17
		$1{\cdot}6 \times 10^5$ type xii
	Vegetative cells	$1{\cdot}0 \times 10^9$ type 18
		$9{\cdot}0 \times 10^9$ type iv
Staph. aureus		$8{\cdot}0 \times 10^9$
E. coli		$7{\cdot}5 \times 10^8$

Method of inoculation

Five samples of beef were inoculated with the above suspension in a longitudinal and vertical plane through the centre of the meat by means of a syringe fitted with with a 6 in. cannula.

Cooking and cooling procedures

Thermocouples were inserted into the centre and outer edge (approximately 6 mm. into the meat) of 15 samples of raw meat. In the moist air oven the meats were cooked for 30 min. per 4·5 kg. weight; meats cooked in the conventional oven were turned, basted and removed from the oven at the chef's discretion in order to simulate a normal cooking procedure.

After cooking, samples of beef were left to cool at room temperature (15° C.), in a cold room (4° C.) and in a blast freezer (−30° C.). Samples cooled in the refrigerator and blast freezer were allowed to stand at room temperature for 15 min. after removal from the oven to avoid condensation.

Throughout the cooking and cooling period, temperatures were recorded with the electronic thermometer; the wires of the thermocouples were long enough to allow the oven and refrigerator doors to remain closed during the experiment.

Sampling and recovery of survivors

After cooking and overnight storage at 15° C. or at 4° C., the five inoculated meat samples were examined bacteriologically. The entire portion of meat could not be cultured, but it was assumed that the majority of surviving organisms would be present around the line of the inoculum and particularly in the centre of the meat. The results obtained, therefore, represent the numbers found in this portion, usually 250–500 g., and not in the entire sample of meat. The portion was chopped into small pieces, mixed with an equal volume of $\frac{1}{4}$-strengh Ringer's solution and homogenized in a blender for 30 sec. The homogenized suspension was passed through sterile gauze to remove coarse material and used for direct viable counts by the surface spread technique, and for enrichment cultures.

RESULTS

Effect of shape and weight of meat sample on heat penetration

The results indicated that during cooking the temperature at any point within the meat was dependent on the distance of that point from the outer surface of the meat. The centres of large bulky cuts of meat will therefore heat more slowly than those of long slim portions of like weight, so that longer cooking times are required to reach the same final temperature. When meats of similar shape and weight were cooked the results were reproducible.

Tables 1 and 2 summarize the data obtained for 15 samples of meat cooked in the conventional hot air and Rapidaire ovens. Figs. 1*a* and 1*b* show in graphic form the relationship of the distance of the thermocouple from the outer surface of the meat to the time taken to reach 60° C. for both cooking methods. A temperature of 60° C. was chosen because vegetative cells of food poisoning bacteria are

Table 1. *Mean heat penetration data at the centre and outer edge of seven samples of beef cooked in a conventional hot air oven at* 213° C.

	4·5 kg. meat cooked for 205 min.				2·7 kg. meat cooked for 165 min.		
Depth of thermocouple (mm.)	76	51	6	3	38	19	6
Time to reach 60° C. (min.)	220	145	40	37	126	115	65
Temperature at end of cooking (°C.)	55	64	90	96	79	79	88
Maximum temperature (°C.)	65	71	90	96	81	80	89

Table 2. *Mean heat penetration data at the centre and outer edge of eight samples of beef cooked in a moist air oven at* 82° C.

	4·5 kg. meat cooked for 315 min.				3 kg. meat cooked for 322 min.			
Depth of thermocouple (mm.)	76	63	19	3	44	32	19	3
Time to reach 60° C. (min.)	230	200	80	43	160	95	65	43
Temperature at end of cooking (°C.)	68	76	77	81	63	78	74	81
Maximum temperature (°C.)	70	77	77	81	66	78	74	81

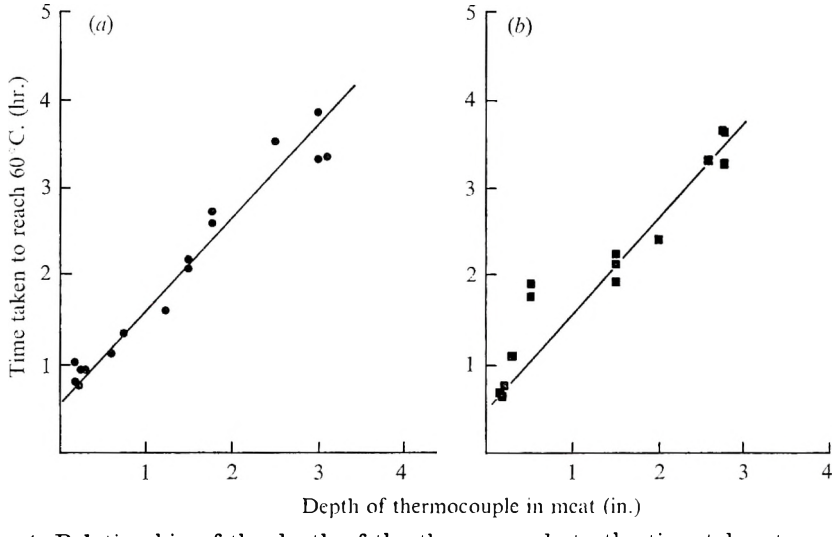

Fig. 1. Relationship of the depth of the thermocouple to the time taken to reach 60° C for (*a*) the moist air oven, and (*b*) the conventional hot air oven.

usually killed and growth no longer occurs at this temperature; it is not implied that a maximum temperature of 60° C. renders the meat bacteriologically safe.

Comparison of the conventional hot air and the moist air ovens

Although both ovens cooked effectively, the results showed that there were differences in the rates of heat penetration in the two ovens. The centres of large portions of meat cooked in the hot air oven were slow to heat up, and a 'lag period' of approximately 1 hr. occurred in which little or no increase in temperature occurred. The lag period did not occur when similar portions of meat were cooked in the moist air oven. This is shown in Fig. 2.

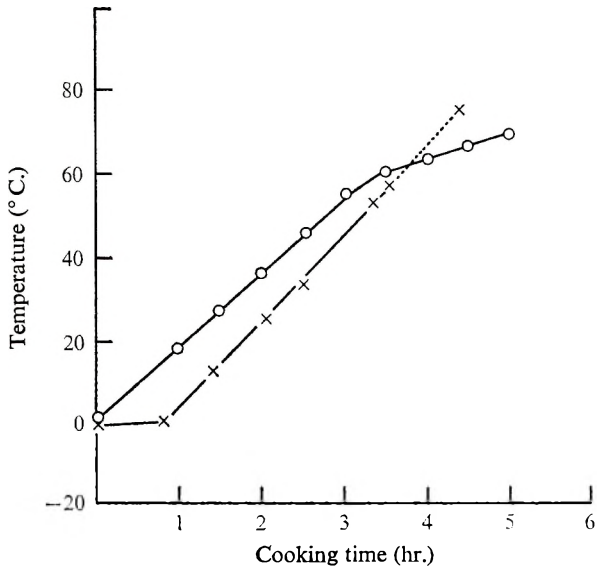

Fig. 2. Heat penetration at the centre of two large samples of meat (*ca.* 4·5 kg.) cooked in the moist air oven (○) and the conventional hot air oven (×). Thermocouples approximately 7·5 cm. from surface of meat. (× - - - - ×) shows the continued rise in temperature recorded after the completion of the cooking time.

All of the samples tested reached a maximum temperature above 60° C. although not always by the end of the cooking time. In the moist air oven the maximum temperature was reached by the end, or within 15 min. of the completion of cooking. With the conventional oven, however, the temperature recorded in the centre of large roasts at the end of the cooking time was considerably lower than the recorded maximum. Temperatures of 55°, 56° and 64° C. were recorded at the end of the cooking time, but there was a continuous rise in temperature for an hour afterwards to reach maximum temperatures of 65°, 68° and 70° C. respectively. This effect was less marked with the smaller roasts.

Cooling of meat

Table 3 summarizes the main features of the results from temperature records of cooling meat. Fig. 3 shows the cooling at the centre of three samples of meat of similar size and weight which were cooked in the hot air oven and then allowed to cool at 15°, 4° and −30° C.

Cooling at 15° *C.* This was effective only in lowering the temperature to approximately 25° C. Loss of heat was very slow, and even after 7 hr. the samples had not reached 15° C., the ambient temperature in the kitchen.

Cooling at 4–5° *C.* This was a more effective method of cooling, although there was little difference in the initial cooling rate for samples cooled at 4° C. or at 15° C. The important difference with refrigeration was that the temperature at the centre of the meat fell to 15° C. or below during the 7 hr. course of the experiment, and would do so irrespective of the room temperature. Fifteen degrees centigrade is taken as the lowest temperature at which *Cl. welchii* will multiply.

Table 3. *Effect of storage at room temperature* (15° C.), *in a refrigerator* (5° C.) *and in a blast freezer* (− 30° C.) *on the cooling of freshly cooked meat*

Weight of sample (kg.)	Temp. of cooling (°C.)	Depth of thermocouple (mm.)	Temp. °C at end of cooling (420 min.)	Time to reach 15 °C. (min.)
	Samples cooked in moist air oven			
4·5–5	4	76	15	420
	− 30	76	− 15	215
2·7–3·2	15	38	21	> 420
	4	44	15	415
	− 30	38	− 28	120
	Samples cooked in hot air oven			
4·5–5	15	51	22	> 420
	4	76	14	395
	− 30	76	− 5	220
2·7–3·2	15	38	17·5	> 420
	4	38	9	235
	− 30	38	− 20	110

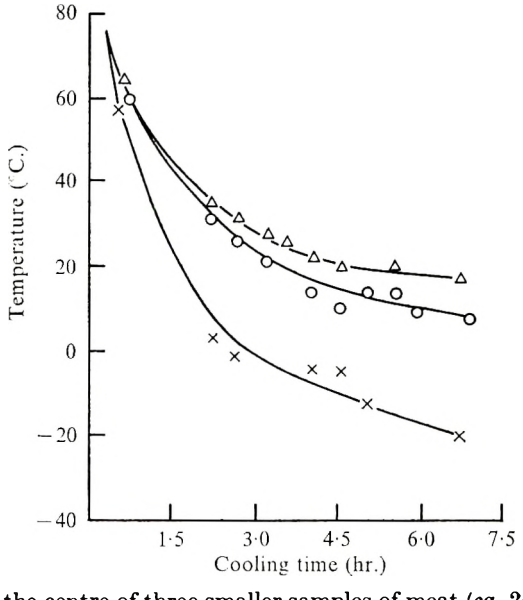

Fig. 3. Cooling at the centre of three smaller samples of meat (*ca.* 2·7 kg.) previously cooked in the hot air oven. (△) at room temperature, 15 °C.; (○) at 4 °C.; (×) at − 30 °C. Thermocouples approximately 3·8 cm. below surface of meat.

Cooling at − 30° *C.* This was naturally the quickest method of cooling. The centre of large (4·5 kg.) samples of beef cooled to below 15° C. in 3 hr. 40 min. – half the time required to reach the same temperature by refrigeration at 4° C.

Effect of shape and weight of meat sample on rate of cooling

There was a relationship between the recorded rate of cooling and the position of the thermocouple. The centre of small roasts reached 15° C. in about half the time required for the larger bulky roasts. When samples were cooked in the moist

Table 4. *Cooking and cooling data for five samples of beef inoculated with viable bacteria before cooking*

Sample	Weight of sample (kg.)	How cooked (oven)	Time for centre to reach 60° C. (min.)	Max. centre temp. (°C)	Temp. of cooling (°C)	Time for centre to reach 15° C. (min.)	Min.* centre temp. (°C)
1	2·7	Rapidaire	130	71	13–15	> 420	21
2	4·5	Hot Air	145	71	13–15	> 420	22
3	2·7	Hot Air	135	71	13–15	> 420	17
4	4·5	Hot Air	220	65	4	420	15
5	5·0	Rapidaire	200	77	4	420	15

* Minimum temperature recorded during the first 7 hr. of cooling, when temperature readings were taken regularly.

air oven the relationship of size to cooling rate was not so marked. The smaller samples (but *not* the larger samples) were cooked and cooled in polythene 'Visicase' bags, which are known to reduce the efficiency of cooling; hot air from the meat condenses on the Visicase and the meat shrinks during cooling so that a layer of air becomes trapped between the meat and the casing.

Survival and multiplication of Cl. welchii *in meat during cooking and cooling*

Table 4 summarizes the results of cooking and cooling for the five inoculated samples of beef.

The results given in Table 5 show that spores of *Cl. welchii* (type 17 and xii) survived cooking and that the outgrowths from the spores could multiply during subsequent cooling. Samples 1–3 were initially cooled at room temperature (15° C.) followed by overnight storage in a room where the minimum recorded temperature was 13° C. These three samples included both small and large roasts and samples cooked by both moist air and conventional roasting methods. The lower count from sample 3 probably reflects the more rapid cooling of this sample.

The two larger samples cooled at 4–5° C. showed only a small increase in the number of cells present, but this was expected. Although both samples were cooled at 4–5° C. the centre temperatures were above 15° C. for 7 hr.; the long cooling would allow germination and some multiplication to occur. However, the number of *Cl. welchii* in the refrigerated samples would be unlikely to give rise to food poisoning, assuming that a dose of 10^8 organisms may be required to initiate infection (Dische & Elek, 1957). The results indicated that the method of cooling and not the method of cooking was the relevant factor with regard to numbers of *Cl. welchii*.

In view of the failure to isolate *Cl. welchii* type iv and 18, it is assumed that all vegetative cells were killed by the cooking. Neither *E. coli* nor *Staph. aureus* were recovered after cooking.

Table 5. *Cell counts obtained after overnight cooling with five samples of beef inoculated before cooking with food poisoning organisms*

Sample no.	Cooled at	Serotype of *Cl. welchii* isolated	Number of orgs/g in* inoculated portion
1	Room temperature	17	$4{\cdot}5 \times 10^6$
		xii	$5{\cdot}3 \times 10^3$
2		17	$8{\cdot}0 \times 10^6$
		xii	$1{\cdot}9 \times 10^3$
3		17	$6{\cdot}2 \times 10^5$
		xii	$3{\cdot}4 \times 10^2$
4	Refrigerator	17	$2{\cdot}2 \times 10^4$
		xii	enrich†
5		17	$3{\cdot}3 \times 10^3$
		xii	enrich

* No *S. aureus*, *E. coli* or cells of *Cl. welchii* type 18 or iv, which together with types 17 and xii made up the inoculum, were isolated.

† Enrich: sample positive by enrichment culture only.

DISCUSSION

One of the objectives of this work was to assess, from a bacteriological standpoint, the safety of the 'Rapidaire' oven for cooking large quantities of meat needed for both traditional methods of feeding a hospital population, and for a frozen meals system which was under investigation for the hospital and for commercial production. The results indicated that the Rapidaire oven was satisfactory, but, like the conventional hot air oven, it could not be relied upon to kill bacterial spores.

The final temperatures reached by cooking in the conventional hot air oven, particularly in the centre of large bulky roasts, were not as high as expected, but this may have been due to the relatively short cooking time. A comparison of this work with that of Sylvester & Green (1961) shows that the two sets of results are similar. These workers recorded a temperature of 79° C. in the centre of a 4–5 lb. roast after cooking for $2\frac{1}{4}$ hr. in an oven heated to approximately 215° C. Although the dimensions of the roasts were not given, it seems likely that the distance of the thermocouple from the surface of the meat was about 1–$1\frac{1}{2}$ in. in a roast of this weight.

The need for refrigeration, if meat is not to be eaten immediately after cooking, has been stressed frequently (Knox & Macdonald, 1943; Hobbs *et al.* 1953). Even with ordinary domestic refrigeration at 4° C., large bulky portions of meat are slow to cool, which re-emphasizes the necessity for using small cuts of meat for efficiency of both cooking and cooling.

The survival of spores from both heat-sensitive and heat-resistant strains of *Cl. welchii* confirms the results of Woodburn & Kim (1966), who found that heat-sensitive spores of *Cl. welchii* in turkey stuffing survived cooking. Several incidents of food poisoning due to heat-sensitive *Cl. welchii* have been reported (Hall, Angelotti, Lewis & Foter, 1963; Taylor & Coetzee, 1966; Sutton & Hobbs, 1968)

and because of the ubiquitous presence of these spores, prevention of this form of food poisoning lies primarily in preventing multiplication of the surviving organisms to the large numbers needed to cause food poisoning. Bryan & Kilpatrick (1971) again draw attention to the necessity for care after cooking meat and poultry in the prevention of *Cl. welchii* food poisoning. The organism was isolated from many meat and environmental samples taken in the kitchen. Useful recommendations are presented at the end of their paper.

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The nature of the toxic reaction of influenza virus towards lung tissue

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SUMMARY

The inoculation of large doses of unadapted influenza virus intranasally into mice results in the production of severe lung lesions. This toxic effect is a result of the entry of virus particles into the lung cells followed by uncoating of the virus ribonucleic acid.

The toxic property of the virus is destroyed by procedures which destroy or modify the nucleic acid such as exposure to monochromatic UV light of wavelength 2537 Å, or treatment with hydroxylamine or Bayer A139. Reagents acting on amino groups are particularly effective as they react with the nucleic acid and probably also interfere with penetration of virus into the cell.

Toxicity is also destroyed by mercurials which probably prevent uncoating of the nucleic acid by union with disulphide bonds, and by oxidizing agents such as iodine, permanganate, osmic acid and hydrogen peroxide under conditions which suggest possible action on some constituent of the virus containing methionine.

The toxic effect produced by the inoculation of large doses of unadapted virus intranasally in mice is associated with the occurrence of an incomplete growth cycle in which there is full production of RNP antigen but no production of haemagglutinin or infective virus.

INTRODUCTION

Inoculation of large doses of influenza virus into animals may cause severe pathological change and even death in the absence of detectable virus multiplication, apparently as a result of the toxic properties of the virus. The effects produced depend on the route of inoculation and toxicity towards lung tissue is of special interest as it is probably one of the major causes of death in human influenza.

Intranasal inoculation of large doses of unadapted virus in mice produces severe lung lesions (Sugg, 1949; Ginsberg, 1954). This paper describes a study of this phenomenon with an attempt to determine the particular virus component responsible for the toxic effect. Egg-adapted virus was treated with chemical reagents reacting with particular virus components or chemical groupings and the resultant effects on the toxicity towards mouse lung determined.

METHODS

Viruses

The DSP (1943) and the A2/Hong Kong (1968) strains of influenza virus were used in these studies. These were originally isolated from man by egg inoculation and had been propagated by egg passage. Virus was precipitated from infected allantoic fluid by 7·5% polyethylene glycol, eluted into phosphate-buffered saline pH 6·5, and purified by adsorption-elution from guinea-pig red blood cells followed by differential centrifugation, the final deposited virus being resuspended in phosphate-buffered saline at pH 6·5 to a concentration of approximately 1% by volume. This virus concentrate was suitably diluted in appropriate phosphate-buffered saline immediately before use.

Chemical treatment

This was similar to the methods used in previous work on the chemical reactions of influenza virus proteins (Hoyle & Hana, 1966). The virus concentrate was diluted 1/20 in phosphate-buffered saline and mixed with an equal volume of reagent in similar buffer. The conditions of reagent concentration, pH, temperature and period of exposure were chosen for maximum possible specificity.

Excess reagent was removed by adsorbing the virus to guinea-pig red blood cells, washing with cold saline and eluting into phosphate-buffered saline at pH 6·5, unless the reagent reacted very rapidly or lysed red blood cells, in which case it was chemically neutralized.

Reagents insoluble in water were added in solution in ether and mixed by rapid shaking to produce an emulsion. Provided the final concentration of ether was less than 5% no destruction of virus properties was caused by the solvent.

In all cases controls were set up in which the virus was exposed to identical procedures and conditions of temperature and pH, but in the absence of reagent or in the presence of previously neutralized reagent.

The choice of suitable chemical reagents was limited to those active between pH 5 and 10, and at a temperature of 37° or less, owing to the instability of the virus outside these limits.

Measurements of virus properties

Haemagglutinin titre

HA titre was measured by titration by the Salk method against a 0·5% (v/v) suspension of guinea-pig red blood cells in saline.

Neuraminidase activity

Neuraminidase activity was determined by measuring the amount of *N*-acetyl neuraminic acid released from an ovomucin substrate under standard conditions by the Aminoff (1961) method and was expressed as the optical density reading at 549 nm.

Complement-fixing antigen

Complement-fixing antigen was measured by the long fixation method, using appropriate human convalescent sera containing antibody predominantly to either the strain specific antigen or to the group antigen.

Toxicity

An intranasal dose of 0·05 ml. of each preparation was given to each of four mice. The mice were killed with chloroform on the third day and their lungs examined for areas of consolidation visible to the naked eye, and lesions recorded as follows.

(i) Mice dying on 2nd or 3rd day with severe lung lesions recorded as D_2, D_3,
(ii) Severe lung lesions affecting all lobes recorded as + +.
(iii) Moderately severe lung lesions recorded as +.
(iv) Small lesions recorded as +/2.
(v) No lesions recorded as 0.

In control experiments with untreated virus it was found that the inoculation of virus preparations with a haemagglutinin titre of 100 or less produced no lesions. With titres ranging from 200 to 2000, lesions of increasing severity were produced, but with inoculum titres from 4000–32,000 a maximal response was attained, the lesions produced by inocula of titre 32,000 being no more severe than with 4000. With these doses variations in the result with individual mice appeared to reflect differences in the degree of inhalation of the inoculum. In most experiments the virus concentrate was diluted to give an inoculum titre of 10,000 but because of differences in purification of the virus samples, and especially in the efficiency of the red cell adsorption-elution used in clearing the treated virus from chemical reagent, titres of actual inocula ranged from 4000 to 32,000.

Infectivity tests

These were carried out by a modification of the 6 hr. soluble antigen production test of Finter & Beale (1956) which although less sensitive than the orthodox infectivity test is more suitable for preparations containing large amounts of virus.

Two eggs were inoculated to the allantoic sac with 0·1 ml. of the virus preparation and after 6 hr. incubation the chorioallantoic membranes were removed, suspended in 1 ml. of phosphate-buffered saline of pH 6·5 containing 1/1000 sodium azide, frozen and thawed three times, centrifuged, and the content of S antigen in the supernatant fluid measured by complement fixation with a human convalescent serum containing antibody to the group antigen.

RESULTS

First experiments were directed to determine if any component of the virus particle was directly toxic or if the toxic effect depended on union of virus with cells. Virus preparations were distintegrated into their component subunits by shaking with ether or dichloromethane, or by alternate freezing in CO_2 actone and

Table 1. *Effect of virus disintegration on toxicity*

Material	Haemagglutinin titre	Neuraminidase activity	S Antigen titre	Toxicity Test	
				Dilution	Result
Hong Kong virus control	32,000	0·40	4	1/1 1/5 1/25 1/125	D_3, + +, + +, 0 D_2, D_3, + +, + + + +, + +, 0, 0 0, 0, 0, 0
Hong Kong virus treated with dichloromethane (aqueous phase)	16,000	0·34	96	1/1 1/5	0, 0, 0, 0 0, 0, 0, 0
D.S.P. virus control	32,000	0·35	6	1/1	D_2, D_2, +, +
Ether treated D.S.P. virus (aqueous phase)	16,000	0·30	128	1/1	0, 0, 0, 0
Hong Kong virus control	8,000	0·375	4	1/1	+ +, + +, + +, +
Hong Kong virus frozen and thawed 5 times	8,000	0·375	3	1/1	D_3, D_3, + +, +/2
Hong Kong virus frozen and thawed 50 times	16,000	0·37	14	1/1	0, 0, 0, 0

Table 2. *The effect of diazotized sulphanilic acid on haemagglutinin and toxicity of influenza viruses*

	D.S.P. Virus		Hong Kong virus	
	Control	Virus + M/400 diazo reagent	Control	Virus + M/400 diazo reagent
Haemagglutinin titre	8,000	< 32	32,000	< 32
Toxicity test result	D_2, D_2, + +, +	0, 0, 0, 0	+ +, + +, +/2, 0	+/2, 0, 0, 0

thawing in water at 20° C. These procedures resulted in release of internal RNP antigen and total loss of toxicity (Table 1), although the haemagglutinating power and neuraminidase activity were not destroyed. Chemical reagents which destroy haemagglutinin do, however, also destroy toxicity. One of the more specific of these reagents is diazotized sulphanilic acid which reacts with histidine and tyrosine, one or other of which appears to be present in the active centre of the haemagglutinin (Hoyle & Hana, 1966). Virus concentrates were diluted 1/10 with borate-buffered saline pH 9·0 and mixed with an equal volume of M/200 diazotized sulphanilic acid and held at 0° C. for 16 hr. The reagent was then neutralized by addition of 2% iminazole. Controls were treated with diazotized sulphanilic acid previously neutralized with iminazole. Toxicity of the DSP virus was completely and that of Hong Kong virus almost completely destroyed (Table 2). Destruction of haemagglutinating activity therefore destroys toxicity, probably by preventing union of virus and cells. There are, however, many chemical reagents which react

with the virus components without destroying haemagglutinin and experiments were set up to determine if any of them would inactivate the toxic property.

Reagents reacting with sulphydryl or amino groups

Results with these reagents are shown in Table 3. Under the condition used dichromate, iodacetamide, *N*-ethyl maleimide and nitroprusside react specifically with the SH group; acrylonitrile reacts strongly with SH and feebly with the amino group; fluorodinitrobenzene and phenyl isothiocyanate react with both groups; while formaldehyde, glyoxal and β-propiolactone are specific for the amino group. The results show clearly that toxicity is unaffected by reaction with SH groups but is destroyed by chemical action on the amino group.

Reagents acting on the disulphide bond

Allison (1962) found that the infectivity of influenza viruses was reversibly inactivated by mercurials and attributed this effect to action on disulphide bonds preventing uncoating of the virus nucleic acid. The combination of urea and dithiothreitol produces an irreversible rupture of the bond. The effect of these reagents on the toxicity of the Hong Kong strain is shown in Table 4. (Reagents acting on the disulphide bond destroy the neuraminidase activity of DSP virus which cannot be recovered after adsorption to red cells.) Toxicity is destroyed by the mercurials but not by urea + dithiothreitol.

Oxidizing agents

Oxidizing agents are very viricidal towards influenza virus and the toxic property can be inactivated without effect on the haemagglutinin. The possible lines of attack of these chemicals include the SH group of cysteine, the CH_3S group of methionine, the unsaturated fatty acids of the lipid and the aromatic rings of the nucleic acid and amino acids.

The action of iodine, permanganate, and osmic acid at concentrations of 1/10,000 at pH 6·0 and 0° C., and of hydrogen peroxide at 1/100, pH 7·0 and room temperature, were investigated. Under these conditions, 1/10,000 iodine is decolorized instantly by a 10-fold excess of cysteine or methionine, within a few seconds by tryptophane and slowly (30–60 min.) by tyrosine and uracil. Histidine and cytosine react in 24 hr. Water soluble agents containing the C=C double bond, such as maleic acid, do not react but iodine dissolves in oils containing unsaturated fatty acids such as arachis oil, and is slowly decolorized (Hoyle, 1964).

Under similar conditions permanganate is decolorized instantly by cysteine and maleic acid and within seconds by methionine and tyrosine. Tryptophane, histidine and uracil react in 10 min. and shaking with arachis oil causes rapid decolorization.

Osmic acid reacts instantly with cysteine and more slowly (2–10 min.) with methionine. There is no apparent reaction with maleic acid but on shaking with arachis oil a brown colour develops fairly rapidly. Osmic acid does not react with the aromatic rings in 1 hr.

Results are shown in Table 5. It is possible that the different oxidizing agents do not act on the same virus component but if they are acting in the same way the

Table 3. *Action of amino and sulphydryl group reagents on the toxicity of DSP and Hong Kong viruses*

(Composite table embodying the results of several experiments.)

Reagent and conditions of reaction	Probable reacting group	Hong Kong virus		DSP virus	
		HA titre	Toxicity test result	HA titre	Toxicity test result
Formaldehyde 1/1000, pH 7, 1 hr. 37° C	NH_2	4,000	0, 0, 0, 0	16,000	0, 0, 0, 0
Glyoxal 1/200, pH 8, 1 hr. 37° C	NH_2	8,000	0, 0, 0	16,000	0, 0, 0, 0
β-propiolactone 1/800, pH 8, 1 hr. 37° C	NH_2	32,000	0, 0, 0	16,000	0, 0, 0, 0
Fluorodinitrobenzene 1/1000, pH 7, 1 hr. 37° C	NH_2 SH	6,000	0, 0, 0, 0	8,000	0, 0, 0
Phenyl isothiocyanate 1/1000, pH 9, 18 hr. 4° C	NH_2 SH	24,000	0, 0, 0, 0	130,000*	0, 0, 0, 0
Acrylonitrile 1/200, pH 7, 1 hr. 37° C	(NH_2) SH	12,000	+ +, +, +	16,000	D_3, D_3, D_3, +/2
Sodium dichromate 1/10,000, pH 6, 2 hr. 37° C	SH	4,000	D_3, D_3, D_3, +	16,000	+ +, + +, +, +
Iodacetamide 1/1000, pH 6, 2 hr. 37° C	SH	8,000	D_3, + +, +, +	16,000	D_2, D_3, +, 0
N-ethyl maleimide 1/500, pH 6, 2 hr. 37° C	SH	32,000	D_3, D_3, + +, +	24,000	D_3, + +, + +, + +
Sodium nitroprusside 1/2000, pH 7, 1 hr. 37° C	SH	24,000	D_3, D_3, + +	8,000	D_3, + +, +, +
Controls	—	4,000	D_3, D_3, D_3, +	8,000	D_3, D_3, +, +/2
Conditions as in tests but without reagent		8,000	D_3, D_3, + +, +	16,000	D_2, D_3, D_3, +
		16,000	D_2, D_2, + +, +	32,000	D_2, D_2, D_3, + +
		32,000	D_3, + +, +, +	130,000*	D_3, D_3, D_3, + +

* High HA titres due to partial disruption of virus at pH 9.

Table 4. *Effect of reagents acting on the disulphide bond on toxicity of Hong Kong virus*

Reagent		Haemagglutinin titre	Toxicity test result
Mercuric chloride	Control	32,000	D_3, ++, ++, +
	Virus + 1/5,000 $HgCl_2$ 1 hr. 37° C pH 6	32,000	0, 0, 0, 0
Parachlormercuribenzoate	Control	50,000*	D_3, D_3, D_3, ++
	Virus + 1/1000 PCMB 1 hr. 37° C pH 8	130,000*	0, 0, 0, 0
Urea + dithiothreitol	Control	32,000	D_2, D_2, D_3, D_3
	Virus + 4M urea + 1/600 DTT 30 min. 20° C. pH 8	50,000	D_3, D_3, D_3, ++

* High HA titres due to partial disruption of virus at alkaline pH.

results suggest action on methionine. Action on SH groups can be excluded because of the results described above and only methionine and tryptophane are attacked by iodine sufficiently rapidly to account for the destruction of toxicity in 10 sec., while the rapid action of osmic acid would appear to exclude action on tryptophane. Hydrogen peroxide destroyed toxicity under conditions in which it was shown to oxidize methionine but other possible actions of peroxide were not studied. The CH_3S group also reacts with alkyl halides to produce sulphonium salts. Exposure of virus preparations to ethyl chloride or ethyl bromide in sealed tubes resulted in loss of toxicity but it was found that the virus particles were disrupted with release of internal S antigen so that loss of toxicity could have been due to virus disintegration.

Agents acting on nucleic acid

All the procedures described above which destroy toxicity also render the virus preparations non-infective. The destruction of toxicity by reagents acting on the amino group may well be due to action on nucleic acid. Agents acting more specifically on nucleic acidi nclude hydroxylamine which attacks the pyrimidines and the ethylene iminoquinone Bayer A 139 which attacks the phosphate sugar backbone (Scholtissek & Rott, 1963).

Virus preparations diluted in phosphate buffer pH 6·5 were mixed with an equal volume of 1/1000 hydroxylamine hydrochloride in buffer pH 6·5. At intervals samples were adsorbed with red cells, the cells washed, the virus eluted and tested for HA titre, toxicity and infectivity by the 6 hr. test. Similar experiments were done with 1/50 Bayer A 139 but in this case TRIS buffer was used in place of phosphate. Results are shown in Table 6. Haemagglutinin titres were unaffected but infectivity and toxicity were destroyed, infectivity as measured by the 6 hr. test being almost as sensitive as toxicity.

Virus preparations diluted in buffer pH 6·5 were exposed in shallow layers to monochromatic UV light of wavelength 2537 Å. At intervals preparations were

Table 5. *Effect of oxidizing agents on toxicity of DSP and Hong Kong viruses*

Reagent and conditions of reaction	Probable reacting groups	Hong Kong virus		DSP virus	
		HA titre	Toxicity test results	HA titre	Toxicity test result
Iodine					
1/10,000, pH 6,	SH				
10 sec., 0° C.	SCH_3	16,000	0, 0, 0, 0	6,000	0, 0, 0, 0
Neutralized with thiosulphate	Tryptophan				
Control (virus + neutralized iodine)	—	16,000	D_3, D_3, D_3, +	6,000	D_3, D_3, +, +
Potassium permanganate					
1/10,000, pH 6, 0° C.	SH				
A 1 min.	C=C (SCH_3, Tyr)	16,000	D_2, D_3, + +	—	—
	SH C=C				
B 10 min.	SCH_3 Tyr Trypt. Hist.	6,000	0, 0, 0	—	—
Neutralized with cysteine	Uracil				
Control (virus + neutralized reagent)	—	16,000	D_3, +/2, 0	—	—
Osmic acid					
1/10,000, pH 6,	SH				
2 min. 0° C	SCH_3	4,000	0, 0, 0, 0	3,000	0, 0, 0, 0
Neutralized with cysteine	C=C				
Control (virus + neutralized reagent	—	3,000	D_3, D_3, +/2, 0	4,000	D_3, D_3, + +, + +
Hydrogen peroxide					
1/100, pH 7, 20° C	SCH_3				
30 min.	Others not tested	12,000	+ +, 0, 0, 0	6,000	0, 0, 0, 0
Control	—	8,000	D_3, + +, +, +	8,000	D_2, D_3, D_3, +

Table 6. *Destruction of infectivity and toxicity of DSP and HK viruses by* 1/2000 *hydroxyalamine hydrochloride and* 1/100 *Bayer A* 139

Experiment	Time of exposure to reagent (hr.)	Haemagglutinin titre	Infectivity test (6 hr.) Egg 1	Infectivity test (6 hr.) Egg 2	Toxicity test result
DSP virus + 1/2000	0	2,000	192	256	D_3, +, +
hydroxylamine	½	2,000	14	12	++, ++, +
hydrochloride	1	2,000	2	2	+, +/2, 0
	2	2,000	0	2	+/2, +/2, 0
	4	2,000	0	0	0, 0, 0
HK virus + 1/2000	0	25,000	112	80	++, +, +/2
hydroxylamine	½	12,000	24	3	+, +, +/2
hydrochloride	1	8,000	4	0	+/2, 0, 0
	2	16,000	0	0	0, 0, 0
	4	16,000	0	0	0, 0, 0
DSP virus + 1/100	0	8,000	12	12	D_3, D_3, D_3, ++
Bayer A139	1	8,000	6	12	D_2, ++, ++, +
	2	8,000	12	24	++, +, +/2, 0
	4	8,000	0	1	+/2, +/2, +/2, 0
	6	8,000	0	0	0, 0, 0, 0
HK virus + 1/100	0	32,000	24	14	D_2, D_3, 0
Bayer A139	1	32,000	24	24	D_3, ++, +/2
	2	32,000	20	28	D_3, +, 0
	4	32,000	4	2	++, +, 0
	6	32,000	0	0	0, 0, 0

removed and tested for HA titre, infectivity and toxicity. Results in Table 7 show that infectivity as measured by the 6 hr. test was slightly more sensitive to UV light than toxicity.

The relation between toxicity and intracellular synthesis of virus components

Relation between toxicity and production of RNP antigen by adapted and unadapted virus

Mice were inoculated intranasally either with a purified non mouse-adapted Hong Kong virus with a haemagglutinin titre of 32,000 or with a mouse-adapted Hong Kong virus in the form of 1 % lung extract.

At intervals three mice from each batch were killed, the lung lesions recorded and a pooled extract of the mouse lungs tested for RNP antigen content by complement fixation with an anti-S serum (Table 8). The unadapted virus produced a full yield of RNP antigen within 8 hr. but consolidated lung lesions did not appear until 48 hr. The amount of RNP antigen detectable in the lungs at 8 hr. was about 20 times the amount present in the inoculated virus.

The mice inoculated with adapted virus did not develop a full yield of RNP antigen until 24 hr. and consolidated lung lesions did not appear until 72 hr.

Table 7. *Effect of exposure to UV light of wavelength* 2537 Å *on toxicity and infectivity of DSP and HK viruses*

Virus	Time of exposure (sec.)	Haemagglutinin titre	Infectivity test (6 hr.) Egg 1	Infectivity test (6 hr.) Egg 2	Toxicity test result
DSP	0	16,000	20	12	D_2, + +, + +, 0
	5	8,000	20	20	D_3, +, +, +/2
	25	8,000	4	4	+, +/2, 0, 0
	125	16,000	0	0	+/2, 0, 0, 0
	625	8,000	0	0	0, 0, 0, 0
	3125	16,000	0	0	0, 0, 0, 0
HK	0	16,000	128	128	D_2, D_3, D_3, + +
	5	16,000	128	64	D_3, + +, + +, +
	25	16,000	128	64	D_3, +, +/2, 0
	125	16,000	0	0	+, +, 0, 0
	625	16,000	0	0	0, 0, 0, 0
	3125	16,000	0	0	0, 0, 0, 0

Table 8. *Production of ribonucleoprotein antigen and development of lung lesions in mice inoculated with adapted and unadapted virus*

Time after inoculation (hr.)	Mice inoculated with unadapted virus of HA titre 30,000: S antigen titre in lung extract	Mice inoculated with unadapted virus of HA titre 30,000: Lesions (3 mice)	Mice inoculated with adapted virus of HA titre 32: S antigen titre in lung extract	Mice inoculated with adapted virus of HA titre 32: Lesions (3 mice)
8	192	0, 0, 0	2	0, 0, 0
16	256	0, 0, 0	48	0, 0, ?+
24	112	0, 0, 0	112	0, 0, 0
48	256	D_2, D_2, D_2	128	0, 0, 0
72	64	D_3, D_3, +	128	+, +, +

Relation between toxicity and haemagglutinin production by adapted and unadapted virus

Mice were inoculated intranasally with unadapted virus with a haemagglutinin titre of 8000. At intervals three mice were killed, the lung lesions recorded and the haemagglutinin titre of pooled saline tracheal washings measured. No haemagglutinin was detected at any stage but severe lesions were found in mice examined at 48 and 72 hr.

When mice were similarly inoculated with adapted virus of haemagglutinin titre 8, haemagglutinin became detectable at 24 hr. and washings attained a titre of 512 at 40 hr.

Relation between virulence of adapted and unadapted virus

The mouse-adapted Hong Kong virus was maintained by serial passage in mice, harvesting the lungs on the 4th day. Of 60 mice inoculated in this way with fully adapted virus 2 died on the 3rd day, 22 on the 4th and 19 had lesions on the 4th day of such severity that death would have resulted on the 5th day.

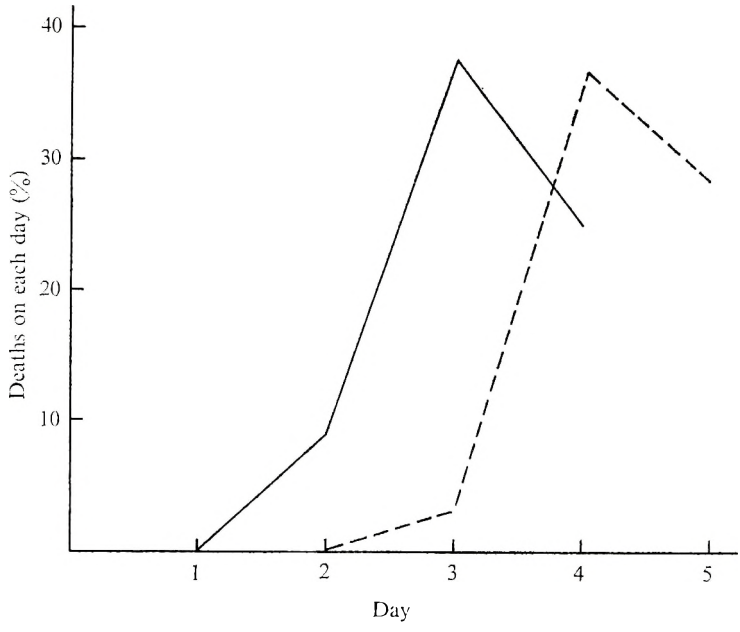

Fig. 1. Mortality of mice inoculated intranasally with adapted and unadapted virus. Mice inoculated with a large dose of unadapted virus (HA titre of inoculum > 4000) ——. Mice inoculated with a small dose of adapted virus (HA titre of inoculum < 4) – – – –.

In tests of the effects of chemical reagents on toxicity, control mice were inoculated with large doses of unadapted virus and examined on the 3rd day. Of 88 such mice eight died on the 2nd day, 33 on the 3rd day and 22 had lesions on the 3rd day of a severity which would cause death on the 4th day (Fig. 1). The lung lesions produced by adapted and unadapted virus were macroscopically indistinguishable.

Figure 1 shows that the mortality curves obtained with the two types of virus were identical in shape and the overall death rate was almost the same, the only difference being that with a large dose of unadapted virus the peak mortality occurred on the 3rd day while with a small dose of adapted virus it occurred on the 4th day. This time difference probably represents the time taken for the adapted virus to multiply to a concentration in the lung equivalent to that produced by the large dose of unadapted virus. If virulence means ability to kill the two forms of virus are equally virulent.

DISCUSSION

The results described in this paper indicate that the toxic effect produced by intranasal inoculation of unadapted virus in mice depends on the penetration of virus into the lung cells and on the release of the virus nucleic acid.

Disruption of the virus particle destroys toxicity as the released nucleoprotein is unable to enter the cell. Destruction of the haemagglutinin prevents the initial union of virus and cell.

Ammonium salts and aliphatic amines are known to interfere with the penetration stage in influenza virus reproduction (Fletcher, Hirschfield & Forbes, 1965);

they possibly act by blocking a cell receptor for the virus amino group, and chemical reagents acting on the amino group are very viricidal and efficient in destroying toxicity. These agents probably have a dual action, reacting with the virus nucleic acid and also interfering with penetration.

Allison (1962) described a reversible inactivation of influenza virus by mercurials which he attributed to union with disulphide bonds preventing uncoating of the virus nucleic acid. The effects on toxicity of agents acting on the disulphide bond may well be explained in this way. Mercuric chloride which prevents uncoupling of the disulphide bond destroys toxicity while toxicity is unaffected by urea + dithiothreitol which uncouples the disulphide bond and would presumably not prevent uncoating of the nucleic acid.

Ackermann & Maassab (1955) showed that influenza virus reproduction was inhibited by the methionine analogue methoxinine which acted at an early stage of the growth cycle immediately after penetration. The effect was not due to interference with incorporation of methionine in protein synthesis but to interference with some other action of methionine. The possibility that the methionine involved was a virus component was not considered. The destruction of virus toxicity by iodine, permanganate and osmic acid may possibly be due to action on the CH_3S group of methionine. The group also reacts with mercuric chloride, hydrogen peroxide and alkyl halides all of which destroy toxicity.

Agents acting on the nucleic acid are particularly effective in destroying toxicity, and hydroxylamine, Bayer A 139 and exposure to UV light destroy toxicity under conditions in which there is no demonstrable action on other virus components. At one stage in the work the possibility was considered that the toxic effect might be due to damage to the cell walls produced during the penetration of the cell by large doses of virus, but the fact that toxicity can be destroyed by action on the nucleic acid shows that the toxic effects are produced at a later stage than penetration. The virus nucleic acid may itself be toxic but more probably the toxic effects are due to synthesis of some protein under the control of the virus RNA. The toxic effect would appear to be associated with the occurrence of some form of incomplete growth cycle (Schlesinger, 1953; Ginsberg, 1954).

When unadapted virus is inoculated intranasally to mice there occurs no demonstrable production of haemagglutinin or infective virus, but a full yield of RNP antigen is obtained after 8 hr. Severe lung lesions follow after an interval of some 40 hr.

With adapted virus also severe lung lesions occur at a similar interval after the production of RNP antigen has reached its peak. It appears that cell damage is induced at an early stage in the growth cycle but lung consolidation takes some time to develop.

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Chemical closet treatment of typhoid carrier faeces

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SUMMARY

An investigation to test the efficiency of chemical closets in treating excreta from typhoid carriers is described. The use of these closets kept a stream, which had in the past frequently contained *Salmonella typhi*, typhoid free for 24 months. Selenite broth as made in this laboratory, containing a final concentration of 0·8 % sodium hydrogen selenite when inoculated with the water sample, was significantly better than commercial selenite brilliant green enrichment broth for the recovery of *S. typhi*.

INTRODUCTION

Disposal of faeces from chronic typhoid carriers in a mental hospital of 1850 beds near Cardiff had posed a problem for many years. This hospital had been previously investigated by Gell, Hobbs & Allison (1945). Disinfection of bedpans with lysol (Harvey, 1957) and sewage disinfection with iodophors had given very unsatisfactory results. In 1963, 'Destrol' chemical closets were installed in a ward toilet block for the exclusive use of the typhoid carriers. 'Destrol' fluid appears from analysis to be a mixture of chlorxylenol, an aniline dye indicator and a zinc compound. All three constituents are emulsified in pine oil.

In this communication we have used the disappearance of typhoid bacilli from brook water sampled below the entry of hospital effluent as an indication of the effectiveness of the 'Destrol' closets. A comparison is also made of S.B.G. enrichment broth used in isolation procedures with our own quadruple strength selenite F broth (Harvey & Price, 1964).

MATERIALS AND METHODS

One litre samples were collected at a convenient point (Point A: Gell *et al.* 1945), and brought straight to the laboratory. The stream was swift running and there was no evidence of pollution on the banks: samples were not obviously contaminated with sewage to the naked eye. The hospital was not informed about the time of the visits.

Enrichment media

Enrichment culture of the stream was essential for the demonstration of *Salmonella typhi*. Previous attempts at isolation by direct plating had been

unsuccessful. Both diluted and undiluted water had been examined using Wilson and Blair's bismuth sulphite agar (de Loureiro, 1942).

Selenite F broths. In 1965 and 1966, the 1000 ml. sample was equally divided between 500 ml. of double strength and 500 ml. of quadruple strength selenite F broth. The quadruple strength enrichment medium gave a final concentration of 0·8 % sodium hydrogen selenite when the water sample was added.

The 0·8 % selenite medium developed from a study of Leifson's (1936) paper, which implied that *S. typhi* might be more resistant to selenite than *S. paratyphi B*. As *S. paratyphi B* was occasionally present in the stream during our early studies and had created cultural difficulties to other workers (Gell *et al.* 1945), the 0·8 % concentration seemed necessary for accurate monitoring of the water. This enrichment broth also aided recovery of *S. typhi* in the presence of other salmonella serotypes and effectively inhibited *B. effluviei* (Wilson, 1928). Presence of competing organisms can be a major problem in investigating sewage, particularly crude effluent. *B. effluviei* is colonially similar to *S. typhi* on Wilson and Blair's medium and can easily confuse attempts to trace typhoid carriers by sewage examination (Harvey, 1957). Concentrations of selenite above 0·8 %, as suggested by Leifson (1936) for sewage examination have not proved valuable in our hands. Callaghan & Brodie (1968) have recently reported favourably on a fluid medium containing 0·8 % sodium hydrogen selenite + streptomycin sulphate.

Selenite brilliant green broth. Selenite brilliant green broth, with and without sulphapyridine, has been suggested for enrichment culture of *S. typhi* by other authors (Pilsworth, 1960; Livingstone, 1965). We decided to compare the two enrichment media using 500 ml. quantities of water sample for each medium. The S.B.G enrichment broth in our investigation was used in accordance with Livingstone's (1965) instructions.

Incubation temperature

Elevated temperature enrichment (incubation at temperatures above 37° C.) is not suitable for isolating *S. typhi* (Harvey & Thomson, 1953), although there are reports where this has been used for plate incubation (Wilson, 1928; Livingstone, 1965). The usual temperature of 37° C was, therefore, used with an incubation time of 24 hr. This timing had been found better than 18 hr. in a previous study (Harvey, 1965). Subcultures were made to de Loureiro's (1942) modification of Wilson and Blair's bismuth sulphite agar. This formula gives very consistent results. It is used unripened. Plates were incubated at 37° C and examined at 24 and 48 hr. Suspicious colonies were picked for further examination.

RESULTS

In 1965, 3/30 samples of brook water contained *S. typhi*. In 1966, however, 21/51 samples were positive for *S. typhi* and we, therefore, believed that the closets were not functioning properly. On inquiry, we learned that, for a period, patients had been responsible for using the closet agitators. This may explain sub-optimal functioning.

Table 1. *Isolation of* Salmonella typhi *from Morfa Brook*

Year	Positive samples	Total samples	Number of regular excreters in hospital
1967	5	42	4
1968	3	46	3
1969	4	49	2
1970	0	49	2
1971	0	14	1

Faeces samples from carriers examined monthly. No *S. typhi* found in stream between 26 March 1969 and 5 April 1971.

Table 2. *Isolation of salmonellas* (*excluding* Salmonella typhi) *from Morfa Brook*

Year	Positive samples	Total samples	Serotypes
1967	0	42	—
1968	4	46	*S. bredeney*
1969	5	49	*S. eimsbuettel* *S. panama* *S. senftenberg* *S. dublin*
1970	6	49	*S. panama* *S. dublin* *S. indiana* *S. eimsbuettel* *S. kiambu* *S. infantis*
1971	3	14	*S. infantis* *S. panama*

Monitoring of the stream continued from 1967, when nursing staff became responsible for supervision of closet use, and has continued without intermission. The results are recorded in Table 1. Other salmonella serotypes were also isolated and are shown in Table 2. The comparison of efficiency of the two enrichment media is given in Tables 3 and 4.

In this investigation, *B. effluviei* was only found in five samples. It was isolated once from 0·8 % selenite broth and five times from S.B.G. enrichment. This supports earlier unpublished observations that 0·8 % selenite enrichment inhibited *B. effluviei*.

DISCUSSION

The Morfa Brook was well known to the laboratory (Gell *et al.* 1945; Harvey, 1957). It was a natural water from which, in the remote past, we had little difficulty in isolating *S. typhi*, phage-type C_1. When, in 1965, we began a regular sampling programme to ascertain the effect of 'Destrol' closets, 3/30 samples contained *S. typhi*. This seemed a reasonable contamination rate compared with

Table 3. *Isolation of* Salmonella typhi *from* 200 *water samples*

Quadruple strength selenite broth	S.B.G. broth	No. of positive samples
+	+	1
+	−	10
−	+	1
−	−	188

The statistical test appropriate is Fisher's exact test $P = 0{\cdot}005$ *significant.*
The quadruple strength selenite broth is significantly superior to S.B.G. enrichment for the isolation of *S. typhi* under these study conditions.

Table 4. *Isolation of salmonellas other than* S. typhi *from* 200 *water samples*

Quadruple strength selenite broth	S.B.G. broth	No. of positive samples
+	+	6
+	−	13
−	+	5
−	−	176

The statistical test appropriate is McNemar's test $\chi^2 = 2{\cdot}72$, $P = 0{\cdot}10$, not significant.
The 200 samples in Tables 3 and 4 were identical and consecutive. This formal media comparison ceased on 5 April 1971.

previous experience. The following year, however, when *S. typhi* was isolated on 21 occasions from 51 samples, we notified the relevant authorities. We were informed of the change in agitation routine and were told that nursing staff would again become responsible for closet supervision. A long-term survey of the qualitative typhoid contamination of the brook was, therefore, planned. The survey covered a period of almost 5 years (Table 1). Twelve isolations of *S. typhi* have been made in that time out of 200 samples. All isolations belonged to phage-type C_1.

The most important information derived from Table 1, was our failure to find *S. typhi* in the brook over the last 24 months of the survey despite the presence in the hospital of chronic typhoid carriers regularly excreting the organism in their faeces. Before the end of the investigation, several of the original carriers had died and this obviously diminished the quantity of faeces requiring treatment.

It would seem that in 1970 and 1971, when agitation was properly performed, 'Destrol' closets successfully rid the hospital effluent of *S. typhi*.

In our hands, quadruple strength selenite F broth proved a more satisfactory enrichment medium for isolation of the typhoid bacillus than S.B.G. enrichment broth. This did not surprise us as *S. typhi* is brilliant green sensitive (Harvey, 1956). The quadruple strength selenite F broth, therefore, has become part of our routine procedure when searching for *S. typhi*. By its use we isolated typhoid bacilli from the River Ogmore near the entry of the Morfa Brook and two miles downstream from the hospital effluent discharge (Harvey, 1957). The laboratory-prepared medium was significantly superior, in this study, to the commercial medium. There was no significant difference between the media when salmonellas other than *S. typhi* were considered.

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Rabies vaccines and interferon

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SUMMARY

Samples of Fermi, Semple, modified Semple, Duck embryo and tissue culture rabies vaccine were inoculated by different routes and in different doses into rabbits, mice and hamsters. The vaccines induced neither detectable interferon nor immediate protection against lethal challenge with CVS rabies virus.

Under similar conditions, high but transient levels of interferon were induced in control animals of the same species with the polynucleotide complex Poly I.C. Hamsters but not mice were protected by Poly I.C.-induced interferon.

No autointerference by vaccine with challenge virus was established. Vaccine-induced protection in mice was directly related to immune response.

INTRODUCTION

Rabies virus, like most other viruses, both induces and is sensitive to interferon. Abundant interferon appears just before death in the brains of hamsters and mice infected with fixed rabies strains and detectable amounts of interferon are found in their blood and other organs (Stewart & Sulkin, 1966; Karakuyumchan & Bektenerova, 1968; G. S. Turner, unpublished results).

Resistance to superinfection mediated by interference or interferon has been induced in cell cultures by live rabies virus (Kaplan, Wecker, Forsek & Koprowski, 1960; Wiktor, Fernandes & Koprowski, 1964; Fernandes, Wiktor & Koprowski, 1964; Selimov, Chuprikova, Kalinina & Sharova, 1965; Depoux, 1965; Yoshino, Taniguchi & Arai, 1966; Barroeta & Atanasiu, 1969). Both live and inactivated rabies virus inhibit the development of Rous sarcomas in fowls by an interferon-like mechanism (Kravchenko, Voronin & Kosmiadi, 1967; Desai, 1970).

Brief protection, mediated by interferon or 'interferon-like' mechanisms, occurs in rabbits or hamsters, challenged with rabies virus after inoculation with the viruses of vaccinia, bovine parainfluenza and Newcastle disease (Levaditi, Nicolau & Schoen, 1926; Vieuchange, 1967; Fayaz, Afshar & Bahmanyar, 1970; Atanasiu, Barroeta, Tsiang & Favre, 1970). High levels of interferon induced in rabbits by the polynucleotide complex (Poly I.C.) protected them for 24 hr. against lethal infection with rabies street virus (Fenje & Postic, 1970, 1971; Janis & Habel, 1970; Postic & Fenje, 1971).

These data add credibility to earlier speculation that similar, non-immune mechanisms might be involved in post-exposure protection by rabies vaccines, although little direct evidence supports these conjectures (Schindler, 1963; Stewart

& Sulkin, 1966; Habel, 1966*a*). In the present study five commonly used rabies vaccines were examined for their capacity to induce interferon in rabbits, hamsters or mice. Resistance to challenge with rabies virus, antibody formation and interferon induction was tested in groups of animals given vaccine by different routes and in different doses. Poly I.C., which induces abundant interferon and protects some animals against lethal challenge was used as control material.

MATERIALS AND METHODS

Vaccines

(i) Conventional Semple type rabies vaccine prepared from infected rabbit brain and inactivated with phenol was obtained from current stocks at the Lister Institute. It had a potency index > 6·0 estimated by the method of Habel (1966*b*).

(ii) Semple vaccine modified by fluorocarbon treatment (Turner & Kaplan, 1967; Kaplan & Turner, 1968) with a potency of 4·8 was also prepared at the Lister Institute.

(iii) Duck Embryo vaccine (DEV) (Eli Lilly & Co.) had a potency of 4·0.

(iv) Tissue Culture vaccine (TCV) (Rabiffa, Institute Merieux, Lyon was a veterinary product with a potency > 6·0.

(v) Fermi type vaccine (Institute of Sera and Vaccines, UFA, USSR) had a potency of > 6·0 and contained $10^{2·7}$ mouse LD 50 of residual live virus.

Viruses

The Lister Institute and WR strains of vaccinia virus were reconstituted for use from freeze-dried stocks kept at 0 to 4° C. 'Standard challenge virus' (CVS) fixed rabies virus was kept at −160° C. as a 10 % suspension of infected mouse brain. Samples when thawed were used immediately and not refrozen.

Polyinosinic-polycytidylic acid

Poly I.C. solution 1 mg./ml. (Microbiological Associates, Bethesda, Md, USA) was kept at 0–4° C.

Animals

New Zealand red rabbits, golden hamsters and T.O. mice were used with initial weights of 1–2 kg., 73 g. and 11–13 g. respectively.

Interference tests

Serial tenfold dilutions of CVS rabies virus were prepared in either undiluted vaccine or in buffer. Five mice were inoculated with each dilution either intramuscularly (0·25 ml.) or intracerebrally (0·03 ml.).

Interferon induction tests

The vaccines were tested in the animals by different routes, doses and numbers of inoculations.

Rabbits. Groups of two to four rabbits were inoculated daily for 14 days, with

subcutaneous (sc) 0·2 ml. doses of Semple vaccine, diluted on a weight basis to correspond with an average human dose (2·0 ml./63 kg.). Modified Semple, DEV and TCV vaccines were administered similarly. Further groups of rabbits received undiluted vaccines subcutaneously either as 14 × 2 ml. daily doses, 6 × 2 ml. doses during 14 days or 1 × 2 ml. dose on each of days 0 and 14. All these rabbits were bled before, during and after immunization on days 0, 2, 4, 7, 10, 14 and 21. Within a group, sera from each day's bleedings were pooled and tested for rabies-neutralizing antibody and interferon.

In other experiments groups of two to four rabbits were bled and inoculated intravenously with 1·0 ml. of modified Semple, DEV or TCV vaccines or Poly I.C. (1 mg./kg.). All were bled 4 and 24 hr. later and their sera were tested for interferon.

Mice. Groups of mice were inoculated with 6 × 0·25 ml. doses of either Semple or Fermi vaccines. Inoculations were given intraperitoneally on days 0, 2, 4, 7, 9 and 11 of two successive weeks (Habel, 1966*b*). On each of these days and on the 14th day, ten mice were challenged intracerebrally with 90 LD 50 of CVS rabies. Ten unchallenged mice were killed at the same time and serum pools and pooled brain tissue extracts (10 %, w/v) were tested for interferon and antibody.

Maximum serum interferon titres are found in mice 2–4 hr. after inoculation with NDV or Poly I.C. (Atanasiu *et al.* 1970; Buckler, du Buy, Johnson & Baron, 1971). Suitable numbers of mice were inoculated with either Semple or tissue culture vaccine. Control mice were inoculated with similar amounts of normal rabbit brain suspension, tissue culture fluid, buffer, or with Poly I.C. (10 μg./g. i.p.). Two hours later mice from each series were inoculated with serial dilutions of CVS rabies, either intramuscularly (0·25 ml.) or intracerebrally (0·02 ml.); five mice were used per dilution. Five unchallenged mice from each group were killed before, then 2 and 24 hr. after vaccine or Poly I.C. treatment; their pooled sera and 10 % brain extracts were tested for interferon.

Poly I.C. (30 μg. in 0·03 ml.) was inoculated intracerebrally into mice in attempts to induce more interferon *in situ*. Groups of these animals were tested for resistance to rabies challenge and for serum and brain interferon titres as described above.

Hamsters. Suitable numbers of this species were inoculated intraperitoneally with undiluted Semple or TCV vaccines (0·2 ml.) or with Poly I.C. (1 mg./kg.). Three doses were given, the first 24 hr. before, the second coincident with, and the final one 24 hr. after challenge. Interferon was estimated in serum or brain extracts of unchallenged animals killed 4 hr. after each dose. Hamsters are highly susceptible to CVS inoculated intramuscularly (Atanasiu *et al.* 1970), and groups of ten treated and ten control animals were challenged by this route with 0·5 ml. CVS calculated to contain 5–50 LD 50.

Interferon assays

Serum or tissue extracts from mice or hamsters were tested for interferon (IF) by applying suitable dilutions to cell cultures prepared from embryos of the respective species (Gifford, 1963). Samples of rabbit origin were tested in the rabbit kidney cell line (RK 13) (Field, Tytell, Lampson & Hilleman, 1967).

Monolayer cell cultures were grown in plastic dishes in Eagle's minimum essential medium (MEM) containing 5% foetal bovine serum and 1% glutamine for RK 13 cells and 10% calf serum for mouse or hamster embryo cells.

Since longer incubation times did not significantly increase interferon titres in this system, six cultures were incubated for 5 hr. at 37° with each dilution of each sample (Subrahmanyan & Mims, 1966). Test material was removed and replaced by maintenance medium (Eagle's MEM + 1% calf serum) and treated and control cells were infected with 50–100 plaque forming units (p.f.u.) of vaccinia virus. Cultures were incubated for 40–48 hr. at 37° C. without agar overlay in 5% CO_2 in air; the monolayers were then stained and plaques were counted.

Interferon titres are expressed as the reciprocal of the dilution reducing vaccinia plaque production by 50% (Wagner, 1961). The method was controlled with internal reference material prepared from the sera of animals treated with Poly I.C.; the sensitivity and reproducibility of rabbit interferon assays was also verified with an international reference preparation (Research Reference Reagents Branch NIH, Bethesda, Md), and that of the mouse assays with material kindly supplied by Dr C. Bradish (Microbiological Research Establishment, Porton). Both standards contained a nominal 1000 international units of interferon. Material reacting positively was identified as interferon by its stability at pH 2·0, resistance to heat (65°) and to nuclease treatment. Further criteria were species but not virus specificity and susceptibility to tryptic digestion (Wagner, Levy & Smith, 1968).

Antibody assays

Rabies antibody was estimated by serum neutralization tests in mice by the method of Atanasiu (1966).

RESULTS

Interferon induction

In rabbits

Rabies vaccines inoculated subcutaneously produced rabies-neutralizing antibody, but no circulating interferon was detected in more than 100 serum samples taken at different times during the several immunization series. No detectable interferon was induced by the rabies vaccines administered intravenously although control animals receiving intravenous Poly I.C. (1 mg./kg.) always responded with serum interferon titres that exceeded 10^3 after 2–4 hr. and declined during the next 24 hr. (Table 1). Our sample of CVS rabies did not regularly kill rabbits by intramuscular injection, and challenge 2 hr. after intravenous administration of vaccine or Poly I.C. was unsatisfactory. In most instances however the mortality in vaccine-treated rabbits exceeded that in controls.

In mice

No interferon was detectable in the sera or brain extracts of mice inoculated intraperitoneally 2 or 24 hr. previously with rabies vaccines. Titres of serum interferon exceeding 10^3 were induced in control mice 2 hr. after inoculation with suit-

Table 1. *Serum interferon in rabbits injected intravenously with rabies vaccine or Poly I.C.* (1·0 *ml.*)

Vaccine	Interferon titres after 0 hr.	2 hr.	24 hr.
Semple*	—	—	—
Arcton treated Semple	< 5	< 5	< 5
Duck embryo	< 5	< 5	< 5
Tissue culture	< 5	< 5	< 5
Poly I.C. (1 mg./ml.)	< 5	1500	15

* Rabbits died within a few minutes of injection even when the dose was reduced four-fold.

able doses of Poly I.C.; much smaller amounts were present in their brains. Treated and control mice did not differ significantly in their susceptibility either to intracerebral or to intramuscular challenge with rabies virus. Median lethal end points determined by titration of challenge virus in both groups were similar, indicating that mice were unprotected by interferon even against minimal challenge doses. Intracerebral inoculation of Poly I.C. induced slightly higher and more persistent titres of brain interferon, but very little circulating interferon (Cathala & Baron, 1970). Again no animals resisted rabies challenge administered by either route.

Serum and brain extracts taken from mice during a six-dose course of Semple vaccine contained no detectable interferon; protection by vaccine appeared to be related entirely to circulating antibody which appeared in increasing amounts after the 4th day after inoculation. Antibody levels in the brain tissue of immunized mice were minimal (Fig. 1). Similar results were obtained when the experiments were repeated with Fermi vaccine containing $10^{2\cdot7}$ LD 50 of residual live virus.

In hamsters

The rabies vaccines tested in hamsters neither induced interferon nor conferred short term protection. Poly I.C. induced substantial amounts of circulating interferon in hamsters but interferon titres in brain extracts were much lower. In both sites, however, interferon increased after the second and third dose of Poly I.C. Fifty per cent of the animals treated with Poly I.C. survived a challenge which killed 90 % of the controls (Table 2).

Auto-interference

When CVS rabies was diluted in either vaccine or buffer and titrated in mice, similar median lethal end-points were obtained. The rabies vaccines did not demonstrably inhibit the replication of homologous live virus in mice whether intracerebral or intramuscular routes of inoculation were used, suggesting that direct interference is an unlikely mode of action for protection by vaccine (Koprowski, Black & Nelsen, 1954; Mitchell, Everest & Anderson, 1971).

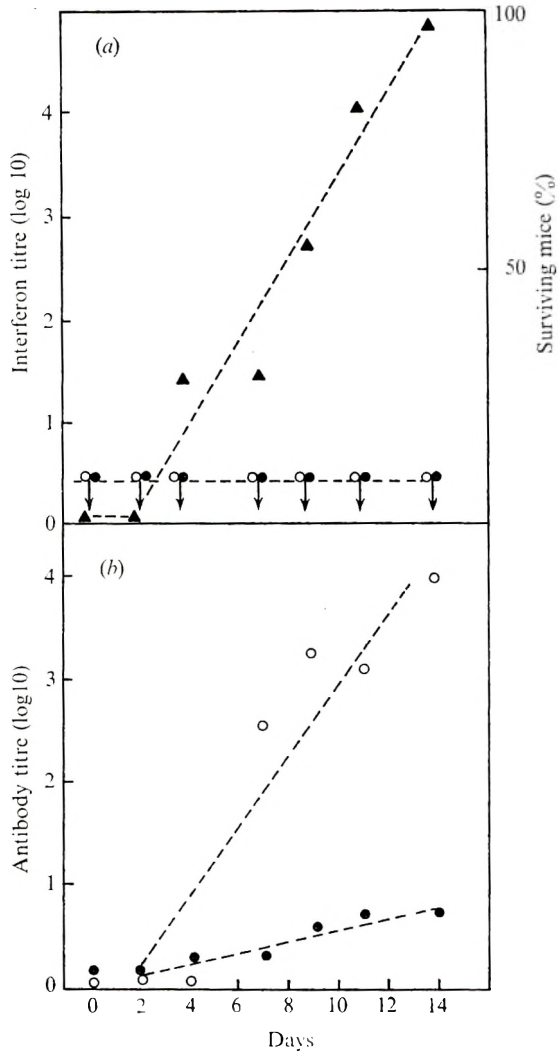

Fig. 1. Protection, antibody response and interferon in mice immunized with rabies vaccine. Groups of 20 mice were inoculated with 0·25 ml. of Semple vaccine diluted 1/10 on days 0, 2, 4, 7, 9 and 11; 10 mice challenged intracerebrally with 90 LD 50 CVS and 10 sampled for interferon or antibody on days 0, 2, 4, 7, 9, 11, 14. (*a*) Surviving mice ▲- - ▲; interferon in brains ●- -●; interferon in serum ○- -○. (*b*) Neutralizing antibody in brains ●- -●; neutralizing antibody in serum ○- -○.

DISCUSSION

The rabies vaccines were tested under conditions and in animals in which interferon is readily induced by Poly I.C. None of the vaccines tested, however, induced either detectable interferon or immediate protection in any of the animals. The virus content of several of the vaccines was similar to that of Newcastle disease virus used for interferon induction by Atanasiu *et al.* (1970). The vaccines were completely or partially inactivated by phenol, β-propiolactone or UV irradiation; the latter method at least is compatible with the retention of interferon-inducing properties. Although other live or killed viruses induce interferon in animals,

Table 2. *Effect of rabies vaccines and Poly I.C. on interferon induction and protection against rabies in hamsters*

Vaccine or inducer*	Interferon† in Serum			Brain			Dead/ challenged
Semple	< 5	< 5	< 5	< 5	< 5	< 5	8/10
Tissue culture	< 5	< 5	< 5	< 5	< 5	< 5	10/10
Poly I.C.	60	87	360	< 5	17	22	5/10
Control	—	< 5	—	—	< 5	—	9/10

* Vaccine or inducer (0·2 ml. ip) given 24 hr. before, at the same time as and 24 hr. after challenge.

† Interferon titres in the sera and brain extracts of pairs of unchallenged hamsters 4 hr. after each dose.

detectable interferon induction by rabies apparently occurs only when large amounts of infective virus are present and is usually highest in the brain just before death (Matsumoto, 1970; Stewart & Sulkin, 1966; Karakuyumchan & Bektenerova, 1968). The live virus in Fermi vaccine apparently neither replicates sufficiently after peripheral inoculation nor is intrinsically enough to induce interferon.

Mice have been protected against several other viral encephalitides by interferon (Field *et al.* 1967; Baron, Buckler, Friedman & McCluskey, 1966; Finter, 1966; Haahr, 1971). Despite the presence of abundant circulating interferon induced by different methods most workers have failed to significantly protect mice against rabies (Baron & Habel, 1967; Atanasiu *et al.* 1970; Soave, 1968; Finter, 1967; Fayaz, Afshar & Bahmanyar, 1970; Hilleman, 1970). Mice are good indicators of the immune response to rabies vaccines but why they are unsatisfactory for testing interferon-mediated protection against rabies is obscure. Little interferon penetrates the central nervous system of mice (Subrahmanyan & Mims, 1966; Finter, 1967). The present results confirm that peak titres of interferon induced in mouse brain are less than 2% of those in their serum, a value only slightly improved by injecting the inducer intracerebrally.

Hamsters, on the contrary, were significantly protected by Poly I.C., despite low titres of brain interferon. Fenje & Postic (1970) also showed that a single dose of Poly I.C. protected rabbits against street virus infection for up to 24 hr., by which time interferon titres in this species have also declined to low values (Cathala & Baron, 1970), (Table 1). The time-limited vulnerability of rabies virus to interferon in some species and the poor penetration of interferon into the central nervous system perhaps indicates that its activity against rabies is exerted in some extraneural cell site. These findings suggest that current concepts of rabies pathogenesis may need reappraisal (Johnson, 1971).

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Anthrax infection in bone meal from various countries of origin

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SUMMARY

Using animal inoculation, three out of six Lebanese and three out of nine Argentinian and two out of two Pakistan separate commercial consignments of bone meal imported during 1970 were found to be infected with anthrax.

INTRODUCTION

It is several years since the danger of anthrax due to imported dried bones from India and Pakistan was first reported by the Chief Inspector of Factories (1939). Consequent upon a decision by a firm of fertilizer manufacturers to cease handling Pakistan bones for safety reasons, the opportunity arose of examining consignments of bone meal from the Lebanon and Argentina for the presence of *Bacillus anthracis*, and also the final Pakistan shipments.

MATERIALS AND METHODS

The test procedure has been detailed in an earlier paper (Davies & Harvey, 1955). Guinea-pigs, protected with gas gangrene and tetanus antitoxin, were inoculated with a suspension of bone meal which had been heated at 65° C. for 5 min. Each consignment tested consisted of two separate samples. From each sample two animals were inoculated.

The presence of anthrax in the inoculated animals was confirmed by post-mortem culture taken from the inoculation site, the spleen and heart blood.

RESULTS

The proportion of bone meal samples found positive for *B. anthracis* is shown in Table 1.

Though the numbers are small, some estimate of the degree of infection of shipments from the various countries was possible. In the infected Lebanese specimens in two instances both samples of the same consignments were positive and once one sample only was positive. In infected Argentine bone meal in no

Table 1. *Isolation of* Bacillus anthracis *in bone meal*

Country of origin	Consignments tested	Consignments positive
Argentine	12	3
Lebanon	6	3
Pakistan	2	2

instance were both specimens from one consignment positive, twice one sample only was positive and once one sample was positive in only one of two animals inoculated. In the Pakistan material, however, both samples of each consignment were positive.

DISCUSSION

Imported bone meal is the only material potentially infected with anthrax to which the general public is exposed. It is also the cause of anthrax among cattle in this country because infected bone meal is incorporated in their feed, high cost greatly limiting its use as a fertilizer in agriculture

There is little published information available concerning anthrax infection in bones and bone meal imported from countries of origin other than India and Pakistan. A positive shipload from Syria has been reported (Davies & Harvey, 1955).

The annual import of dried bones and bone meal into this country was no less than 68,000 tons in 1967 (Lovett & Falconer, 1969). As Pakistan bones constitute more than half of this importation (Veterinary Record, 1969) it would therefore seem impracticable to switch over to other sources of supply owing to world demand. It is now apparent that this alternative would not eliminate the risk of anthrax infection anyway. This depends on whether the bones originated from slaughterhouses or from fallen animals or were a mixture of the two.

The practical difficulties and cost of adequately heat treating all the bone and bone products entering this country would be very great. The total result of this trade is a fair but fluctuating amount of anthrax among cattle, and about ten or fewer human cases of infection reported every year in the United Kingdom. This is the price which has to be paid for this particular import. Human risks might be reduced further by such measures as the issue of warning notices with bone meal used as fertilizer, by the destruction of sacks employed in its conveyance instead of their repeated use and by more widespread immunization of workers at risk.

The difficulties of adequate sampling in the screening of bones and bone meal for *B. anthracis* is implicit in the instance of one Argentine consignment quoted, only one of four inoculated animals yielding a positive result. This irregularity in the distribution of anthrax spores has been noted by us before (Davies & Harvey, 1955). Inherent sampling errors would render it impracticable to select certain heavily infected bone meal batches for heat treatment, or indeed for the monitoring of treated bone as a test of safety. Perhaps a trial might be made of the South African technique (De Kock, Sterne & Robinson, 1940) in which the detection of clostridial spores served as indication that the material originated from fallen animals and might therefore be anthrax infected.

We are grateful to Mr F. N. Wilson for his co-operation, and to Mr A. R. Wood and Mr T. H. Price for their technical assistance.

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Osmotic injury in rapidly thawed T4 bacteriophage

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SUMMARY

Osmotic injury in frozen-thawed T4 phage was caused by the sudden and large fall in the electrolyte concentration of the unfrozen aqueous phase during rapid thawing of frozen samples. In accordance with the classical interpretation of osmotic shock it was found that DNA was quantitatively liberated from T4 phage inactivated by the osmotic injury of rapid thawing.

The degree of inactivation of osmotically shocked T4 phage was temperature dependent, being much increased by lowering the temperature, but was independant of the pH of the suspending medium. The T4Bo osmotic shock-resistant phage was refractory to the osmotic injury of rapid thawing.

INTRODUCTION

Previous studies on the effects of freezing and thawing suspensions of T4 and T4Bo bacteriophages (Greaves, Davies & Steele, 1967; Steele, Davies & Greaves, 1969*a*, *b*) showed that the phage were inactivated by three different mechanisms of injury. These can conveniently be termed 'osmotic injury', 'ionic injury' and 'eutectic injury'.

Osmotic injury to the T4 phage was believed to be responsible for most of the inactivation of rapidly thawed samples. It was avoided by slow thawing of frozen samples. Leibo & Mazur (1969) have also implicated osmotic injury as a cause of inactivation of frozen-thawed T4 phage.

Ionic injury resulted from the dual effects of electrolyte concentration and lowered temperature in frozen samples, while eutectic injury occurred when phage suspensions were frozen to temperatures below the eutectic temperature of the suspending medium.

Ionic and eutectic injuries were dealt with in the earlier studies. It is the purpose of this communication to clarify the correlation between osmotic injury in frozen-thawed phage and the phenomenon of 'osmotic shock' which is demonstrable in unfrozen phage suspensions.

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MATERIALS AND METHODS

The methods of phage preparation and purification, and the techniques used for freezing, thawing and titre determinations were the same as those described previously. Phosphate buffer was added to the suspending media of samples subjected to sub-zero treatments to prevent the ionic injury due to electrolyte concentration (Steele *et al.* 1969*b*).

DNA determinations

DNA determinations were obtained using the ethidium bromide fluorimetric technique of Le Peck & Paoletti (1966). When the dye ethidium bromide (2,7-diamino-9-phenylphenanthridine-10-ethyl bromide) binds to nucleic acids, for a suitably chosen wavelength of fluorescence excitation the intensity of fluorescence emmission increases by a factor of 50-fold to 100-fold. This increase in fluorescence is directly proportional to the nucleic acid concentration and is not affected by the presence of protein.

In this study fluorescence measurements were obtained with an Aminco–Bowman Spectro-fluorimeter with the wavelengths set at λEx. 524 mμ and λEmm. 587 mμ.

A preliminary experiment showed that there was no increase in the fluorescence of ethidium bromide solutions when intact T4 phage were added at a concentration of 2×10^{12} p.f.u./ml. Presumably the ethidium bromide molecule is too large to penetrate the phage head membrane and thus cannot bind to the internal DNA.

T4 phage DNA was prepared and purified by the phenolic extraction procedure (Kaiser, 1960) and standardized in terms of 'phage equivalents DNA/ml.' by colorimetric comparison with intact purified phage of known plaque titre using the diphenylamine reaction (Burton, 1956).

A calibration graph was then constructed by adding known concentrations of purified T4 phage DNA in phosphate buffer (KH_2PO_4-Na_2HPO_4 0·13 M – pH 7) to an equal volume of ethidium bromide solution (10 μg./ml. in phosphate buffer), and plotting the fluorescence readings of the mixtures.

Experimental frozen-thawed or osmotically shocked phage samples were added to an equal volume of ethidium bromide solution (10 μg./ml. in phosphate buffer) and their fluorescence intensities determined. The concentrations of liberated DNA as 'phage equivalents DNA/ml.' were then read off the calibration graph.

RESULTS AND DISCUSSION

Osmotic injury during freezing and thawing

Samples of T4 phage or T4Bo (osmotic-shock-resistant) phage suspended in phosphate-buffered saline (KH_2PO_4-Na_2HPO_4 0·13 M, NaCl 0·15 M – pH 7) were frozen and equilibrated at $-5°$ C. for 10 min. and then cooled at 1° C./min. to temperatures down to $-20°$ C. On reaching the desired temperature the samples were immediately rewarmed either rapidly (450° C./min.) or slowly (5° C./min.) until thawing was complete, and the viable titres determined (Fig. 1).

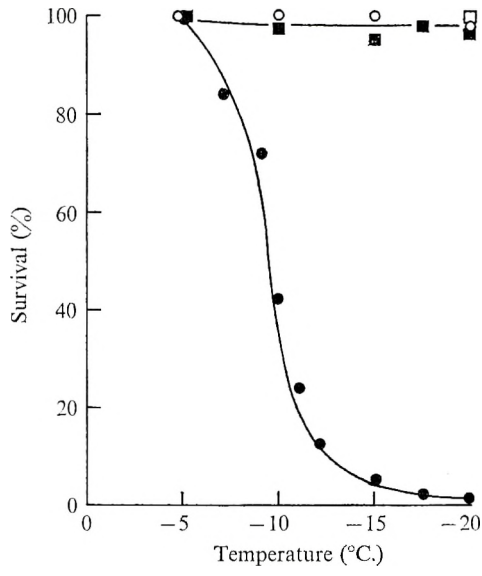

Fig. 1. The percentage survival of frozen-thawed T4 and T4Bo phages suspended in 0·13 M phosphate buffer + 0·15 M-NaCl. Samples were cooled at 1° C./min. ○, T4 phage, slow thaw; ●, T4 phage, rapid thaw; □, T4Bo phage, slow thaw; ■, T4Bo phage, rapid thaw.

The only significant inactivation which occurred was in the T4 phage samples which were rewarmed rapidly. In the classical osmotic inactivation of T4 phage – 'osmotic shock' – first described by Anderson (1953) and later by Leibo & Mazur (1966), suspensions of phage in concentrated salt solutions are rapidly diluted with distilled water. The resulting inactivation of the phage is believed to occur because the diffusion of water into the phage head is more rapid than electrolyte loss. This transient osmotic pressure gradient across the head membrane causes its subsequent rupture. Rapid thawing of frozen samples produces a similar rapid dilution of electrolytes, which are concentrated by freezing.

Effect of pH and temperature

Before identifying the osmotic injury of rapid thawing with osmotic shock, two important factors must be considered. First, there is a pH difference since phosphate-buffered saline becomes acidic during freezing (Van den Berg, 1959) and secondly there is the temperature difference.

Table 1 shows that osmotic shock sensitivity (T4 phage) and osmotic shock-resistance (T4Bo phage) are not affected by the pH of the suspending medium.

The effect of temperature was investigated by rapid 100-fold dilution in distilled water of T4 phage suspended in concentrated NaCl solutions, the NaCl solutions and distilled water being maintained at 25° C. or 4° C. (Fig. 2). There was a considerable decrease in the viability of the samples osmotically shocked at 4° C. compared with 25° C. The experiment was repeated using NH_4Cl suspending media and osmotically shocking the phage at 56° C., 25° C. or 4° C. (Fig. 3). Once again a definite temperature effect was observed. There was little inactivation of the

Table 1. *The effect of pH on osmotic shock and osmotic shock-resistance. Samples were rapidly diluted* 100*-fold from* 3 M*-NaCl at adjusted pH (using* N*-HCl or* N*-NaOH) into distilled water*

pH	T4 phage survival (%)	pH	T4Bo phage survival (%)
3·7	1·3	4·6	102
4·6	1·9	7·0	96
5·5	1·6	7·6	93
6·5	0·7	10·1	95
7·9	1·3	—	—
8·8	0·9	—	—

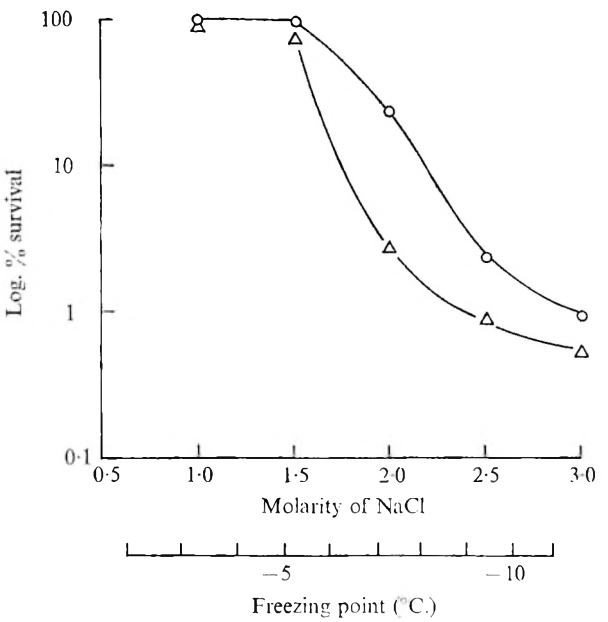

Fig. 2. The effect of temperature on the survival of osmotically shocked T4 phage. Samples were osmotically shocked by a rapid 100-fold dilution from NaCl solutions at the indicated molarity into distilled water. ○, 25° C.; △, 4° C. The lower scale shows the freezing points of the solutions for comparison with Fig. 1.

phage samples osmotically shocked at 56° C. Obviously the osmotic transients last longer and are more severe at lower temperatures owing to the slower rates of diffusion of the ions.

This decreased survival of phage osmotically shocked at lower temperatures explains why the degrees of inactivation produced by osmotic shock at 25° C. and osmotic injury during rapid thawing are very similar for a given salt concentration even though rapid thawing is a slower dilution process than the osmotic shock procedure.

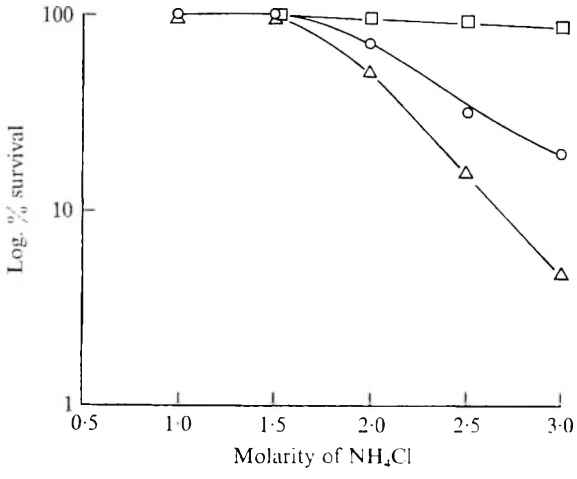

Fig. 3. The effect of temperature on the survival of T4 phage osmotically shocked from NH_4Cl solutions. The procedure was the same as for Fig. 2. □, 56° C.; ○, 25° C.; △, 4° C.

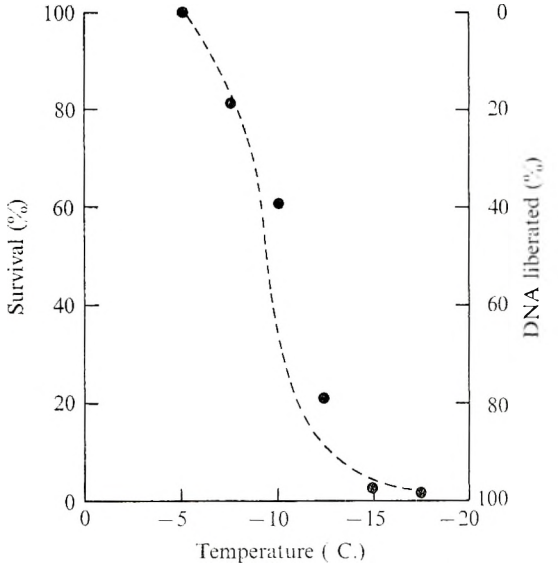

Fig. 4. Samples of T4 phage suspended in 0·13 M phosphate buffer + 0·15 M-NaCl were cooled at 1° C./min. and thawed rapidly. The percentage DNA liberated (●) was compared with the viability as determined by plaque assay (dotted line – taken from Fig. 1).

Liberation of DNA

It has been demonstrated both microscopically (Kleinschmitt, Lang, Jacherts & Zahn, 1962) and chemically (Hershey & Chase, 1952) that DNA is released from the heads of osmotically shocked T-even phage.

Using the ethidium bromide estimation it was found that 99% of the T4 phage DNA was liberated after osmotic shock by rapid 100-fold dilution of a suspension in 3 M-NaCl with distilled water, at 25° C. This exactly correlated with the 1%

viability as determined by plaque assay. Fig. 4 shows the excellent agreement between the viability of rapidly thawed T4 phage and the percentage DNA liberated.

Leibo & Mazur (1966) found that approximately half of their T4B phage inactivated by osmotic shock retained their DNA, as measured by CsCl density gradient profiles. They stored their purified phage stocks in 1·5% nutrient broth, whereas the stocks used in the present experiments were stored in phosphate buffer. We have previously shown (Steele *et al* 1969*a*) that compounds, probably peptides, present in broth media substantially alter the sensitivity of T4 phage to the effects of freezing and thawing. This raises the interesting possibility that peptides adsorbed onto the T4 phage might partially prevent the release of DNA during osmotic shock without altering the degree of inactivation. Such an explanation is especially likely in the light of the results of Panijel, Huppert & Barbu (1957) who showed that the proportion of T2r phage which liberated their DNA during freezing and rapid thawing could be significantly increased by purifying the phage with trypsin (1·5 μg./ml.) thereby removing proteins and peptides adsorbed from their broth medium, although this treatment did not alter the sensitivity of the phage to freezing and thawing as measured by plaque assay.

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Protection of T4 bacteriophage against inactivation during freezing and thawing by addition of peptides

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SUMMARY

Peptides of rabbit globin produced by tryptic digestion, were found to be highly protective against inactivation of freeze-thawed T4 phage. In concentrations of about 10^{-3} M the peptides protected the phage against inactivation by both concentrated NaBr in the unfrozen aqueous phase and the eutectic phase change.

Fractionation of the peptides by G 25 Sephadex showed that peptide concentration rather than peptide size was the more important factor in determining the degree of protection of the phage by electrolyte effects. In contrast, protection against eutectic injury was strongly dependent on peptide size.

Possible mechanisms of action of the protective peptides are discussed.

INTRODUCTION

Peptone additives in concentrations of 5–10% (w/v) have been widely used as protectives against freeze-thawing and freeze-drying injury to micro-organisms and viruses, although the mechanism of the protective effect is unknown. Recently, Steele, Davies & Greaves (1969*a*) showed that separation of a 1% peptone solution on a G 25 Sephadex column yielded some fractions which were highly protective for T4 bacteriophage against freezing injury. The protective compounds were believed to be small peptides. The peptone preparation being used ('Bacteriological Peptone' – Evans Ltd.) was an unspecific papaine digest of muscle and unsuitable for further investigations. What was required was a pure protein which could be split enzymically into peptides of known composition. The amino acid sequence and positions of tryptic cleavage of rabbit globin have been determined by Von Ehrenstein (1966) and Braunitzer, Best, Flamm & Schrank (1966), and the details of G 25 Sephadex separation of the tryptic digest peptides have been published by Hunt, Hunter & Munro (1968). This paper reports the preliminary findings of using globin peptides as protective additives.

MATERIALS AND METHODS

The methods of phage preparation and purification, and the techniques used for freezing, thawing and titre determinations were the same as those described previously (Steele *et al.* 1969*a*, *b*). Percentage survivals were determined by plaque assay.

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Preparation of purified rabbit globin and tryptic digestion

The red cells of 30 ml. rabbit blood were washed six times in saline and then lysed in distilled water. The red cell membranes were removed by centrifugation at 15,000 *g* for 15 min. The haemoglobin solution was then dialysed against distilled water for three days at 4° C. The haem was removed by spraying the dialysed haemoglobin solution into acetone containing 2% (v/v) concentrated HCl at −20° C. with fast magnetic stirring (Rossi-Fanelli, Antonini & Caputo, 1958). The resulting white precipitate of globin was washed in acid-acetone, acetone, acetone-ether 50% (v/v) and ether (twice) and then dried in a current of air.

The globin was dissolved at 10 mg./ml. in distilled water + 2% trypsin (Bovine Pancreatic Trypsin – Sigma & Co., U.S.A.). The globin was precipitated by adding NH_4HCO_3 to a final concentration of 0·1 M, and digestion then carried out for 2 hr. at 37° C. The pH was maintained at 8·2 by addition of 0·1 M-NH_4OH. After digestion the solution was neutralized with acetic acid. Insoluble material was coagulated by freezing and thawing and then removed by centrifugation. The supernatant was freed from volatile salts by triple freeze-drying.

Sephadex fractionation of the globin tryptic digest

Hunt *et al.* (1968) analysed the peptide composition of the fractions obtained by G 25 (fine grade) Sephadex fractionation of globin tryptic digest (G.T.D.). They found that the fractions fell into eleven peaks whose position could be determined by measuring the O.D. at 280 mμ. 250 mg. of G.T.D. was fractionated through the Sephadex column of Hunt *et al.* (160 cm. long, 800 ml. bed-volume) using 1·0 M acetic acid as eluent. The O.D. at 280 mμ was continuously monitored during fractionation by a Uvicord LKB recorder (LKB Produkter, Stockholm, Sweden). The 10 ml. fractions were freeze-dried twice and redissolved in 2·5 ml. distilled water. The relative peptide concentration of the fractions was measured by O.D. at 215 mμ. The optical densities and peak positions are shown in Fig. 2 A. Although the absolute concentration of the peptides was not measured, 250 mg. of digested globin fractionated into 2·5 ml. fractions is equivalent to a maximum concentration of 2×10^{-3} M for each peptide in any fraction.

RESULTS

Protective effect of globin tryptic digest.

Two test systems were used to determine the protective effect of the G.T.D. (i) Protection of the T4 phage against the denaturing effect of electrolytes concentrated during freezing was investigated by adding G.T.D. to T4 phage suspended in 0·1 M-NaBr. The samples were cooled at 1° C./min. to −17·5° C. and thawed slowly (to avoid osmotic injury) – control survival 1%. (ii) Protection against inactivation of the T4 phage at temperatures below the eutectic temperature of the suspending medium was investigated by adding G.T.D. to T4 phage suspended in 0·13 M phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 7). The samples were cooled at 1° C./min. to −45° C. and then thawed slowly – control survival 11%.

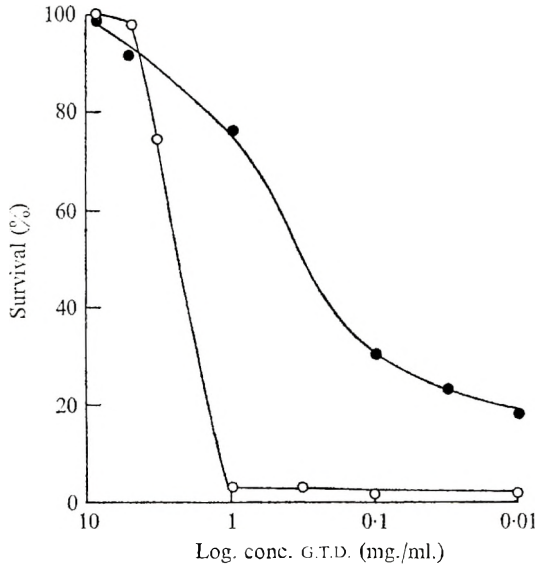

Fig. 1. Protection of T4 phage with globin tryptic digest (G.T.D.) against ionic (○) or eutectic (●) injury during freezing and thawing.

The protective action of different concentrations of G.T.D. against the two types of inactivation are shown in Fig. 1. The graph shows a sigmoid relationship between the G.T.D. concentration and protection of the T4 phage against inactivation below the eutectic temperature ('eutectic injury'), whereas the protective effect against the inactivation by electrolyte concentration ('ionic injury') is curiously discontinuous.

Inhibition of protection by added amino acids

Earlier work with peptone fractions (Steele *et al.* 1969*a*) had shown that fractionation of peptone actually increased the protective action of some of the fractions above that observed for whole unfractionated peptone. The removal of inhibitory compounds by fractionation was suggested as the explanation for this effect. The report also showed that amino acids had no protective effect by themselves and might have been the inhibitors removed from the peptides by fractionation. To test this possibility, pure amino acids were added in concentrations of 0·01 M to T4 phage samples suspended in phosphate buffer containing 5 mg./ml. added G.T.D. The samples were cooled at 1° C./min. to −45° C. and then thawed slowly. The added G.T.D. was omitted in a control series of samples. The results are shown in Table 1. The added amino acids had no significant effect of their own, with the exception of phenylalanine which lowered survival, but all of them considerably inhibited the protective action of the G.T.D.

Protective effect of Sephadex fractions

The Sephadex fractions were tested for protective effect against ionic injury and eutectic injury in the same way as was used to test the whole digest (Figs. 2B, C).

Many of the fractions were highly protective against ionic injury, the degree of

Table 1. *The inhibitory effect of amino acids when added to T4 phage frozen with 5 mg./ml. added globin tryptic digest* (G.T.D.). *The samples were cooled at 1° C./min. to −45° C. and thawed slowly*

	Survival (%)	
Added amino acid 0·01 M	0·13 M buffer	0·13 M buffer + 5 mg./ml. G.T.D.
None (control)	12	90
Glycine	8	51
Alanine	11	29
Valine	8	20
Leucine	4	18
Iso-leucine	6	18
Serine	11	49
Threonine	13	27
Methionine	6	18
Phenylalanine	0·6	18
Tryptophane	10	35
Histidine	11	37
Arginine	14	33
Lysine	24	36
Aspartic acid	12	35
Glutamic acid	12	35
Proline	—	19

protection appearing to depend on the relative peptide concentration (O.D. 215 mμ) rather than on the peptide size (fraction number).

In contrast the protection against eutectic injury increased with increasing fraction number, indicating that smaller peptides gave greater protection. The peaks of protection also matched fairly well with the positions of the peptide peaks. Table 1 showed that unprotective amino acids blocked the effect of the larger protective peptide molecules. By analogy it is possible that small peptides exert a dominant action over larger peptides. On the basis of this consideration a plot was made, using the data of Hunt *et al.* (1968), of the smallest peptide of each peak (as measured in amino acid units) against the maximal survival associated with each peak (Fig. 3). This showed a possible correlation with peptide length and indicated that maximal protection was associated with di- or tri-peptides.

Protection by dilysine and trilysine

The peptide bonds split by tryptic digestion have a C-terminal basic peptide of arginine or lysine. Since Fig. 3 indicated that di- or tri-peptides might give the best protection against inactivation of T4 phage by eutectic injury, a tryptic digest of polylysine was prepared as this results in an equal mixture (by weight) of dilysine and trilysine (Waley & Watson, 1953).

Polylysine with an average molecular weight of 175,000 (Sigma & Co., U.S.A.) was dissolved at 10 mg./ml. in 0·1 M-NH_4HCO_3 and digested for 2 hr. at 26° C. with 0·2 mg./ml. added trypsin. The resulting digest was freeze-dried twice. Paper chromatography confirmed that it contained roughly equal proportions of dilysine and trilysine but was essentially free from lysine.

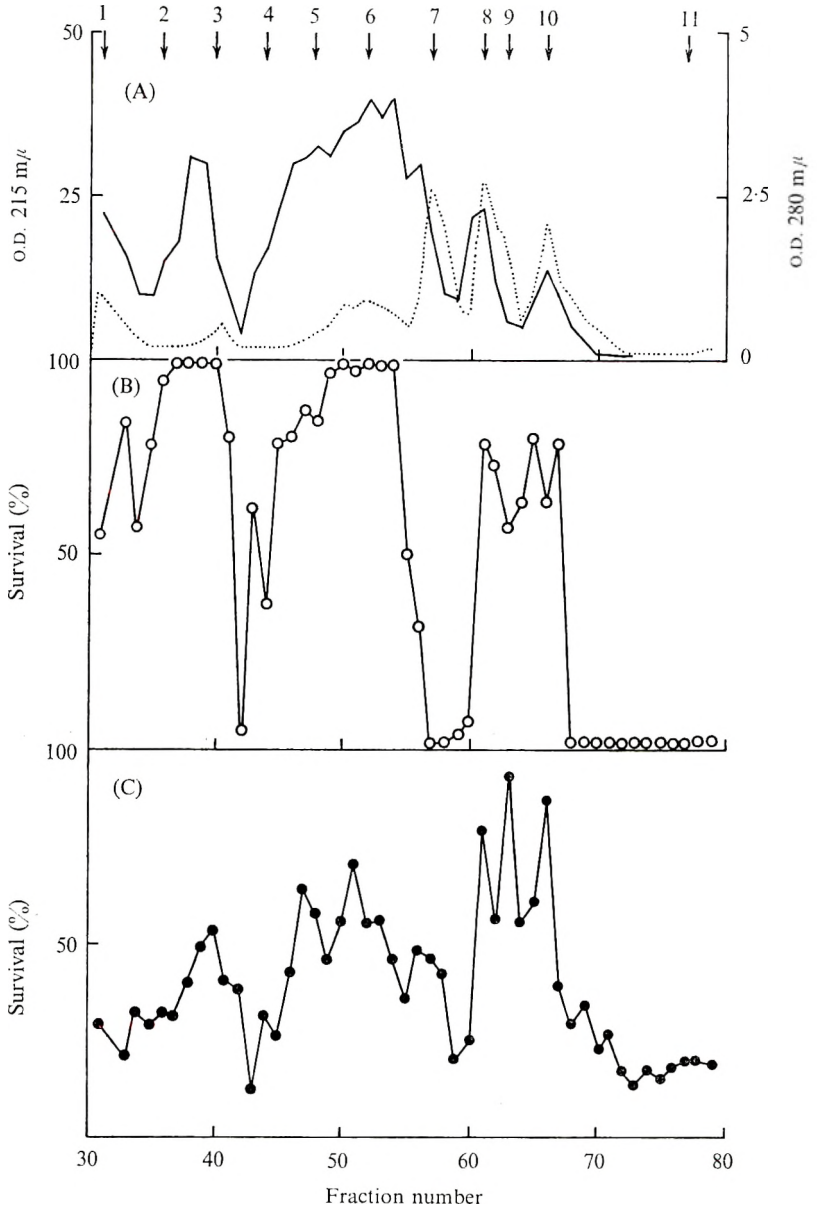

Fig. 2. The protective effect of G 25 Sephadex fractions of globin tryptic digest on the viability of freeze-thawed T 4 phage.

(A) The optical densities of the fractions at 280 mμ (dotted line) and 215 mμ (solid line). The positions of the peptide peaks analysed by Hunt *et al.* (1968) are also shown.

(B) Protection against ionic injury. Samples of T4 phage suspended in 0·1 M-NaBr + fraction were cooled at 1° C./min. to − 17·5° C. and then thawed slowly. The control survival (no added fraction) was 1 %.

(C) Protection against eutectic injury. Samples of T4 phage suspended in 0·13 M phosphate buffer + fraction were cooled at 1° C./min. to − 45° C. and then thawed slowly. The control survival (no added fraction) was 11 %.

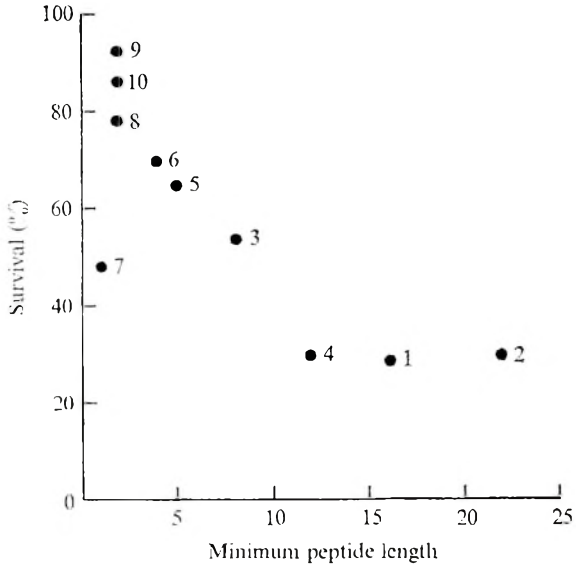

Fig. 3. A possible correlation between peptide length (in amino acid units) and protection of T4 phage against eutectic injury during freezing and thawing. The peaks of protection of Fig. 2(C) are shown as a function of the shortest peptide present in the respective fraction (data from Hunt *et al.* 1968).

The digest was added at 1 mg./ml. (equivalent to $1{\cdot}5 \times 10^{-3}$ trilysine $+ 2 \times 10^{-3}$ dilysine) to T4 phage suspended in 0·13 M phosphate buffer. Samples were cooled at 1° C./min. to $-45°$ C. and then thawed slowly. There was 100% survival of these protected samples compared with the control survival of 11% in samples in which the polylysine digest was omitted.

The digest was also tested for protective activity against ionic injury: 1 mg/ml. of the digest was added to T4 phage suspended in 0·1 M-NaBr. Samples were cooled at 1° C./min. to $-17{\cdot}5°$ C. and then thawed slowly. There was 62% survival of these protected samples compared with the control (no added digest) survival of 1%.

Undigested polylysine at a concentration of 2 mg./ml. gave no protection against ionic or eutectic injury, when tested in the same way as the tryptic digest.

DISCUSSION

The mechanism by which strong solutions of electrolytes denature proteins is still a matter of controversy. The degrees of denaturation produced by different salts is highly dependent on the species of ions, and in the present context the mechanism of inactivation of T4 phage by high electrolyte concentrations is called 'ionic injury' for simplicity and convenience. The protective effect of peptides against ionic injury was extremely powerful, and their mode of action is therefore of considerable interest. If ionic injury is due to the effects of ions on the solvent properties of water, especially in regard to the hydrophobic interactions which stabilize the native protein configurations (Von Hippel & Schleich, 1969), it is difficult to believe that such low concentrations of added peptides (10^{-3} M)

could neutralize the effect of a 100-fold greater concentration of NaBr in the test system. This leaves the alternative explanation that the peptides protected through an interaction with the phage proteins, which would at first seem improbable since no correlation was observed between peptide size and protective effect. However, it is well known that molecules strongly bound to proteins (e.g. substrate added to an enzyme) give good protection against denaturation. Tanford (1968) shows that even one molecule strongly bound to a protein molecule might decrease the rate of denaturation by a factor of 1000-fold. Hence peptide molecules which bind to phage proteins could protect against denaturation whatever their length.

In contrast, the results indicated that the protection afforded by peptides against eutectic injury was dependent on peptide size (as measured by amino acid units and Sephadex filtration), smaller peptides (e.g. dilysine and trilysine) giving the best protection. When the eutectic phase transition occurs, the suspended T4 phage particles are suddenly transferred from a liquid phase to a solid phase and inactivation results ('eutectic injury'). The mechanism of eutectic injury is unknown but perhaps it is caused by removal as ice of water essential for the native protein configurations. On such a hypothesis the protective peptides could block the removal of protein-bound water from labile sites by an interaction with the protein, or alternatively they might stabilize the proteins against conformational alterations even if some essential structural water were removed. Such a hypothesis would explain the dependence on peptide size since only those peptides which bind to the labile sites could be protective. Also peptides which protect the phage against eutectic injury should protect against ionic injury as well, although the converse would not be true. This consideration is in fact well shown in Fig. 2.

I would like to thank Professor R. I. N. Greaves and Dr J. D. Davies for their advice and encouragement during this work. I would also like to thank Dr A. Munro and his colleagues at the Biochemistry Department, Cambridge, for their help and advice with the Sephadex fractionation procedures and for making their analytical data available to me. This work was supported in part by a grant from the Medical Research Council.

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IgG, IgA and IgM responses in acute rubella determined by the immunofluorescent technique

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SUMMARY

The indirect immunofluorescent technique has been used to study the specific immunoglobulin responses in twelve adult cases of acute uncomplicated rubella. IgG, IgA and IgM antibodies increased virtually simultaneously. IgG antibody persisted throughout the period of study but showed a slight tendency to fall in titre after 7 months. IgM antibody was detected in nine cases. In these patients it was present in high titre 5–15 days after the rash but was not detected after 20 days. IgA antibody was detected in all cases. It was present in high titre 5–20 days after the rash but was no longer detectable after 29 days except in one patient who had a very low titre at 78 days. The presence of specific IgA and IgM indicates recent rubella in uncomplicated cases, and if the immunofluorescent method is used both types of antibody should be sought.

INTRODUCTION

A rise in antibody titre detected by haemagglutination-inhibition (HAI), neutralization or complement fixation is the most satisfactory serological proof of the diagnosis of acute rubella. Unfortunately this is often impossible to demonstrate in those cases which present more than 4 to 5 days after the onset of the rash, when the initial rise in titre has already occurred. In such patients, and in many symptomless contacts, only serum of high titre may be available. The importance of confirming rubella in a woman who has developed a rash in early pregnancy has led many workers to devise tests which would identify antibody formed in response to acute infection and distinguish it from antibody resulting from infection in the more distant past.

Schluederberg (1965), studying the serological response to acute infection with mumps, measles and Coxsackievirus, showed that the initial antibody contained

a proportion of IgM which was not detectable in late convalescent sera taken after several weeks, nor in sera from healthy adults with no history of recent infection.

Subsequent workers have shown that a temporary IgM response occurs also in rubella and that the presence of rubella-specific IgM indicates recent infection. Banatvala *et al.* (1967) and Best, Banatvala & Watson (1969) demonstrated rubella-specific IgM in early convalescent sera by observing a fall in HAI titre after treatment with 2-mercaptoethanol which reduces IgM. Vesikari & Vaheri (1968), Best *et al.* (1969) and Desmyter, South & Rawls (1971), demonstrated HAI activity in the IgM-containing fractions obtained from early convalescent sera by centrifugation on sucrose density gradients. The indirect fluorescent antibody technique has been used to study immunoglobulin responses in acute rubella by Baublis & Brown (1968), Cohen, Ducharme, Carpenter & Deibel (1968) and Haire & Hadden (1970), all of whom showed that the IgM response was temporary in uncomplicated cases. The presence of HAI activity has also been demonstrated in the IgM fractions obtained from early convalescent sera by gel filtration through Sephadex G-200 (Gupta, Peterson, Stout & Murphy, 1971) and agarose (Bürgin-Wolff, Hernandez & Just, 1971). More recently Ogra *et al.* (1971) have combined density gradient centrifugation with the technique of radio-immunodiffusion in order to measure the immunoglobulin responses in acute rubella and following the administration of attenuated rubella vaccine.

The evidence from several techniques indicates that IgG and IgM antibodies in acute rubella appear rapidly within a few days of the rash and reach their highest titres within 1 or 2 weeks. IgG antibody persists, but IgM rapidly disappears and its detection after more than a month becomes increasingly unlikely in uncomplicated cases.

Virus-specific IgA was demonstrated in acute rubella by Baublis & Brown (1968) and was still present 22 days after the rash, but no subsequent sera were tested. Bürgin-Wolff and her colleagues showed that IgA followed the same course as IgM in acute rubella and was likely to be useful as an index of recent infection. However, Ogra and his colleagues showed that IgA appeared later and increased more slowly than IgG and IgM and that it persisted without appreciable loss for at least a year.

We have used the indirect immunofluorescent technique, based largely on the methods of Haire & Hadden (1970), to study the specific IgG, IgA and IgM levels in patients with acute rubella and in patients with no recent history of the disease. Because of the demonstration by Fraser, Shirodaria & Stanford (1971) that the presence of rheumatoid factor with anti-IgG activity can cause apparent staining of IgM by the fluorescent method we have also studied a small number of patients with rheumatoid arthritis.

MATERIALS AND METHODS

Patients with acute rubella

An outbreak of rubella affecting eleven university students aged 19 to 22 years occurred in February and March, 1971. Ten students were male, nine of whom lived in the same hall of residence. Prodromal symptoms were more severe than is usual in rubella and included headache, malaise, running nose, sore throat, pain on movement of the eyes, conjunctivitis and aches in the joints and limbs. They were followed after 1–5 days by the appearance of the rash which was finely maculopapular or erythematous and involved the face, trunk, arms and legs. Most patients had enlargement of the lymph nodes, particularly in the occipital and cervical groups, which persisted in some cases for 7 days after the appearance of the rash. Of the seven patients who were seen within 3 days of the onset of the rash all had rises in HAI titre of at least four-fold. Rubella virus was isolated from the throats of four of these. Sera from the first four cases were tested for liver function and for the presence of heterophile antibody, but showed no abnormality.

Twenty-three specimens of serum were taken from the eleven cases at times ranging from the day of the rash to the twenty-ninth day thereafter. Twenty-one further specimens were taken between 45 and 256 days after the rash.

Nine specimens of serum were also taken from a medical colleague who developed rubella, at times ranging from two to 153 days after the rash.

None of these patients with acute rubella possessed rheumatoid factor detectable by the latex agglutination test.

Patients with no history of recent rubella

Serum samples were obtained from 30 pregnant women who were attending the antenatal clinic and were undergoing routine screening for rubella antibody. Sera containing rheumatoid factor were obtained from seven out-patients with rheumatoid arthritis and one patient with suspected lupus erythematosus. The ages of these eight patients ranged from 37 to 75 years.

Absorption of sera

All sera were absorbed at least once with washed BHK21 cells in order to reduce non-specific fluorescence. At a dilution of 1/4 0·8 ml. of serum was absorbed with about 50 million cells at 4° C. overnight. Absorption was particularly necessary for sera with low fluorescent titres. Some sera required two absorptions and a few required three separate absorptions before nonspecific fluorescence had been reduced sufficiently for a final reading of the IgM or IgA titre to be made. Conjugates were absorbed once only, usually at a dilution of 1/10.

Rubella virus

The Judith strain of rubella virus was grown at 35° C. in monolayers of Vero cells in rotating 500 ml. flat glass prescription bottles. Vero cells used for this purpose were grown in medium 199 containing 7% (v/v) fetal calf serum and maintained in the same base with 1% serum. The tissue culture fluid was harvested

4–8 days after infection and yielded virus titres of 10^5 to 10^6 infectious particles per ml. when titrated in RK13 cells.

Cover-slip preparations for fluorescent staining

Cover-slip cultures were made with BHK21 (clone 13) cells and with LLC.MK2 cells. BHK21 cells, free from mycoplasma, were grown in minimum essential medium (as modified by Macpherson & Stoker, 1962) containing 10% (v/v) fetal calf serum and 10% (v/v) tryptose phosphate broth. They were maintained in the same base with 1% serum and 5% tryptose phosphate broth. LLC.MK2 cells were grown in Eagle's basal medium (Grand Island Biological Company) containing 2% (v/v) calf serum and were maintained in the same base without serum.

Fragments of cover-glass measuring 22×5 mm. were inserted into $4 \times \frac{1}{2}$ in. tissue culture tubes to which 1 ml. volumes of growth medium containing between 50,000 and 90,000 cells were then added. The tubes were incubated at 37° C. in a sloped position for 24–48 hr., when they were infected by replacing the growth medium with tissue culture fluid from the Vero bottles diluted with an equal volume of the appropriate maintenance medium. The tubes were re-incubated at 30° C. for 3 days and the medium was replaced by fresh maintenance medium on the day after infection. Uninfected cover-slip cultures were prepared for use as controls. Preliminary experiments showed that incubation of infected cover-slips for 3 days at 30° C. was the optimum combination of time and temperature. Fluorescence was less bright when higher temperatures were used and at 37° C. was often barely visible. After incubation the cover-slips were removed, rinsed in phosphate-buffered saline (PBS), fixed in acetone for 5 min. at room temperature and then allowed to dry in air for at least half an hour. For storage the cover-slips were fixed to microscope slides with adhesive tape so that about 12 mm. of each cover-slip protruded over the edge of the slide. Up to six cover-slip fragments were attached to each slide in this way, in the form of a rake. The slides with cover-slips attached were then stored in airtight polythene containers at -20° C. until required.

Fluorescent staining

Fluorescein-conjugated globulins prepared against individual classes of human immunoglobulin were obtained from commercial sources. The anti-IgG conjugate (Behringwerke*) used for staining LLC.MK2 cell preparations was used at a dilution of 1/80. The conjugates prepared against human IgG, IgA and IgM (Wellcome Reagents Limited) which were used to stain BHK21 cell preparations were used at dilutions of 1/160, 1/25 and 1/15 respectively. A single batch of each type of conjugate was used throughout. The cover-slips, still attached to slides, were stained by applying the reagents to their protruding portions. Serum dilutions were allowed to act for one hr. at 37° C. After three 10 min. washes in separate changes of PBS the conjugate dilution was applied and allowed to act for 45 min. at 37° C. After three more washes the preparations were counterstained in

* Obtained from Hoechst U.K. Limited, Hoechst House, Salisbury Road, Hounslow, Middlesex.

1/100,000 Evans Blue for 20–30 sec., passed briefly through distilled water and allowed to dry in air at 37° C. The cover-slips were finally mounted in glycerol buffered at pH 8·5.

Microscopy

A Reichert Zetopan microscope with a toric lens glycerol-immersion dark-ground condenser was used throughout. LLC.MK2 preparations were illuminated by ultraviolet light from an HBO 200 mercury vapour lamp, using a UG1/1·5 mm. exciter filter and GG13/2 mm. + Wratten 2B barrier filter. BHK21 preparations were illuminated by a 100 watt quartz-halogen bulb, using an interference exciter filter of the type described by Rygaard & Olsen (1969) and a matched OG 530 barrier filter.*

Haemagglutination-inhibition titrations

Sera were inactivated at 56° C. for 30 min., absorbed with kaolin, and titrated in plastic trays by the method in routine use in this laboratory (Thompson & Tobin, 1970). When the endpoint appeared to fall between two doubling dilutions the titre was recorded as the arithmetic mean.

Sucrose density centrifugation

A density gradient was prepared consisting of five layers of sucrose solution, each of 0·9 ml. volume, ranging in concentration from 12·5 to 37·5% (w/v). The gradient was left to stand for 5 hr. at 4° C. A 1/2 dilution of serum was absorbed with chick red cells for at least 1 hr. at 4° C, and 0·5 ml. was layered on the top of the gradient which was then centrifuged overnight at 35,000 rev./min. in the SW 50 rotor of a Beckman 'Spinco L' centrifuge. No absorption with kaolin was done because the non-specific inhibitors, which are β-lipoproteins, remain at the top of the gradient. About 12 fractions consisting of five drops each were collected after piercing the bottom of the tube. The HAI titres of the fractions were determined in microtitre trays, starting with undiluted material. The presence of separate classes of immunoglobulin in the fractions was detected by double diffusion in agar gel, using antisera specific for human IgG, IgA and IgM (Wellcome Reagents Limited).

RESULTS

Microscopic appearances

In positive preparations finely granular cytoplasmic fluorescence was seen in a variable proportion of cells. Nuclear fluorescence was not observed. In serum titrations the number of fluorescent cells diminished with increasing dilution of the serum, as did the brightness of individual cells. The end-point was taken as the last dilution in which specific fluorescence could clearly be seen. In the early stages of this work we used cover-slip cultures of LLC.MK2 cells, because these cells had previously been used successfully by Baublis & Brown (1968). In preparations of

* The interference and barrier filters were obtained from Polaron Equipment Limited, 4 Shakespeare Road, Finchley, London, N3 1XH.

these cells stained for IgG and illuminated by ultraviolet light the fluorescent material occupied a hemispherical position adjacent to the nucleus. In such preparations we obtained titres up to 1024 with early convalescent sera (Table 1). However, we were unable to obtain satisfactory staining of IgM by the use of this system. Considerably better results were obtained by the use of BHK21 cells illuminated by a quartz-halogen lamp. Although the proportion of fluorescent cells appeared to be somewhat less than with LLC.MK2 cells the contrast between the fluorescence and the background was superior. Intracellular fluorescence was more easily observed, finer detail could be seen and higher end-points in titrations were obtained (Table 1). Examples of specific staining of IgG, IgA and IgM using the latter system are shown in Plates 1 and 2. In BHK21 cells stained for IgG the fluorescent material showed a tendency to be concentrated near the nucleus, whereas in preparations stained for IgA and IgM it was usually distributed more uniformly throughout the cytoplasm. However, this difference was not consistently observed in all preparations.

Immunoglobulin responses in patients with acute rubella

The titres of rubella antibody in the IgG, IgA and IgM classes of immunoglobulin in eleven students with acute rubella are shown in Table 1, together with the HAI titres. All three classes of antibody increased virtually simultaneously within three days of the rash. In only one patient in this group (case 1, 2 days after the rash) was IgM detected without IgG, and then only in low titre. IgG antibody persisted throughout the period of study, but in some cases declined after 7 months. HAI antibody followed a similar course. IgA antibody was demonstrated in all patients and reached titres of 128 to 2048 between 5 and 20 days after the rash. It then rapidly declined, and was not detected after 29 days except in case 6 in whom it was still present 78 days after the rash in very low titre. IgM antibody was demonstrated in titres of 64 or more in seven cases, and reached maximum levels 5–15 days after the rash. It then rapidly disappeared and was not detected in sera taken after 15 days. In one patient (case 2) very little specific IgM was detected, and in three patients (cases 3, 6 and 10) none at all. No IgM response was detected in case 6, but no serum was available between the day of onset and the twentieth day thereafter.

The immunoglobulin responses are typified by the results from a colleague who experienced an attack of rubella and from whom it was possible to obtain numerous specimens of serum. These results are shown separately in Table 2. In this case low titres of rubella IgA and IgM were found on the second day in the absence of detectable IgG.

Immunoglobulins in sera from patients with no history of recent rubella

Serum samples were examined from 30 pregnant women who were attending the antenatal clinic and who gave no history of recent rubella or recent contact with the disease. In eleven consecutive sera without rubella antibody (HAI < 20) no specific IgG, IgA or IgM was detected at a serum dilution of 1/4. In 19 consecutive sera with HAI titres ranging from 20 to 480 IgG antibody was detected

Table 1. *Haemagglutination-inhibition and immunoglobulin antibody titres in 44 sera from 11 patients with acute rubella*

Case no.	Days* after onset	HAI titre	Immunoglobulin titre obtained by immunofluorescence: LLC.MK2† preparations IgG	BHK21 preparations‡ IgG	BHK21 preparations‡ IgA	BHK21 preparations‡ IgM
6	0	< 20	< 4	< 4	< 4	< 4
2	1	< 20	< 4	< 4	< 4	< 4
5	1	< 20	< 4	< 4	< 4	< 4
7	1	< 20	< 4	< 4	< 4	< 4
10	1	120	32	128	64	< 4
1	2	< 20	< 4	< 4	< 4	16
9	3	320	64	128	64	64
4	8	1280	16	128	512	512
11	5	2560	256	2048	128	128
10	8	1280	256	4096	512	< 4
9	10	1280	128	2048	512	512
7	11	⩾ 2560	64	2048	2048	128
8	11	⩾ 2560	128	2048	2048	2048
5	13	480	64	2048	512	256
2	14	960	128	1024	1024	8
1	15	320	64	2048	512	1024
3	16	⩾ 2560	256	2048	2048	< 4
6	20	640	128	⩾ 8200	512	< 4
4	26	1280	64	4096	< 4	< 4
7	26	⩾ 2560	256	4096	128	< 4
5	28	1280	128	1024	4	< 4
1	29	960	180	4096	< 4	< 4
3	29	⩾ 2560	256	⩾ 8200	256	< 4
3	45	⩾ 2560	256	1024	< 4	< 4
11	56	320	512	1024	< 4	< 4
10	57	1280	1024	⩾ 8200	< 4	< 4
9	68	320	512	2048	< 4	< 4
8	77	640	512	⩾ 8200	< 4	< 4
6	78	640	512	⩾ 8200	8	< 4
5	79	1280	512	1024	< 4	< 4
2	80	320	256	1024	< 4	< 4
4	84	160	.	512	< 4	< 4
7	84	1280	512	⩾ 8200	< 4	< 4
1	87	⩾ 1280	128	⩾ 8200	< 4	< 4
3	95	1280	512	⩾ 8200	< 4	< 4
9	221	320	.	4096	< 4	< 4
11	227	80	.	1024	< 4	< 4
5	239	320	.	2048	< 4	< 4
2	240	160	.	256	< 4	< 4
6	243	320	.	4096	< 4	< 4
4	244	160	.	256	< 4	< 4
1	246	120	.	512	< 4	< 4
10	247	⩾ 1280	.	1024	< 4	< 4
3	256	640	.	1024	< 4	< 4

* Day 0 = day of onset of rash.

† LLC.MK2 cell preparations were examined by ultraviolet illumination.

‡ BHK21 cell preparations were examined by quartz-halogen illumination.

Table 2. *Serial haemagglutination-inhibition and immunoglobulin antibody titres in a patient with acute rubella*

Days after onset	HAI	Immunoglobulin titre obtained by immunofluorescence on BHK21 cell preparations		
		IgG	IgA	IgM
2	20	< 4	16	8
4	160	512	128	128
6	960	1024	256	128
10	1280	1024	512	512
12	960	2048	256	512
20	640	2048	64	16
33	960	2048	< 4	< 4
75	960	2048	< 4	< 4
153	480	2048	< 4	< 4

by immunofluorescence in all cases in titres ranging from 8 to 2048. The number of cases in this group was too small for any consistent relationship to be detected between the HAI and IgG titres. In 18 cases in this group no IgA or IgM was detected at a serum dilution of 1/4. One patient with an HAI titre of 120 and no detectable IgM showed IgA staining at a dilution of 1/4, which was not removed after three successive absorptions with BHK21 cells.

Centrifugation on sucrose density gradients

Because of the failure to detect specific IgM in cases 3 (day 16) and 10 (day 8) and the low titre in case 2 (day 14) we centrifuged these three sera on sucrose density gradients together with three other sera which showed high titres of IgM by the fluorescent method (case 1, day 15; case 8, day 11; case 9, day 10). In all six sera rubella HAI activity was detected in similar amounts in the IgM-containing fractions. The three sera in which the fluorescent method had detected little or no specific IgM in fact contained as much IgM as those which had shown high fluorescent titres. The fractions were then tested for the presence of rubella-specific immunoglobulin by the fluorescent method. The fractions were applied undiluted for 1 hr. to infected BHK21 cover-slip preparations which were then washed with PBS and stained with conjugates in the normal manner. All six sera gave similar results and the findings from two sera are shown in Tables 3 and 4.

IgM antibody was detected by immunofluorescence in the heavy fractions from all six sera, and there was very little overlap with IgG which was present in the lighter fractions. IgA antibody was present in the IgG-containing fractions, but was also detected in lesser amounts in the IgM-containing fractions, although the latter fractions showed no detectable IgA by gel diffusion.

Competition between IgM and other immunoglobulins

In three cases the fluorescent method had failed to detect specific IgM in unseparated serum but had demonstrated it successfully in the heavy fractions obtained by ultracentrifugation. We therefore tried to detect blocking of IgM

Table 3. *Rubella antibodies in serum fractions obtained by centrifugation on a sucrose density gradient*

Patient no. 10, 8 days after rash

Fraction no.	HAI titre of fraction	Immunoglobulin detected in fraction by gel diffusion			Rubella-specific immunoglobulin detected in fraction by immunofluorescence		
		IgG	IgA	IgM	IgG	IgA	IgM
1	4	−	−	+	−	+	+ +
2	4	−	−	+	−	+	+ +†
3	4	−	−	+	−	+ +	+ +
4	8	tr*	+	−	+	+ + +	+
5	32	+	+	−	+ +	+ +	−
6	⩾ 64	+	+	−	+ +	+ +	−
7	⩾ 64	+	+	−	+ +	+ +	−
8	16	+	+	−	+ +	+	−
9	4	tr	−	−	+ +	+	−
10	8	−	−	−	+	tr	−
11	32	−	−	−	.	.	.
Titre in unseparated serum	1280				4096	512	< 4

* tr = Trace.
† Fluorescent titre of this fraction = 32.

Table 4. *Rubella antibodies in serum fractions obtained by centrifugation on a sucrose density gradient*

Patient no. 8, 11 days after rash

Fraction no.	HAI titre of fraction	Immunoglobulin detected in fraction by gel diffusion			Rubella-specific immunoglobulin detected in fraction by immunofluorescence		
		IgG	IgA	IgM	IgG	IgA	IgM
1	4	−	−	−	−	tr	+ +
2	8	−	−	tr	−	+	+ + +
3	32	−	−	+	−	+	+ + +†
4	8	−	−	tr	−	+	+ +
5	4	−	−	−	tr	+ +	+
6	16	+	+	−	+ +	+ + +	−
7	32	+	+	−	+ +	+ +	−
8	⩾ 64	+	+	−	+ +	+	−
9	⩾ 64	+	+	−	+ + +	+	−
10	⩾ 64	+	+	−	+ + +	+	−
11	4	tr*	−	−	.	.	.
12	2	−	−	−	.	.	.
Titre in unseparated serum	⩾ 2560				2048	2048	2048

* tr = Trace.
† Fluorescent titre of this fraction = 64.

Table 5. *Fluorescent staining reactions in rubella-infected BHK21 preparations treated with immunoglobulin-containing fractions applied together or sequentially and stained with anti-IgM conjugate*

Case no.	Days after rash	Fraction (or mixture of fractions) used for staining: IgG + IgA + IgM together	IgG + IgA together, followed by IgM	IgM alone
3	16	tr*	+	+ +
2	14	tr	+ +	+ +
10	8	+	+	+ +
1	15	+	+	+ + +
8	11	+	+ +	+ +
9	10	+	+ +	+ +

* tr = Trace.

antibody by IgG or IgA which may have been competing for the same antigenic sites. Coverslips were stained with separate fractions, applied in mixtures or sequentially, from the six sera which had been centrifuged. For each serum one cover-slip was treated with a mixture of fractions designed to include IgG, IgA and IgM in approximately equal amounts. A second cover-slip was treated with a mixture of IgG and IgA, washed, and then treated with IgM alone. A third cover-slip was treated with IgM only. The final dilution of each fraction was kept constant in the staining mixtures by appropriate dilution with PBS. All cover-slips were finally stained with anti-IgM conjugate. The results are shown in Table 5. As is usual in blocking experiments the results were not clear-cut. Nevertheless, there was a marked reduction in the intensity of staining of IgM when IgG, IgA and IgM were applied together, as compared with control preparations which were treated with IgM alone (contaminated only with small amounts of IgA). This reduction in the intensity of staining either did not occur, or was less marked, when treatment with IgG and IgA together was followed in a separate stage by treatment with IgM. In a corresponding experiment in which cover-slips treated with IgG, IgA and IgM together were then stained with anti-IgG and anti-IgA conjugates, no evidence of blocking of IgG or IgA by IgM was obtained.

Immunoglobulins in sera containing rheumatoid factor

Sera were examined from seven patients with rheumatoid arthritis and one patient with suspected lupus erythematosus. None of the patients gave any history of recent rubella or recent contact with the disease. All eight sera contained rheumatoid factor, with high titres in the sensitized red cell agglutination test or the latex agglutination test or both. All contained rubella antibody from past infection, with HAI titres from 40 to 480 and fluorescent IgG titres from 64 to 2048. Only one serum failed to show staining when tested for rubella-specific IgA and IgM. Three sera showed apparent rubella IgM titres of 32 but no detectable IgA at a dilution of 1/4. The remaining four sera showed apparent IgM titres from 64 to 256 and IgA titres from 16 to 64.

DISCUSSION

Our results by the fluorescent method confirm that the IgM response is temporary in acute rubella and that if only a single serum is available the presence in it of specific IgM should indicate recent infection. Our results also confirm the findings of Bürgin-Wolff and her colleagues by showing that IgA antibody follows a course similar to that of IgM and may be equally valuable as an index of recent infection. Our results differ, however, from those of Ogra and his colleagues who used density gradient centrifugation followed by radio-immunodiffusion and found that IgA appeared in only about 50 % of children with acute rubella but persisted for at least a year.

In three of our cases little or no specific IgM was detected in whole serum by immunofluorescence although centrifugation on sucrose density gradients showed that it was present. Occasional failure of the fluorescent method has also been reported by Vesikari, Vaheri & Leinikki (1971) who were unable to demonstrate specific IgM by fluorescence in six out of 22 early convalescent sera, although they detected it by centrifugation in all cases. When they examined sera which were taken after the administration of attenuated vaccine and which had HAI titres much lower than those occurring after natural disease, they detected specific IgM by centrifugation in 50 % of cases but could demonstrate only traces of IgM in occasional sera by fluorescence. Vesikari and his colleagues considered the fluorescent method to be less sensitive than the density gradient technique, and attributed their failures to the relative insensitivity of the method and the difficulty of distinguishing weak specific fluorescence from the background. In our work occasional failure to demonstrate IgM by fluorescence could not have been due to insensitivity of the method because the latter was able to detect IgM in the heavy fractions obtained by centrifugation, even after considerable dilution (see Tables 3 and 4). It is possible that failure to detect IgM in the unseparated sera may have been due to blocking of IgM by other immunoglobulins. Competition between IgG and IgM for antigenic sites has been demonstrated by Cohen, Norins & Julian (1967) who studied IgG and IgM antibodies to *Neisseria gonorrhoeae* by the immunofluorescent technique. Our experiments suggest that blocking may occur in the rubella system described here, but there was no conspicuous difference in this respect between the sera in which the fluorescent method failed to detect IgM and those sera in which high fluorescent IgM titres were obtained. Because of the failure to detect specific IgM in some cases it seems advisable, if the fluorescent method is used, to test for IgA in addition.

Although the presence of specific IgM indicates recent infection in uncomplicated acute cases, positive fluorescent staining of IgM may occur in the absence of recent infection if a serum containing virus-specific IgG also contains IgM globulins such as the rheumatoid factor (RF) with anti-IgG activity (Fraser *et al.* 1971). Fraser and his colleagues distinguish between 'primary' staining, which is directly due to virus-specific IgM, and 'secondary' staining which is due to IgM anti-globulins and which can be eliminated by previous treatment of the serum with aggregated IgG.

In seven out of eight sera containing rheumatoid factor we obtained positive IgM staining which we assume is secondary, although we have not treated the sera with aggregated IgG. In four of these specimens we also obtained positive IgA staining. Rheumatoid factor is predominantly IgM, but some anti-IgG activity may occur in other immunoglobulin classes and may have accounted for the IgA staining in these cases. The results of IgM and IgA staining on single specimens of serum should therefore be interpreted with caution, and consideration should be given to possible causes of antiglobulin activity in the serum such as rheumatoid arthritis, infectious mononucleosis and hepatitis. The early convalescent sera studied in this work contained no RF and there seems little doubt that the IgM and IgA-staining in these specimens was primary.

Immunofluorescence is more sensitive than gel diffusion and our results therefore provide some information on the purity of the fractions obtainable by sucrose density gradient centrifugation. The latter method was criticized by Newman, Horta-Barbosa & Sever (1969) who found when studying cord sera from congenitally infected infants that the heavy fractions were consistently contaminated with IgG which they thought might contribute to the HAI activity. In the sera from acute adult cases described here there was negligible overlap between IgG and IgM and no IgG could be detected by fluorescence in the heavier fractions which were richest in IgM. Possibly the globulins in cord sera may behave differently from those in adult sera on centrifugation. However, the IgM-containing fractions did contain IgA, presumably in polymeric form, which was detectable by fluorescence although not by gel diffusion. The work of Bürgin-Wolff and her colleagues shows that IgA may contribute to the HAI activity, but if the IgA response is temporary, as our work indicates, then its presence in the IgM fractions obtained by centrifugation need not interfere with the diagnosis of recent infection.

All methods in current use for the detection of rubella-specific immunoglobulins require specialized apparatus and expertise, the availability of which is likely to determine the choice of technique in any individual laboratory. Each method has certain shortcomings and it is possible that a combination of methods, such as fluorescent staining of serum fractions, might be the most sensitive technique for the study of immunoglobulins in rubella and other viral infections, although such a combination would probably be too laborious for routine use.

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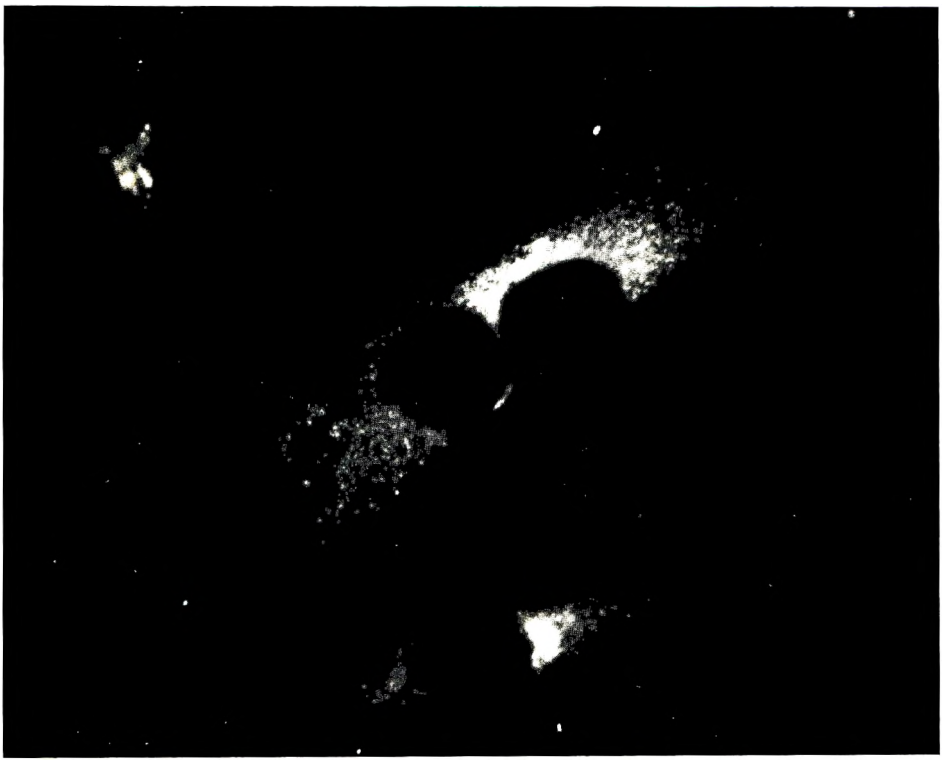

Fig. 1

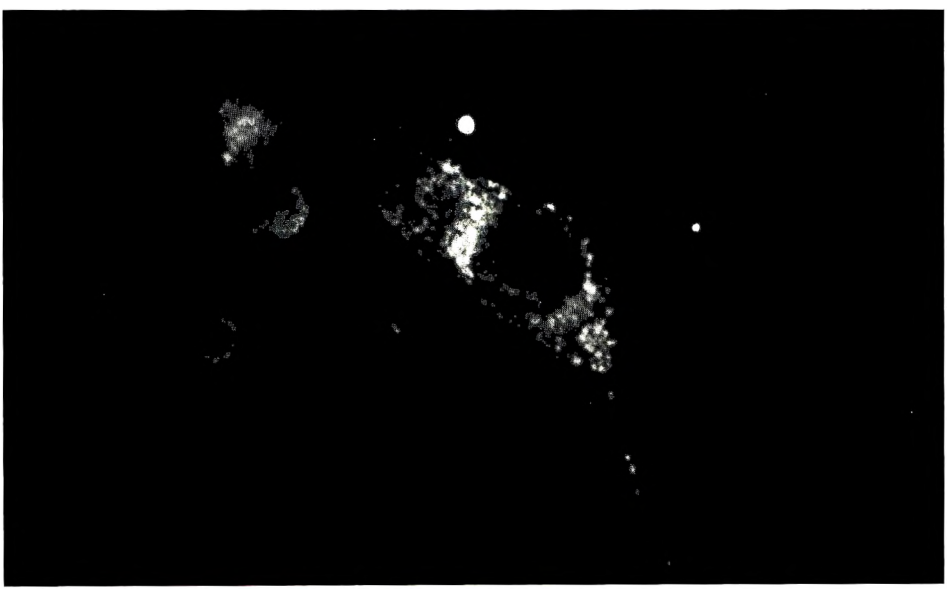

Fig. 2

Fig. 3

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EXPLANATION OF PLATES

Fig. 1. Immunofluorescent staining of rubella-specific IgG. × 900. Serum from case 6, 20 days after the rash, at a dilution of 1 in 16.

Fig. 2. Immunofluorescent staining of rubella-specific IgA. × 750. Serum from case 8, 11 days after the rash, at a dilution of 1 in 20.

Fig. 3. Immunofluorescent staining of rubella-specific IgM. × 750. Serum from case 8, 11 days after the rash, at a dilution of 1 in 20.

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The influence of the route of immunization on the protection of mice infected intracerebrally with *Bordetella pertussis*

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SUMMARY

The development of immunity in mice to *Bordetella pertussis* induced by intracerebral, intravenous or intraperitoneal vaccination was analysed in terms of the viable bacteria in the brain after intracerebral challenge, the serum antibodies, and protection against the sublethal infection of the lung that follows intranasal inoculation.

A vaccine introduced intracerebrally was five to ten times more effective than that given intraperitoneally or intravenously, as measured for each route by the amount of vaccine required to protect half the mice against an intracerebral challenge 14 days later (ImD50). Intracerebral vaccination induced higher antibody titres than vaccination by the other two routes. The survival of infected mice given 1–3 ImD50 doses of vaccine intracerebrally 14 days before, followed a pattern similar to that after intraperitoneal or intravenous vaccination with up to 10 ImD50 of vaccine: the numbers of organisms increased for 3 days and then declined. Injection of about four ImD50 of vaccine intracerebrally produced a local immunity, resulting in an immediate kill of challenge organisms given 14 days later. Such an effect following intraperitoneal vaccination was achieved only against challenges with an avirulent strain. It is suggested that better stimulation of circulating antibody and local immunity in the brain together account for the better protection induced by intracerebral vaccine.

Immunity to an intracerebral infection appears therefore to have at least three components, each specific for pertussis. The first, like that induced by intraperitoneal and intravenous vaccination, reaches a maximum in 2 or 3 weeks and is probably an expression of a general response by the animal operating not earlier than 3 days after infection. The second is a local immunity, appearing after the same interval. The third is a short-lived local immunity which has been described by previous workers; it immediately follows the injection intracerebrally of ten times less vaccine than that needed to protect against a challenge 14 days later and lasts only 2–3 days. The second and third types result in immediate sterilization of the infection.

Mice recovering from sublethal brain infection with avirulent organisms were immune to a second infection with a virulent organism, but this was achieved not by the ability to kill the re-infecting organisms immediately on injection into the brain, but only after the 3–4 days lag such as follows intraperitoneal vaccination.

INTRODUCTION

Throughout this paper, vaccination and challenge routes are referred to by the abbreviations IP (intraperitoneal(ly)); IV (intravenous(ly)); and IC (intracerebral(ly)). The vaccination route is given first and the challenge route second: IP/IC indicates IP vaccination followed by IC challenge.

The protective property of a *Bordetella pertussis* vaccine is assayed in mice, 14 days after a single IP injection of vaccine, by an IC challenge with infecting organisms (Kendrick, Eldering, Dixon & Misner, 1947). Experience has shown that the results are reasonably correlated with those in children in the field (Medical Research Council, 1956). The test, however, is artificial in the sense that *B. pertussis* is not a natural pathogen of mice, and protection against an IC infection has little obvious connexion with the disease in children.

The course of infection in the brains of IC infected mice, unvaccinated and IP vaccinated, was described by Dolby & Standfast (1961); even in mice completely protected by the vaccine, the number of living organisms in the brain increases for 3–4 days before falling. From a comparison of active and passive protection, it seemed to us that the IP vaccine stimulated the production of antibody that reached the brain tissue 3–4 days after infection, when the blood–brain barrier became 'leaky' (Holt, Spasojević, Dolby & Standfast, 1961).

Doubt has been cast on the role of antibody in this mouse protection test by several workers. Evans & Perkins (1954*a*, 1955) and Wardlaw & Jakus (1968) could find no protective circulating antibody from 5 hr. to 14 days after one strongly protective IP dose of vaccine.

Evans & Perkins (1954*b*) and Andersen (1957) used very small IC doses of vaccine to protect mice against IC challenge given immediately or up to 3 days later, suggesting some form of local immunity which may not be mediated by antibody. Evans & Perkins suggested that two types of immunity were operative in the mouse potency assay of vaccine. Blake & Wardlaw (1969), however, quote an experiment of Dr Fischel's in which this short-lived immunity was abolished after body irradiation and have shown that the immunity present one day and 14 days after vaccination IP is inhibited by the immuno-suppressive drug, cyclophosphamide.

The experiments described below were done to compare the course of infection in the mouse brain after IC, IP and IV vaccination. An antigen introduced into the brain tissue leaks into the circulation (Cairns, 1950; Mims, 1960) and so stimulates a general immune response as well as having a local effect.

The general immune response to vaccination was investigated by measuring (i) serum antibodies *in vitro* by assay or by passive protection against sublethal lung infections and (ii) the active protection against a sublethal lung infection, a state that may be independent of circulating antibody.

METHODS

Mice

Four strains of mice (Theiler's Original, Schneider–Webster, ICI and a National Institutes of Health strain, NIH-BS, derived from Webster–Swiss mice) were used, but there was no difference in the results obtained attributable to mouse strain. Male and female mice were used, but only one sex in any experiment.

Vaccination

Mice were vaccinated at 16–17 g. in weight. The ImD 50 values were estimated by the method of Reed & Muench (1938).

The Lister Institute Reference pertussis vaccine, K 278, formalin-killed and resuspended in thiomersal saline at 2×10^{10} organisms/ml., was used in all experiments. This vaccine was about ten times more potent when given IC than IP or IV (Table 2). As a non-specific control, a batch of Lister Institute alcohol-killed TABC vaccine was used.

Vaccine was given IP either neat or diluted in sterile buffered saline in a volume of 0·2 ml.; the large dose of 10^{10} organisms was given in 0·5 ml.

For IC use, 0·03 ml. volumes were injected. The inoculum was concentrated by centrifugation from the thiomersal saline suspension and resuspended in buffered saline. This material was diluted suitably for other routes of injection in experiments where direct comparison was being made.

For IV vaccination, 0·2 ml. volumes were given into the tail vein.

Challenge infections

All mice were infected under ether anaesthesia. For lethal brain infections, strain 18-323, strain 2 atox. from Dr E. K. Andersen (derived from Albany 40103), and strain B 5533 from Dr M. Haire (passaged through mice to make it virulent) were used. For intranasal, sublethal infections, a strain, originally from Glaxo laboratories, mouse-passaged at this Institute and referred to as Gl. 353, was used. Strain L 84, from the Lister Institute collection, was used for sublethal brain infections. Growth was harvested from 20 hr. Bordet-Gengou plates, suspended in 1 % Casamino Acids (Difco), and the suspension first adjusted until equal to an opacity of 10 international units, approximately equivalent to 10^{10} total organisms/ml., of which 10–20 % were viable. Further dilutions were made in 1 % Casamino Acids as required.

Mice were challenged with strain 18-323 in 0·03 ml. volumes containing 50,000 organisms, representing a challenge of about 100 LD 50. Strain L 84 grew in mice, but did not kill them in IC doses up to 10^6 organisms in 0·03 ml., and was used to determine the immunity induced by the cerebral growth of an avirulent strain and the effect of vaccination immunity on a non-virulent strain.

Sublethal lung infections, used to determine the general immune response in contrast to local brain immunity, were established by instilling strain Gl. 353 in 0·04 ml. volumes containing 10,000–50,000 organisms.

Brain and lung counts

Animals were killed with coal gas, and brains or lungs removed aseptically into 2 oz. universal bottles containing 9 ml. Casamino Acids and 2 ml. of 5 mm. diameter glass beads. The bottles were shaken – 3 min. for brains, 10–15 min. for lungs – on a vertical shaker, throw $2\frac{3}{4}$ in., 325 rev./min. The homogenates were diluted tenfold in 1% Casamino Acids and viable counts were estimated by the method of Miles & Misra (1938) on Cohen & Wheeler blood plates (see below); when the count was expected to be low, 0·5 ml. of brain homogenate was spread over one plate.

The Cohen & Wheeler blood plates were made from medium as described by Cohen & Wheeler (1946), but with 0·1% acid casein hydrolysate (Oxoid), 0·5% glutamic acid, 5% blood and 1·3% New Zealand agar.

Passive protection tests; protective index for lung infection

Equal volumes of antiserum and double-strength challenge suspension of strain Gl. 353 were mixed, and 0·04 ml. instilled into the noses of 40 mice. A similar number of control mice received the same challenge dose, without antiserum, at the same time. Ten mice from each group were killed after 2 hr. or 1, 4 or 6 days, and viable counts made of the organisms in the lungs of each mouse. The protective index of the serum is calculated by subtracting the mean viable count of the 40 serum-treated mice from the mean viable count of the 40 control mice; thus the higher the figure, the greater the protection.

In vitro *tests for antibodies*

Agglutinin titrations. Dilutions of serum in saline and living organisms of strain 18–323, freshly harvested from Bordet–Gengou plates into saline at a concentration of 5×10^9 organisms/ml., were incubated in Dreyer tubes at 37° C. for 4 hr.; the results were read after a further 20 hr. at room temperature (*ca.* 18° C.). Eight doubling dilutions of serum were put up, the final dilution in the first tube being 1/40. The degree of agglutination in each tube was given a numerical value: 4, 3, 2, 1 where 4 = complete agglutination and 1 = smallest trace distinguishable from no agglutination. The figures were added together and called the agglutinin rating. It was felt that this rating gave a better evaluation of two sera which gave agglutinations: 4, 4, 4, 3, 0, 0, 0, 0 and 2, 1, 1, 1, 1, 1, 0, 0. The first serum had a rating of 15; the second of 7. The 'titre' of the first serum would usually be regarded as 1/320 and of the second as 1/1280.

Antihaemagglutinin and bactericidal antibody. These were measured as described by Dolby (1965) and Dolby & Vincent (1965).

RESULTS

Comparison of intraperitoneal and intracerebral vaccination

Figures 1–5 illustrate the courses of infection in the brain resulting from an IC challenge 14 days after IC or IP vaccination. Ninety per cent of the organisms, both vaccine and challenge, injected IC are lost very quickly from the brain by

draining away through the cerebrospinal fluid (Cairns, 1950; Mims, 1960). The vaccine 'lost' to the brain is, however, available for antigenic stimulation in the rest of the body. Nevertheless, there is a pronounced difference between the two results.

Titrations showed that, IC/IC, one ImD 50 of vaccine was 1×10^8 organisms with the infection curve shown in Fig. 2; this dose of vaccine had no effect IP/IC and the mice behaved as the uninfected controls in Fig. 1. One ImD 50 of vaccine IP/IC was 1×10^9, or ten times as great, with a similar infection curve (Fig. 4). In these three curves, Figs. 1, 2 and 4, the viable counts rose to the 4th or 5th day; the counts in mice destined to die continued to rise as in the controls, but the counts in recovering mice fell and by the 9th or 10th day surviving mice had sterile brains (Figs. 2, 4).

The course of infection following 10 ImD 50 was quite different with the two routes. In the IC/IC series there was an immediate fall and by the 3rd day most of the brains were sterile (Fig. 3). In the IP/IC series, the curve (Fig. 5) resembled the one ImD 50 dose curves (Figs. 2, 4) and, though all the mice survived, there were no sterile brains before the 6th day.

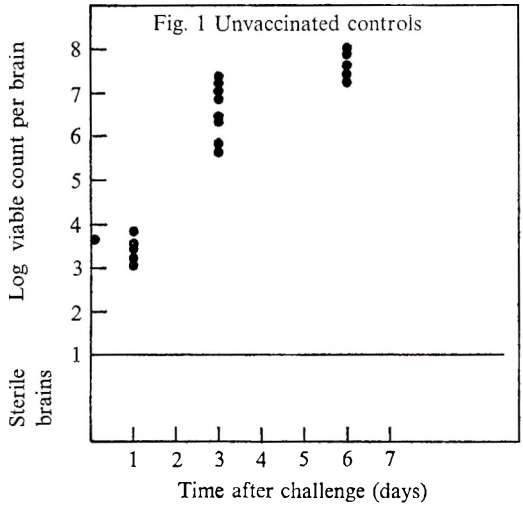
Bacterial growth curves following infection 14 days after IV vaccination were similar to those of IP vaccinated mice. The ImD 50 was a little lower, $0{\cdot}8 \times 10^9$ instead of 1×10^9.

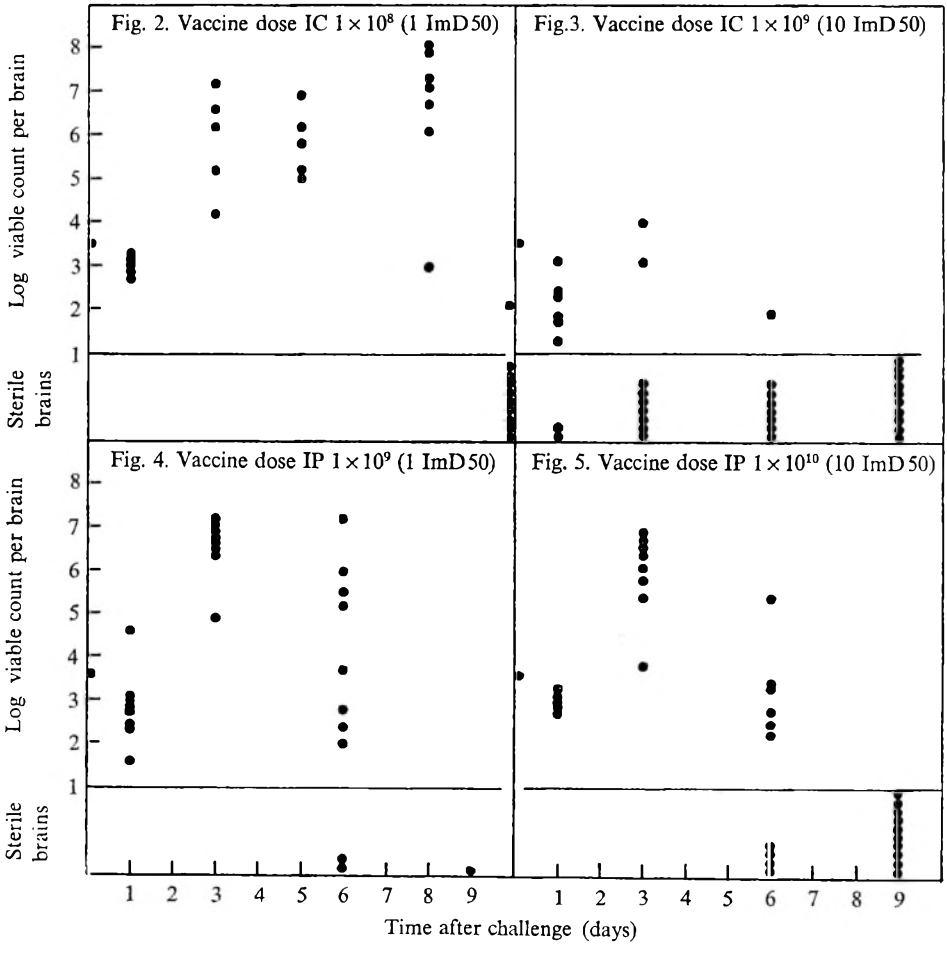
The possibility of a local non-specific effect of vaccine given IC was tested by estimating protection in terms of lowered brain counts following a small dose of TABC vaccine given IC 14 days before the *Bordetella pertussis* challenge. The counts obtained were indistinguishable from the unvaccinated controls in Fig. 1. A second experiment, in which TABC vaccine IC was combined with one ImD 50 pertussis vaccine IP, gave a result similar to Fig. 4. From these experiments we conclude that a measurable local non-specific immunity was not elicited by IC vaccination, or, if it were, it had disappeared by the 14th day when the IC challenge took place.

The pronounced difference between 10 ImD 50 of vaccine IC and IP on the ensuing IC challenge was not therefore non-specific. Although the ImD 50 values differed tenfold, one ImD 50 for each route had the same effect on infection. Figure 6 illustrates that, at about 3 ImD 50, the difference between IC and IP vaccination becomes apparent. The one-day lowering of count in mice given 2·5 ImD 50 by the IC route was extended to 2 days. Increasing the dose of vaccine IC increased the initial bactericidal effect until a curve like Fig. 3 was reached.

The mechanisms of the greater efficacy of intracerebral over intraperitoneal or intravenous vaccination

General immunity. The vaccine introduced into the brain and leaking into the circulation makes an IC vaccination equivalent to an IV injection of perhaps 80% of the IC dose. To determine the general immune response 14 days after IC vaccine, lung counts were made at intervals after a sublethal (intranasal) infection. The animals were also bled out and the serum tested for antibodies. These tests were chosen as indicators of a host response and no assumption is made as to lung protection or positive antibody response being correlated with the protection of mice in the routine assay of potency.





For figure legends 1–5 see opposite page.

The effect of IC vaccine on lung infection is shown in Figs. 7–10. Doses of 10^5 and 10^6 were inactive and the mice behaved like the controls (Fig. 7); 10^7 had a very slight effect (Fig. 8) and 10^8 organisms had a pronounced effect (Fig. 9): 10^9 organisms almost sterilized the lung (Fig. 10). A dose of 10^8 was equivalent to about one ImD 50 IC/IC and affects a brain infection as in Fig. 2. Thus, doses of IC vaccine effective against IC challenges were similarly effective against lung infections.

The effects of a 2×10^8 dose of vaccine by the three routes IP, IC and IV on a sublethal, intranasal infection 14 days later were next compared. In the IP vaccinated mice, the lung counts were only slightly lower than the controls; mice on this dose were not protected against an IC challenge. After IC and IV vaccination the lung infection was suppressed to about the same extent and lung counts were similar to those shown in Fig. 9. Increasing the dose of vaccine to 1×10^9 increased the protection against lung infection so that the IP route was now effective, but the relative efficacies by the three routes were the same as for the smaller dose. In spite of the similarity of lung counts in IV and IC inoculated mice, IV vaccination protected mice against an IC challenge less well than IC vaccination; this is reflected in the ImD 50 values (Table 1). Thus, although IC and IV vaccination were both far more efficient than IP, the IV vaccination route occupied a more intermediate position in protecting mice against an IC challenge.

The antibody response in mice vaccinated by the three routes was measured in groups of five or ten mice 14 days after 10^9 organisms. The sera were tested for antihaemagglutinin, total agglutinins, complement-mediated bactericidal antibody, and potency in protecting mice passively against a sublethal lung infection (Table 1). Vaccine given IC was the most efficient as an antibody stimulus and IP vaccine the worst; IV vaccine was intermediate in two of the antibody tests, but similar to the IP vaccine in the other two. The ImD 50 values for each route of vaccination against an IC challenge fall in the same order as the average figures for antibody assays, with IV vaccine in an intermediate position.

There is no doubt that vaccine inoculated IC stimulates a better response in the

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Figs. 1–5. Viable counts of *Bordetella pertussis* strain 18-323 in the brains of mice vaccinated with large or small doses of pertussis vaccine either IP or IC 14 days before they were challenged with 50,000 organisms (*ca.* 5000 viable). Each point represents one mouse.

Fig. 1. Unvaccinated controls.

Fig. 2. Vaccine dose 1×10^8 organisms (= 1 ImD 50) IC.

Fig. 3. Vaccine dose 1×10^9 organisms (= 10 ImD 50) IC.

Fig. 4. Vaccine dose 1×10^9 organisms (= 1 ImD 50) IP.

Fig. 5. Vaccine dose 1×10^{10} organisms (= 10 ImD 50) IP.

Similar groups of 10 mice were kept for 14 days after challenge to determine the number of mice dying on each vaccine. These were: unvaccinated controls, 10/10 (number dead/total number at risk); vaccine dose 1 ImD 50 IC, 6/10; 10 ImD 50 IC, 1/10; 1 ImD 50 IP, 5/10; 10 ImD 50 IP, 0/10.

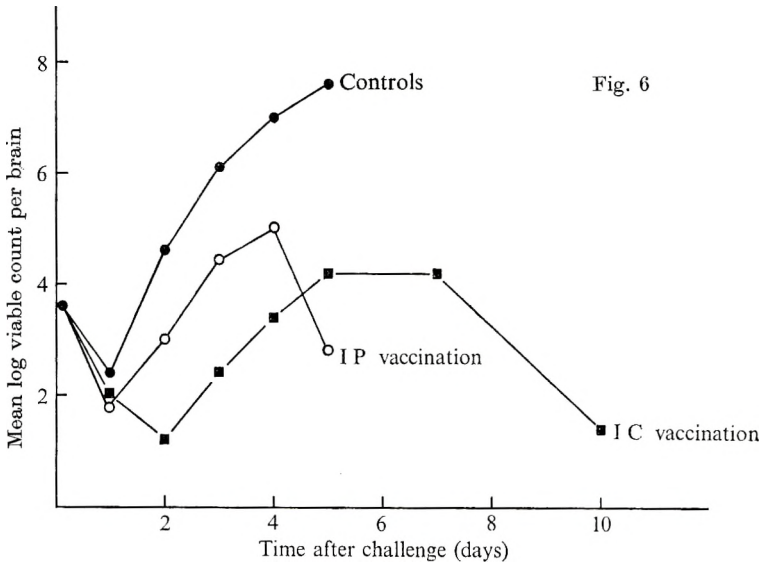


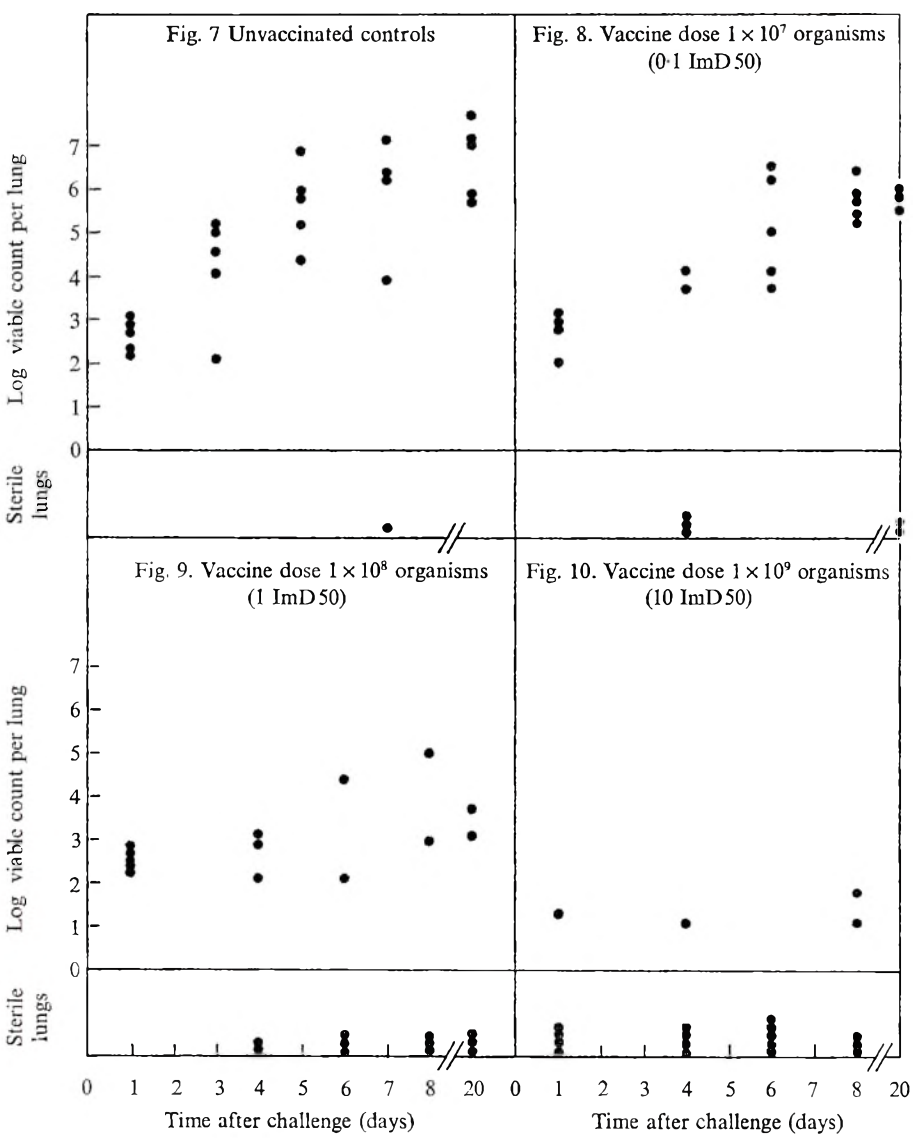
Fig. 6

Fig. 7 Unvaccinated controls

Fig. 8. Vaccine dose 1×10^7 organisms (0·1 ImD50)

Fig. 9. Vaccine dose 1×10^8 organisms (1 ImD50)

Fig. 10. Vaccine dose 1×10^9 organisms (10 ImD50)



body as a whole than vaccine given IP. The question to be considered is whether this alone accounts for the dramatic effect on IC infection.

Local immunity in the brain. We have shown above that one ImD 50 of vaccine IC/IC (10^8 organisms) (Fig. 2) had the same effect on brain infection as one ImD 50 IP/IC (10^9 organisms) (Fig. 4). All the mice were infected, the organisms first grew and were then killed. With larger doses of vaccine, the course of infection diverged in IC and IP vaccinated mice (Fig. 6); with an IC vaccination of 10 ImD 50, which protected all the mice, the brains were sterilized within 3 days or so (Fig. 3), whereas the organisms grew in IP vaccinated mice given 10 ImD 50 for 3–4 days before being killed (Fig. 5).

It is to be expected that the ability of IC vaccine to stimulate a better immune response than IP vaccine would be reflected in differences in ImD 50 in the IC/IC and IP/IC tests; and indeed the characteristic courses of infection after 10 ImD 50 doses of vaccine IC and IP (an immediate decline in numbers IC/IC (Fig. 3) and a decline only after 3 days IP/IC (Fig. 5)) suggest different mechanisms of overcoming the brain infection. Figure 3 suggests an immediate specific local action.

Evans & Perkins (1954*b*), Blyth (1955) and Andersen (1957) demonstrated a type of immunity in the brain, the so-called interference effect of vaccine given 3 hr. to 3 days before challenge. We have confirmed their results. This effect is, however, quite separate from that illustrated in Fig. 3. Doses of IC vaccine between 10^7 and 8×10^7, too small to produce a response in any of the tests we have used or to protect against IC challenge at 14 days, protected mice against a challenge 3 hr. later. This ability had disappeared in 3 days, even with bigger doses of vaccine, to be replaced, provided the dose was above 10^8 organisms, by the usual specific immunity to the challenge as described above.

The effect of a live IC vaccine was next investigated using L 84, an avirulent strain of *Bordetella pertussis* known to establish itself in the mouse brain, multiply, and then die out. With an inoculum of 10^6 organisms of L 84, the brain counts increased tenfold by the 3rd–4th day after infection and then declined; by 20 days the brains were sterile. We re-infected these mice with 18-323 3–4 weeks after the primary L 84 infection. The course of the 18-323 infection in the twice-infected and

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Fig. 6. Mean viable counts of *Bordetella pertussis* strain 18-323 in the brains of mice vaccinated with $2{\cdot}5 \times 10^8$ organisms (2·5 ImD 50) IC or 5×10^9 organisms (5 ImD 50) IP 14 days before they were challenged with 50,000 organisms (3980 viable). Each point represents the geometric mean brain count of five mice. ●–●, Unvaccinated controls; ■–■, IC vaccinated mice (2·5 ImD 50); ○–○, IP vaccinated mice (5 ImD 50).

Figs. 7–10. Viable counts of *Bordetella pertussis* strain Gl. 353 in the lungs of mice vaccinated with various doses of pertussis vaccine IC 14 days before they were challenged with 10,000 organisms intranasally. Each point represents one mouse.

Fig. 7. Unvaccinated controls.

Fig. 8. Vaccine dose 1×10^7 organisms (0·1 ImD 50) IC.

Fig. 9. Vaccine dose 1×10^6 organisms (1 ImD 50) IC.

Fig. 10. Vaccine dose 1×10^9 organisms (10 ImD 50) IC.

Vaccine doses of 1×10^5 and 1×10^6 were similar to the unvaccinated controls.

Table 1. *Effect of vaccination route on serum antibody and resistance to IC challenge 14 days after* Bordetella pertussis *vaccine*

	Pooled serum of 10 mice 14 days after vaccination with 10^9 organisms				
Route of vaccination	Protective index* lung infection	Antihaemagglutinin titre	Agglutinin rating†	Bactericidal index‡ at 1/30 dilution	ImD 50 against an IC challenge of 50,000 organisms at 14 days
IC	1·6	27	22	1·6	200×10^6
IV	1·1	0	8	0·6	800×10^6
IP	0·6	0	7	0·3	1000×10^6

* See p. 490. † See p. 490.

‡ Difference between log number of organisms in control tube (no killing) and log number of organisms with antiserum and complement.

control mice is shown in Fig. 11. The brains were not immediately sterilized. The immunity to 18-323 was well over one ImD 50 since nearly all the mice survived. Yet the course of infection was similar to that after one ImD 50 of dead vaccine IC (Fig. 2) and not like a bigger protective dose (Fig. 3). The counts increased for 3 days and then decreased, suggesting the operation of a general immunity stimulated by antigen leaked-out from the brain, rather than a local brain immunity. If such were the case, it might be expected that the mice would also be immune to a sublethal lung infection, as after one ImD 50 of dead vaccine IC (Fig. 9). However, the L 84-infected mice were not immune to sublethal doses of Gl. 353 intranasally.

Immunization with lower doses of live L 84 were less effective: 50,000 organisms protected 26 % and 500,000 protected 60 % of mice against 18–323.

Intraperitoneal vaccination and infection with other strains

As already described, an IC vaccination of more than 5 ImD 50 produced an immediate effect on virulent *Bordetella pertussis* 18-323 introduced into the brain 14 days later, whereas 10 ImD 50 of vaccine IP did not do so; at most, there was a slightly lower infection curve for the vaccinated mice than for the controls, but always with a rise and fall (Fig. 6). Challenge with another virulent challenge strain, 2 atox., produced similar curves.

The course of infection in IP vaccinated mice after challenge with three other strains was different, however, from that after 18-323 and 2 atox. infection. Counts were held down at the challenge level or actually reduced from it as early as one day after infection. For example, when mice were vaccinated IP with about one ImD 50 of vaccine as measured IP/IC against an 18-323 challenge, and challenged with the avirulent but establishing strain L 84, there was no dramatic immediate sterilization (Fig. 12), but the bacterial count fell to 100 and was held at this level for 4 days until the usual IP/IC effect operated and clearance was achieved faster than in non-vaccinated mice. The ImD 50 could not, of course, be measured for the L 84 infection because non-vaccinated mice recovered eventually without vaccination.

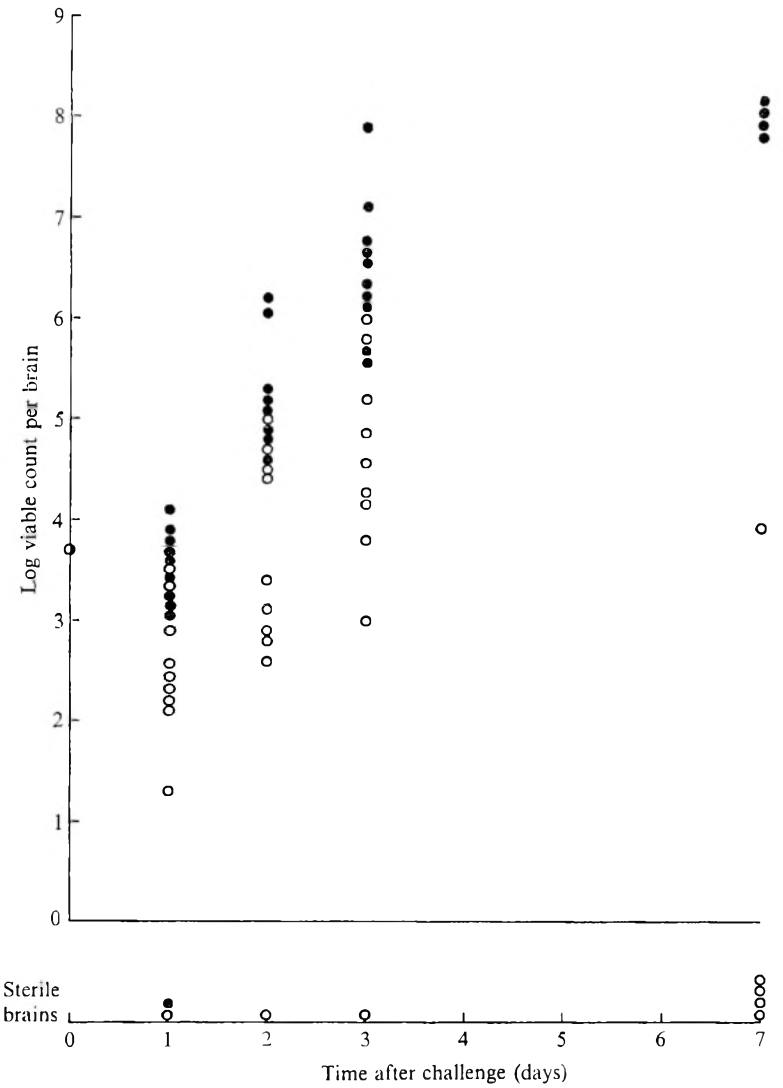

Fig. 11. Viable counts of *Bordetella pertussis* strain 18-323 in the brains of mice vaccinated with a living vaccine IC $3\frac{1}{2}$ weeks before they were challenged with 50,000 organisms (5000 viable). Vaccinated mice were injected with 5×10^6 total organisms of the avirulent strain of *B. pertussis*, L 84; a group of mice from the 90 % or more survivors from this treatment had sterile brains 10 days after vaccination. Each point represents one mouse. ●, Unvaccinated controls; ○, Vaccinated mice. In groups of mice kept 14 days after challenge to determine the number of mice dying, 0/10 survived in the control group and 19/21 in the vaccinated mice.

Two other strains, this time virulent, were next used for challenge, following IP vaccination, and the course of infection measured. These were Gl. 353 and a strain made virulent by mouse passage, B5533 P5. The LD 50 for both of these in non-immune mice was about the same as that of 18-323; and the organisms grew similarly to 18-323 in non-immunized mice; Adams (1970) illustrated the growth curve for Gl. 353. The ImD 50 of vaccine against these strains was, however, very

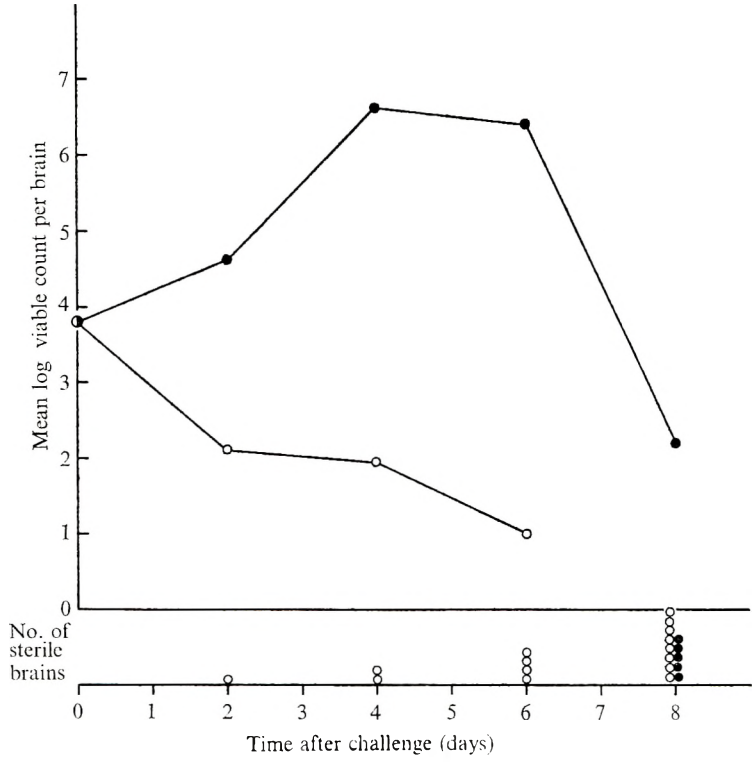

Fig. 12. Mean viable counts of *Bordetella pertussis* strain L84 in the brains of mice vaccinated with 1×10^9 organisms IP 14 days before challenge with 100,000 organisms (6300 viable). Each point represents the geometric mean brain count of infected mice in each group of eight (sterile brains shown separately). ●–●, Unvaccinated controls; ○–○, Vaccinated mice.

Table 2. *Course of infection in mice vaccinated IP with one level of vaccine and challenged IC, various strains*

Challenge strain 5×10^4 total (= log 3·5/brain)	LD50	ImD50 of vaccine IP	Day after infection	Viable count of organisms per brain: Log count (geometric mean, 5 mice) Vaccinated	Non-vaccinated
Gl. 353	320	20	1	3·8	3·4
			3	4·2	6·1
			6	1·4	7·8
B5533 P5	1050	100	1	2·9	nd
			3	2·9	5·2
			4	2·5	nd
			5	2·2	nd
18–323	1500	6	1	2·8	2·9
			3	4·8	5·2
			6	1·8	7·3

nd = not done.

low and a dose equivalent to 6–10 ImD 50 against 18-323 was equal to one of 20–100 ImD 50 against these strains.

Viable counts in vaccinated mice are shown in Table 2 and are similar to those after challenge with the avirulent strain. Brains were not sterilized immediately but the counts were held down until clearance began at about 3 days. Bactericidal antibody levels in the serum of the three groups of mice could not be correlated with the events in the brain.

DISCUSSION AND CONCLUSIONS

In spite of objections based on the absence of direct evidence (Wardlaw & Jakus, 1968), protection by IP vaccination against brain infection 14 days later with 18-323 *could* be due to circulating antibody leaking into the brain when the blood–brain barrier becomes permeable 4 days after infection (Holt *et al.* 1961). A curve of intracerebral growth similar to that for mice adequately protected in the IP/IC active immunization test and illustrated in Fig. 5 was obtained in mice passively protected against IC infection by IP antiserum (Dolby & Standfast, 1961, Figs. 7 and 8). The experiments of Blake & Wardlaw (1969) raise the question whether anything other than antibody is involved.

The order of efficacy of the three vaccination routes for producing (i) protection against IC challenges at 2 and 14 days, (ii) an immediate reduction in bacterial count of an IC challenge at 14 days, and (iii) suppression of sublethal lung infections, is shown in Table 3. This order was the same for protection against IC challenge as for induction of serum antibodies (Table 1). Vaccination IC was the most effective way of inducing high titre antisera, with IV vaccination intermediate and IP vaccination the least effective. Circulating antibody entering the brain could therefore account for the drop in the brain count 3–4 days after a challenge given 14 days after vaccination by any of the three routes, and the better stimulus of IC injected vaccine (although for what reason?) could explain its efficacy against IC infection reflected in ImD 50 values. There is also, however, the *shape* of the infection curves in vaccinated mice to be considered. For an 18–323 challenge these are different in IC vaccinated mice from those given equivalent ImD 50 of vaccine IP or IV in all doses greater than 2 ImD 50. Beginning with 2–3 ImD 50, it is clear that the drop in bacterial numbers in the IC vaccinated mice is greater during the first two days after challenge than in the IP vaccinated mice, as shown in Fig. 6. This becomes more exaggerated with higher doses until, with 10 ImD 50, the local effect in the IC vaccinated mice is so strong that sterilization begins at once and is complete at a time when the brain count has not yet started to fall in IP and IV vaccinated mice.

We have not investigated whether the same protective dose of vaccine IC and IP produces the same amount or the same kind of circulating antibodies, bearing in mind that ten times less vaccine is needed to immunize mice by the IC route. The Table 1 data were collected on sera of mice immunized with one given number of organisms. The greater efficacy of the IC route of vaccination might reflect the induction of different classes or kinds of antibody globulin (Dolby & Dolby, 1969) or the production of a local immune response.

Table 3. *A comparison of IC, IV and IP vaccinations*

Route of vaccination	Minimum number of organisms in killed vaccine inducing: Local interference phenomenon 2 days later	50 % protection against IC challenge with strain 18-323	Suppression of sublethal lung infection	Immediate bactericidal effect against IC challenge with strain 18-323
IC	2×10^7	1×10^8	1×10^8	4×10^8
IV	—	8×10^8	3×10^8	$> 2 \times 10^9$
IP	—	1×10^9	1×10^9	$> 1 \times 10^{10}$

In contrast to IC vaccination, the infection curve produced by 18-323 in the brain two weeks after IP vaccination suggested that no recovery began until 3 days after challenge. Use of an avirulent challenge, L84, or two other virulent strains, exposed an earlier antibacterial effect; the mechanism was there, but unable to operate against 18-323 (or 2 atox.) until 3 days after challenge.

The shape of the infection curve after IC challenge with 18-323 3 weeks after primary IC infection with an avirulent strain was surprising: we had expected an immediate reduction in count in the protected mice similar to that shown after IC vaccination, instead of the rise and fall typical of IP vaccination and subsequent IC challenge. Adams (1970) had demonstrated the slowing down of the growth of 18-323 introduced into the brain 2 days after an avirulent infection, but had found no effect $4\frac{1}{2}$ days after such an infection, whereas re-infection with an avirulent strain at 2 and 4 days after the primary infection had been suppressed with an immediate drop in count. No inhibitory effect on the re-infecting strain was observable at 1 hr. after the primary infection. This suppression resembled the Evans & Perkins interference phenomenon except in its absence at 1 hr.

The Medical Research Council trials (1956) showed that the IP/IC mouse test correlates well with protective potency in the child. Clearly therefore any test that might replace it must correlate in the same way. Table 3 suggests that more factors are operating to overcome infection in the IC/IC vaccination test than in the IP/IC test, although one of the factors may be common to both. It seems to us that the analysis of the mechanism promoting recovery only 3 days after infection is still of great importance.

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Diphtheria infection in North West Canada, 1969, 1970 and 1971

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SUMMARY

In three years, *Corynebacterium diphtheriae* was isolated from 1238 people, consisting of 820 North American Indians or Metis, 318 people of Caucasian origin, 97 Eskimos and 3 Asiatic Indians. Diphtheria infection of the throat, nose, ears and skin was common in the North American Indian and Metis people, but rarely caused severe symptoms. The infection occurred less often in white people but was more serious; of 27 cases of toxic respiratory diphtheria, 25 were white people. The public health significance of the endemic infection of the North American Indian and Metis people is discussed.

INTRODUCTION

When mass prophylactic immunization against diphtheria was introduced, there was concern that it would result in an increased number of carriers (Topley, 1933). Instead of this, *Corynebacterium diphtheriae* has almost disappeared from most countries where there is an immunization regime. This has not happened in North West Canada where, in spite of a high prophylactic immunization rate, diphtheria infections continue to occur.

A description of diphtheria infection diagnosed bacteriologically is given in this paper. Some features of the disease, which is widespread and endemic in this region, are unusual and of epidemiological interest.

The region and the people

Specimens come to this laboratory from an area extending northwards from latitude 52° N in Alberta, to the Arctic Coastal districts of the Northwest Territories. The climate is dry, with a long cold winter and short warm summer; the southern part of the region is in the cool temperate zone, but the winter is longer and colder to the north.

North West Canada is inhabited by the native people, which include pure bred and half bred (Metis) North American Indians, and white people of Caucasian origin. This region is very sparsely populated, with the exception of the city of Edmonton, population 435,500, where most of the inhabitants are white. Most of the native population live in scattered, often remote and isolated, villages, or in communities on reserves; in contrast to the white people, their socio-economic

conditions and standards of hygiene are low; they live in close personal contact in overcrowded houses without adequate washing facilities. Approximately 18,000 Indians live on reserves in Northern Alberta and an additional 6200 in the North-west Territories. Population figures for Metis are not available. Diphtheria-positive Eskimos are also included in this paper, but they are too few to be representative, and are not discussed as further investigations are being made. The mode of life and environment of the Eskimos are very different from those of the Indians and Metis.

MATERIALS AND METHODS

The results of bacteriological cultures reported in this paper were from unselected swabs, taken and submitted by physicians or nurses for diagnostic or public health purposes. Serum-coated cotton wool swabs were used for specimens from nose, throat, skin lesions, and ear discharges. Swabs from distant places were sent immersed in a modification of Stuart's transport medium (Amies, 1967). The swab was inoculated on 10% sheep blood nutrient agar, Hoyle's lysed sheep blood tellurite agar, and Billings' modification of Tinsdale's serum cystine tellurite agar. The procedures for culture and identification of *Corynebacterium diphtheriae* have been described (Jellard, 1971). All strains of *C. diphtheriae* were tested for toxin production by a modification of the plate diffusion test of Elek (1948); the technique was that described by King, Frobisher & Parsons (1949), except for the use of the serum substitute medium of Hermann, Moore & Parsons (1958). The tests were examined at 24, 48 and 72 hr. incubation. Fifty-nine strains of *C. diphtheriae* were also tested for toxin production by subcutaneous inoculation of guinea-pigs, which confirmed the results of the plate test on every occasion.

RESULTS

Corynebacterium diphtheriae was cultured from nose, throat, skin or ear swabs from 1238 people; of these 820 were North American Indian or Metis, 318 were white, 97 were Eskimo, and 3 were Asiatic Indian. Altogether there were 1272 cultures of *C. diphtheriae*, whose type and toxigenicity are given in Table 1; the additional 34 cultures came from infections of more than one site, simultaneous infection with more than one strain, or subsequent reinfection with a different strain of *C. diphtheriae*. The primary site of isolation and ethnic group are shown in Table 2. All cultures from Eskimos were non-toxigenic. No relationship between the site and the type or toxigenicity of the infecting strain of *C. diphtheriae* was found. (When a toxigenic strain was found, nose and throat swabs from contacts were taken, which would account for a higher proportion of toxigenic strains from the nose and throat, than from the ear or skin.) More ear and skin swabs from natives were diphtheria-positive than from white people.

It was not possible to determine the race of every person who was swabbed, but of 4948 consecutive nose, throat, skin and ear swabs 402 were from natives and 4546 from whites. Of the 402 swabs from natives, 19 (4·7%) were positive, whereas

Table 1. *Type and toxigenicity of* 1272 *cultures of* Corynebacterium diphtheriae

Type of *C. diphtheriae*	Toxigenic	Non-toxigenic
Gravis	274	193
Intermedius	261	23
Mitis	31	415
Atypical	19	56
	585 (46 %)	687 (54 %)

Table 2. *Primary site of isolation of* Corynebacterium diphtheriae *and the ethnic group of* 1238 *persons*

Site	Indian/Metis	Eskimo	Caucasian	Asiatic Indian	Total
Nose/throat	389	25	277	2	693
Ear	224	65	14	0	303
Skin	207	7	27	1	242
Total	820	97	318	3	1238

3 out of 4546 (0·07 %) of swabs from white persons were positive. In this small sample the incidence of diphtheria-positive swabs was more than 60 times greater in native than in white persons.

Age of diphtheria-positive persons

The age of 1197 diphtheria-positive persons is shown in Fig. 1. The highest incidence of diphtheria isolation was in the first year of life, and there was a transient rise at the age of 6 years, the school entry age.

Prophylactic immunization

An inquiry showed that the immunization rate of children from the different areas was satisfactory and that immunization was carefully maintained. This was confirmed by a small investigation of 208 Indian children aged 1–12 years, who were infected with toxigenic *C. diphtheriae*; 160 (77 %) had had a primary series of three inoculations, with or without a reinforcing dose, completed within 7 years of infection, and were therefore fully immunized; 19 were inadequately immunized; 29 were unimmunized.

Seasonal incidence of isolation of C. diphtheriae

In each autumn and winter there was an increase in the number of positive cultures (Fig. 2, Table 3). *C. diphtheriae* was isolated each week of the 3-year period.

Nose and throat swabs

Of 727 cultures of *C. diphtheriae* isolated from nose or throat swabs, 381 (52·4 %) were toxigenic and 346 (47·6 %) were non-toxigenic.

Haemolytic streptococci, mostly group A, were isolated from many of the diphtheria-positive nose and throat swabs. It is often impossible to determine the

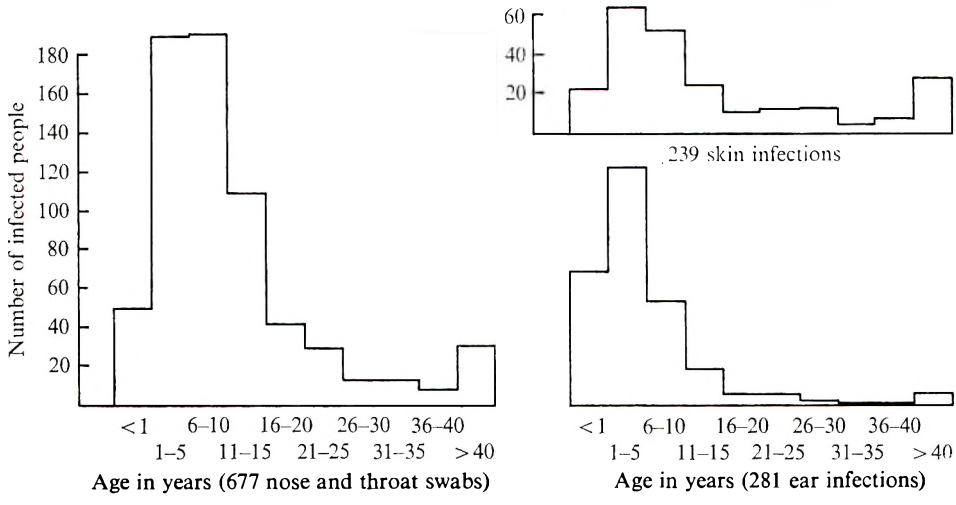

Fig. 1. *Corynebacterium diphtheriae* infections by age in swabs from skin, ear, nose and throat.

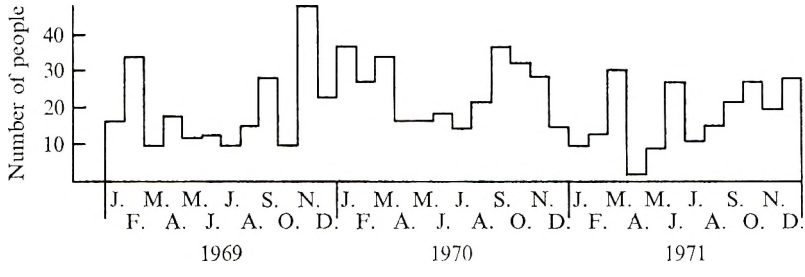

Fig. 2. 1969–1971, monthly isolations of *Corynebacterium diphtheriae* from nose and throat swabs of 693 persons.

causative or primary pathogen in a mixed streptococcal and diphtheritic infection. For the purpose of this paper, the diagnosis of toxic diphtheria of the upper respiratory tract is restricted to those people who were bacteriologically positive for *C. diphtheriae*, who had a diphtheritic membrane or clinical signs of diphtheria. Details are given in Table 4 of the 27 cases of toxic respiratory diphtheria; 25 of these people were white. In addition, an unimmunized white girl aged 2 years died from acute clinical diphtheria which was not confirmed by culture, because tonsillar tissue, excised at autopsy, was immersed in formalin; toxigenic *C. diphtheriae* mitis was isolated from two sibling carriers. All these cases came from rural areas, where white people were more liable to have contact with Indians or Metis than in urban districts.

Skin lesions

The skin lesions of 242 persons were diphtheria-positive; 27 of these were white. Eighty-six (35·5 %) were infected with toxigenic *C. diphtheriae*, and 156 (64·5 %) were infected with non-toxigenic strains. There were no toxic effects from these infections. Of the 242 lesions, 230 (95 %) were associated with haemolytic strepto-

Table 3. 1969–1971, *monthly isolations of* Corynebacterium diphtheriae *from (a) skin lesions:* 242 *people and (b) ear swabs:* 303 *people*

	1969		1970		1971	
	(*a*) Skin	(*b*) Ear	(*a*) Skin	(*b*) Ear	(*a*) Skin	(*b*) Ear
Jan.	3	9	5	7	4	10
Feb.	4	7	4	5	6	8
Mar.	5	8	5	7	7	20
Apr.	2	5	4	9	2	5
May	5	2	5	5	6	6
June	2	4	6	8	9	6
July	7	6	1	4	5	4
Aug.	6	5	6	11	6	5
Sept.	5	11	13	16	16	18
Oct.	7	13	12	15	18	11
Nov.	5	11	9	9	14	12
Dec.	9	6	11	7	9	8

Total (*a*) skin: 242.
Total (*b*) ear: 303.

Table 4. *Twenty-seven cases of toxic respiratory diphtheria*

Race	Age	Sex	Type of *C. diph.*	Toxigenicity	Prophylactic Immunization State
White	2	F	Intermedius	+	Nil
White	2	M	Gravis	+	Nil
White	2	M	Intermedius	+	Nil
White	4	F	Gravis	+	Nil
White	5	M	Intermedius	+	Nil
White	5	M	Intermedius	+	Nil
White	6	F	Intermedius	+	Nil
White	6	M	Gravis	+	Incomplete
White	7	M	Mitis	+	Incomplete
White	7	F	Mitis	+	Nil
White	7	M	Gravis	+	Nil
White	7	M	Intermedius	+	Incomplete
Indian	10	F	Gravis	+	Not known
White	10	F	Mitis	+	Full
White	12	M	Gravis	+	Not known
Metis	12	M	Gravis	+	Full
White	13	F	Intermedius	+	Full
White	13	M	Mitis	+	Incomplete
White	13	M	Gravis	+	Not known
White	14	F	Intermedius	+	Full
White	32	F	Gravis	+	Not known
White	42	M	Gravis	+	Not known
White*	45	M	Intermedius	+	Not known
White	47	M	Intermedius	+	Not known
White	50	M	Gravis	+	Nil
White	50	M	Gravis	+	Not known
White	52	F	Gravis	+	Not known

* Fatal.

Table 5. *Clinical diagnosis of* 198 Corynebacterium diphtheriae *positive skin lesions*

Impetigo	102
Trauma	39
Ulcer, leg	23
Burn	16
Eczema	15
Ulcer, hand	3

cocci of groups A, B, C or G and 209 (86·3%) with *Staphylococcus aureus*. The clinical diagnosis of 198 persons with diphtheria-positive skin lesions is given in Table 5. With the exception of the ulcers, the lesions were superficial and did not suggest diphtheria infection. The ulcers were on the leg or arm, oval or circular, up to 4 × 3 cm. and 1 cm. deep, with a sero-purulent base and well-defined raised margin.

Ear swabs

All 303 diphtheria-positive ear swabs were from persons with acute or chronic otitis media and aural discharge; 14 were from white people; 102 (33·7%) were infected with toxigenic *C. diphtheriae*, and 201 (66·3%) with non-toxigenic strains. Information was available for 281 persons, and of these 130 (46·3%) were children in the first 2 years of life (Fig. 1); the youngest was 4 weeks old. Toxic symptoms due to diphtheria were not found. Haemolytic streptococci of groups A, B, C or G were present in 247/303 (81·1%) of these swabs, *Streptococcus pneumoniae* in 14/303 (4·6%), *Staph. aureus* in 234/303 (77·2%) and on only two occasions was there a pure growth of *C. diphtheriae*.

DISCUSSION

These findings confirm the observation of Dixon & Thorsteinson (1969) that diphtheria infection is common in the native and Metis people of North West Canada. It is much less common in white people, though when they are infected they may be seriously ill. The difference in the disease in native and white people is very striking. In the natives, the infection is endemic and may present as uncomplicated infection of the nose and throat, superficial skin infection, and ear infections in babies and young children. Classical toxic diphtheria nearly always occurs in white people, and with them skin and ear infections are rare.

The different nature of the disease in white people and the natives of tropical countries has been known for many years. It was suggested that racial factors contributed to the difference (Frost, 1928; Dudley, 1929), but this has been difficult to prove. Dudley thought that the domestic environment was important, and that the rarity of clinical diphtheria in natives was due to natural infection immunity, acquired early in life, in the close contact of the home. This was supported by Liebow, MacLean, Bumstead & Welt (1946) in the South Pacific Islands, and by Gunatillake & Taylor (1968) in Ceylon, who found that skin diphtheria was common and led to an effective herd immunity. The epidemiological signifi-

cance of diphtheritic skin lesions has also been shown by Belsey, Sinclair, Roder & LeBlanc (1969).

The endemic skin diseases of the natives of tropical countries and North West Canada are comparable; conditions in the overcrowded homes are suitable for cross-infection with diphtheria bacilli and the associated pathogenic haemolytic streptococci; this may occur by skin-to-skin contact or indirectly by articles contaminated with the discharges from infected superficial skin lesions and nasopharyngeal secretions. The conditions in North West Canada are probably more favourable for cross-infection; the severe northern winters confine the people indoors and prolong the time of exposure to infection, and the discharging ears of the babies and young children also contribute to the high incidence of the disease.

Because of the frequent association of non-toxigenic and toxigenic *C. diphtheriae* with haemolytic streptococci and *Staphylococcus aureus*, the role of *C. diphtheriae* as the primary pathogen in the skin and ear infections is questionable. The elimination of this mixed endemic infection is difficult, but the aim should be an improvement in the living conditions and education in personal hygiene. Previous immunization had no apparent effect on the skin and ear infections. In fact, Grasset (1952) doubted the need for prophylactic immunization of African natives, while he stressed its importance for white people who lived in contact with them. It is just as important that white people in North West Canada should continue to be fully immunized against diphtheria.

Little is known about the relationship of toxigenic and non-toxigenic strains of *C. diphtheriae* in nature, but the scarcity of non-toxigenic strains in many places where diphtheria does not exist does suggest a close association. The coexistence of non-toxigenic and toxigenic strains in endemic areas is interesting; non-toxigenic strains should not be ignored, but regarded as an indication that the conditions are also suitable for toxigenic strains. In the laboratory, non-toxigenic *C. diphtheriae* has been converted to toxigenicity by the acquisition of lysogenic bacteriophage (Freeman, 1951; Groman, 1953), but no such transformation has yet been shown to occur in natural conditions.

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The production of mastitis in cows by the intramammary inoculation of T-mycoplasmas

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SUMMARY

Six milking cows were inoculated with bovine and human T-mycoplasmas and control materials into the udder via the teat canal. Control materials produced only a slight transient cell response in the milk. Bovine T-mycoplasmas produced clinical mastitis in nine out of ten quarters inoculated. Seven developed clinical mastitis together with visible changes in the milk, excretion of T-mycoplasmas and greatly increased cell counts in the milk. In three of these quarters, in two different cows, milk secretion ceased completely. Two quarters in a different cow showed visible milk changes, excretion of T-mycoplasmas and increased cell counts. Two quarters were inoculated with human T-mycoplasmas and neither produced any signs of mastitis.

Infection of the udder with T-mycoplasmas did not stimulate high-titre serum antibody levels as measured by the metabolic inhibition test, but whey samples gave high titres in two of the cows that were able to control and resolve the infection.

INTRODUCTION

T-mycoplasmas were isolated first by Shepard (1954) from the human urogenital tract. Since then they have been isolated from the human oropharynx (Taylor-Robinson & Purcell, 1966), from the urogenital tract of cattle (Taylor-Robinson, Haig & Williams, 1967), from pneumonic calf lungs (Gourlay, 1968), from eyes in cases of infectious bovine keratoconjunctivitis (Gourlay & Thomas, 1969), from the throats of cats (Tan & Markham, 1971), from the genital tract of dogs and the throats of squirrel monkeys (Taylor-Robinson, Martin-Bourgon, Watanabe & Addey, 1971).

The role of T-mycoplasmas in disease is not clear: they have been incriminated in non-specific urethritis in man (Shepard, 1969), abortions and premature births in women (Knudsin, Driscoll & Ming, 1967) and pneumonia in calves (Gourlay & Thomas, 1970). In the last instance pneumonic lesions were produced in calves following the endobronchial inoculation of T-mycoplasmas.

In view, however, of the difficulty of obtaining calves of an appropriate gnotobiotic specification for further experimental work on pneumonia, and the difficulty of monitoring the progress of pneumonia, we decided to investigate whether the bovine mammary gland was an alternative site for studies of T-mycoplasma

infection. An advantage of the bovine udder is that it comprises four separate quarters; all of which are accessible for clinical examination, easy to sample and have a limited and readily determined bacterial flora. This paper reports on the production of mastitis in cows by the intramammary inoculation of T-mycoplasmas and on the potential value of the udder for studying the pathogenesis of bovine T-mycoplasma infections and the immune response of the host.

MATERIALS AND METHODS

Cows

Six Ayrshire or Friesian Cross Ayrshire cows, 3–5 years of age, were used. They were each giving about 3 gal. of milk a day at the time of inoculation, except cow M 153 which was giving only $1\frac{1}{2}$ gal. Total cell counts were performed on samples of milk from each quarter for a few days before inoculation and only quarters that had a cell count of less than $10^{5\cdot2}$ cells/ml. were used. Milk smears were stained by the 'Single Dip' method of Broadhurst & Paley (1939) and cells were counted by the technique described by Pattison & Holman (1951). By cultural methods no large-colony or T-mycoplasmas were demonstrated in the pre-inoculation milk samples.

After the afternoon milking the cows were inoculated in each quarter with 10 ml. of either T-mycoplasma broth culture or control broth material. The inoculum was inserted via the teat canal with a syringe. Sixteen hours after inoculation, and at daily intervals thereafter, milk samples were examined from each quarter for abnormal appearance, T-mycoplasmas, cells and bacteria. The quarters were also examined clinically for signs of mastitis.

Strains of T-mycoplasma

Four strains of T-mycoplasmas were used: the A 417 strain that had been isolated from a pneumonic calf lung (Gourlay & Thomas, 1970), the D 32 strain that had also been isolated from a pneumonic calf lung (calf 24, table 1, Gourlay, Mackenzie & Cooper, 1970), the REOW strain isolated from the human urogenital tract and supplied by Dr D. Taylor-Robinson, and the M 126/68 strain also isolated from the human urogenital tract and supplied by Dr B. E. Andrews. Two different substrains of the A 417 strain were used; the first was at the 10^{-11} dilution from the original lung tissue while the second – 'cloned' – had undergone six subcultures in broth of which three had been from the terminal dilution, as a means of purification, and was at a 10^{-25} dilution from the original lung. The D 32 strain had undergone eight subinoculations, three of them from the terminal dilution, and was at a 10^{-23} dilution from the original lung.

The T-mycoplasmas were grown in U-broth. This was similar to GS broth (Gourlay & Leach, 1970) except that it also contained 0·1 % urea, 5 % yeast extract (Difco) and 0·05 M Hepes (Sigma). Glucose, lactalbumin hydrolysate and DNA were omitted and the pH adjusted to 6·0. The titre of viable organisms in cultures or milk was estimated by making duplicate serial tenfold dilutions in U-broth. Growth produced an alkaline shift of pH as a result of the metabolism of urea and was indicated by a colour change of phenol red in the medium. Viability was esti-

mated as colour change units (c.c.u.). The titre was the highest dilution that produced a colour change after incubation at 37° C.

Control inocula were either uninoculated broth or broth culture of T-mycoplasmas which had been incubated at 37° C. until viable organisms could no longer be demonstrated (dead organisms). The pH was then adjusted to 7·0.

Histopathology

Representative portions of mammary tissue, taken at autopsy, were fixed in neutral buffered formalin. Paraffin sections were stained by haematoxylin and eosin.

Serology

Sera and whey were examined for antibodies to T-mycoplasma (A 417 strain) by the metabolic inhibition (MI) test of Purcell, Taylor-Robinson, Wong & Chanock (1966). Titres are given as the reciprocal of the highest dilution of serum that inhibited growth.

Sera were also examined for the presence of haemoglobin reactive protein (HRP) as described by Spooner & Miller (1971).

Bacteriology

The number of bacteria was estimated in milk samples by spreading 0·1 ml. of milk over the surface of an ox-blood-agar plate and counting the number of colonies after incubation at 37° C. for 24 hr. The bacterial count in milk was not considered significant if there were fewer than 1000 colony-forming units per ml. and there was an absence of obvious mastitis pathogens.

RESULTS

Udder response

Details of the inocula used for the six cows are given in Table 1. The results obtained from cows A 5, L 629 and L 686 are given in Figs. 1–3. All three cows developed mastitis in the quarters inoculated with 10^6 or more viable A 417 T-mycoplasmas, as indicated by an increase in milk cells, and visible milk and udder abnormalities. T-mycoplasmas were also isolated from these quarters. Plate 1A shows the changes in appearance of milk from the LF quarter of cow L 629. Injection of control materials into the udder produced only a transient cell response in milk. Uninoculated broth gave only a slight cell response but the injection of dead organisms produced a greater though still transient cell response. Cow L 686 reacted slowly and less severely than the others and eventually overcame the infection. The RH and LH quarters of this cow developed only transient cell responses. T-mycoplasmas were not isolated at any time from the milk from the RH quarter even though the control 'dead' inoculum unfortunately contained 10^3 viable organisms. T-mycoplasmas were isolated from milk of the LH quarter (inoculated with REOW organisms) only on the day after inoculation.

Cow L 91 reacted only slightly to the intramammary inoculation of mycoplasmas and only the LF quarter developed more than a transient cell response. It is of

Table 1. *Details of inocula*

Cow	Quarter	Inoculum
A 5	LF	10^6 A 417
	LH	Uninoculated broth
	RF	NI
	RH	NI
L 629	LF	10^7 A 417
	LH	Dead A 417
	RF	10^7 A 417
	RH	Uninoculated broth
L 686	LF	10^7 A 417 'cloned'
	LH	10^7 REOW
	RF	10^6 A 417
	RH	10^3 'dead' A 417 'cloned'
L 91	LF	10^7 A 417 'cloned'
	LH	10^6 M 126/68
	RF	10^6 A 417
	RH	Dead A 417
M 153	LF	10^8 A 417
	LH	NI
	RF	NI
	RH	NI
M 626	LF	NI
	LH	10^6 D 32
	RF	NI
	RH	10^6 A 417 'cloned'

NI = not inoculated.

particular interest that the RF quarter did not develop mastitis, considering the results with the inoculation of A 417 organisms into the first three cows. The milk from the LF quarter had an initial cell response, in which T-mycoplasmas were isolated, but the cell response had returned to normal by the 5th day. On the 6th day, a second response occurred which became moderately severe and persistent. T-mycoplasmas were still being excreted in the milk at a low titre after 41 days. Milk and udder abnormalities were observed at the height of the second response.

Cows M 153 and M 626 were killed before the full extent of their infection could be assessed. The LF quarter of cow M 153 reacted severely and, at slaughter on the 4th day after inoculation, the T-mycoplasma titre was 10^6/ml. and the cell count was $10^{7\cdot7}$ cells/ml. Udder abnormalities were evident, manifest by induration and a drop in milk yield, while the milk became watery and separated. Cow M 626 was killed 9 days after inoculation. The two inoculated quarters both reacted; the RH one more severely than the LH one. T-mycoplasmas were isolated from both quarters up to the time the cow was killed. The maximum titres were 10^6 organisms/ml. in the RH quarter and 10^5 organisms/ml. in the LH quarter. The cell counts in the RH quarter reached a maximum of $10^{8\cdot2}$ cells/ml. and in the LH quarter $10^{7\cdot3}$ cells/ml. Both quarters produced abnormal milk but showed no obvious udder abnormalities. The milk from the RH quarter became watery and separated whereas the milk from the LH quarter contained only clots. Small

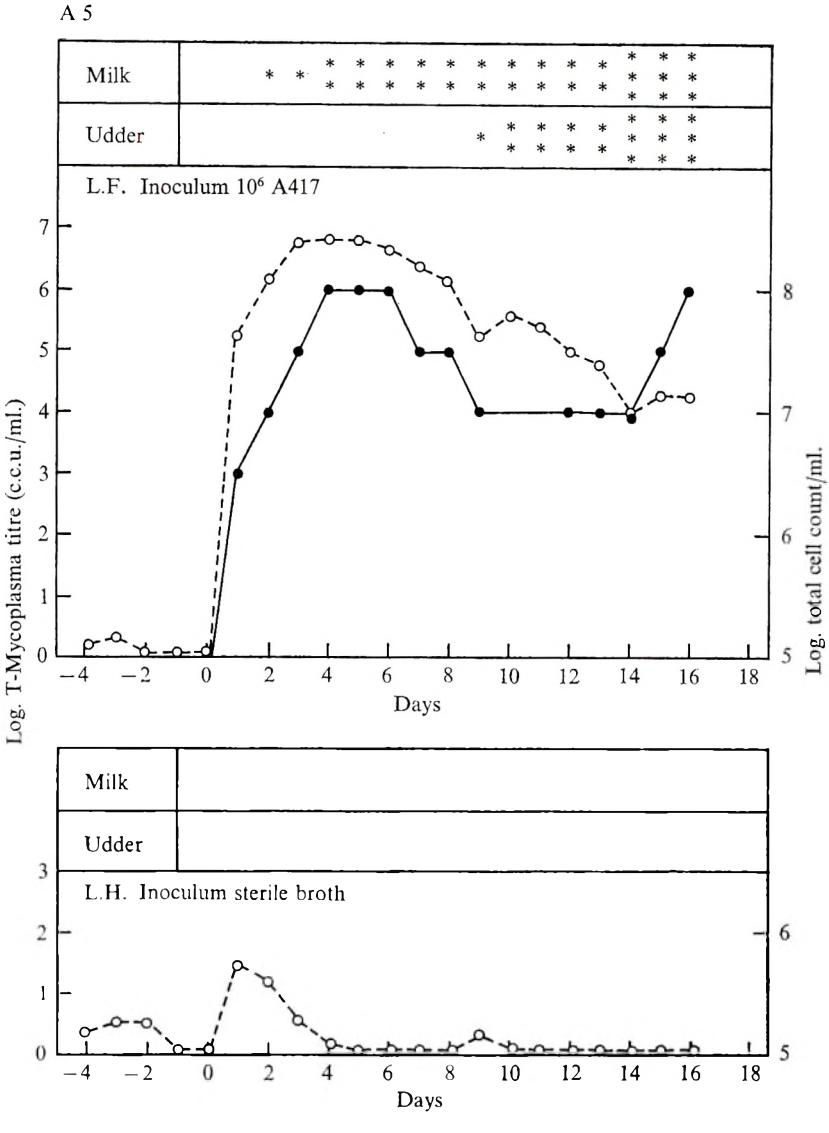

Fig. 1. Cow A 5. Daily milk cell count ○– –○, T-mycoplasma titre in milk ●——●, and milk and udder abnormalities. Inocula: L.F. quarter, 10^6 A 417; L.H. quarter, uninoculated broth. Milk abnormalities: * dark yellow, ** containing clots, *** separated – clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

pieces of udder tissue were taken from these latter two cows for histopathological examination.

Bacteriology

Bacterial counts in milk were monitored throughout the course of mycoplasma infections, as a guard against the possibility of a concurrent bacterial infection causing the mastitis, coincident with high mycoplasma titres. There was no increase in the bacterial count in any of the experiments reported. In fact, the

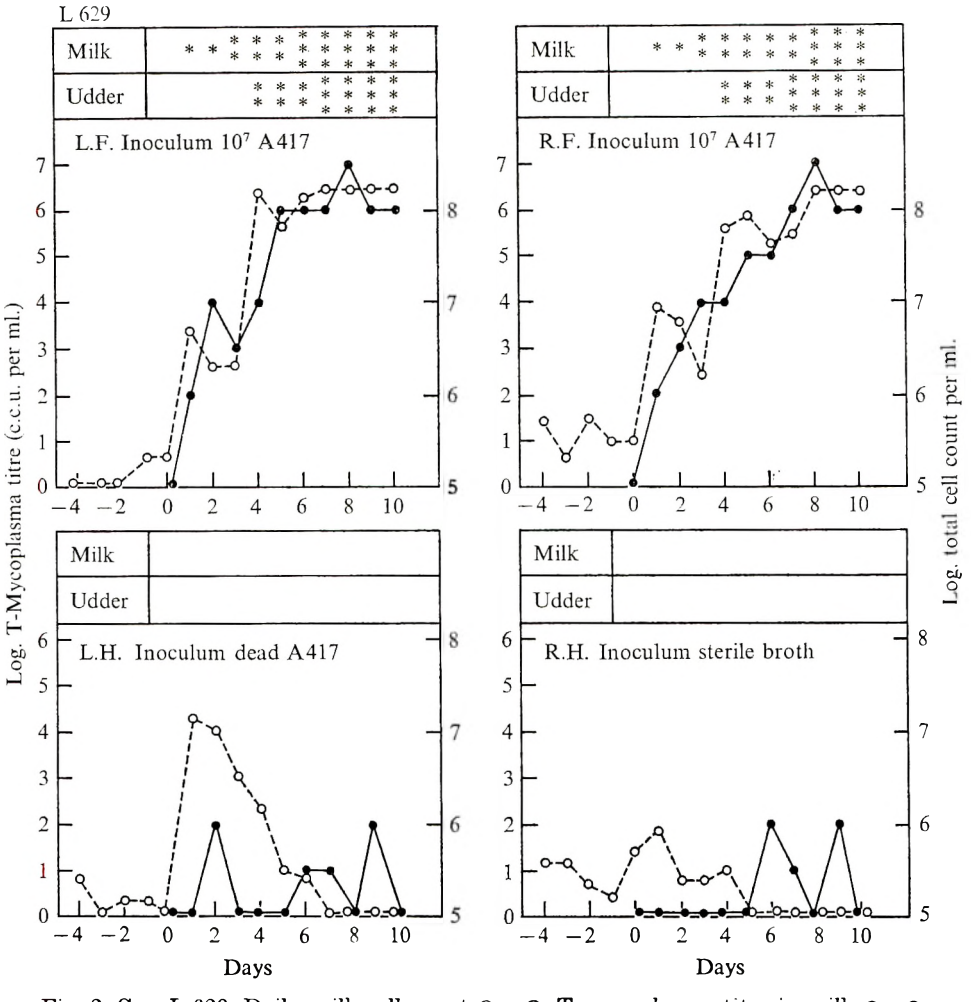

Fig. 2. Cow L 629. Daily milk cell count ○--○, T-mycoplasma titre in milk ●—●, and milk and udder abnormalities. Inocula: L.F. and R.F. quarter: 10^7 A 417; L.H. quarter: dead A 417; R.H. quarter: uninoculated broth. Milk abnormalities: * dark yellow, ** containing clots, *** separated – clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

bacterial count noticeably decreased in quarters with high mycoplasma titres and, in many instances, the milk appeared bacteriologically sterile.

Serology

No serum samples were obtained from cow M 153 as it was slaughtered after only 4 days. All the other serum samples tested before inoculation had an antibody titre of < 10. MI antibody titres of 20 were found in the sera of three of the five cows after infection. These were cows A 5, L 629 and L 686.

All whey samples taken before inoculation showed MI antibody titres of < 10. The whey from cow L 91 appeared to stimulate the growth of the T-mycoplasmas. After inoculation, MI antibody (titre > 640) was found in whey samples from all

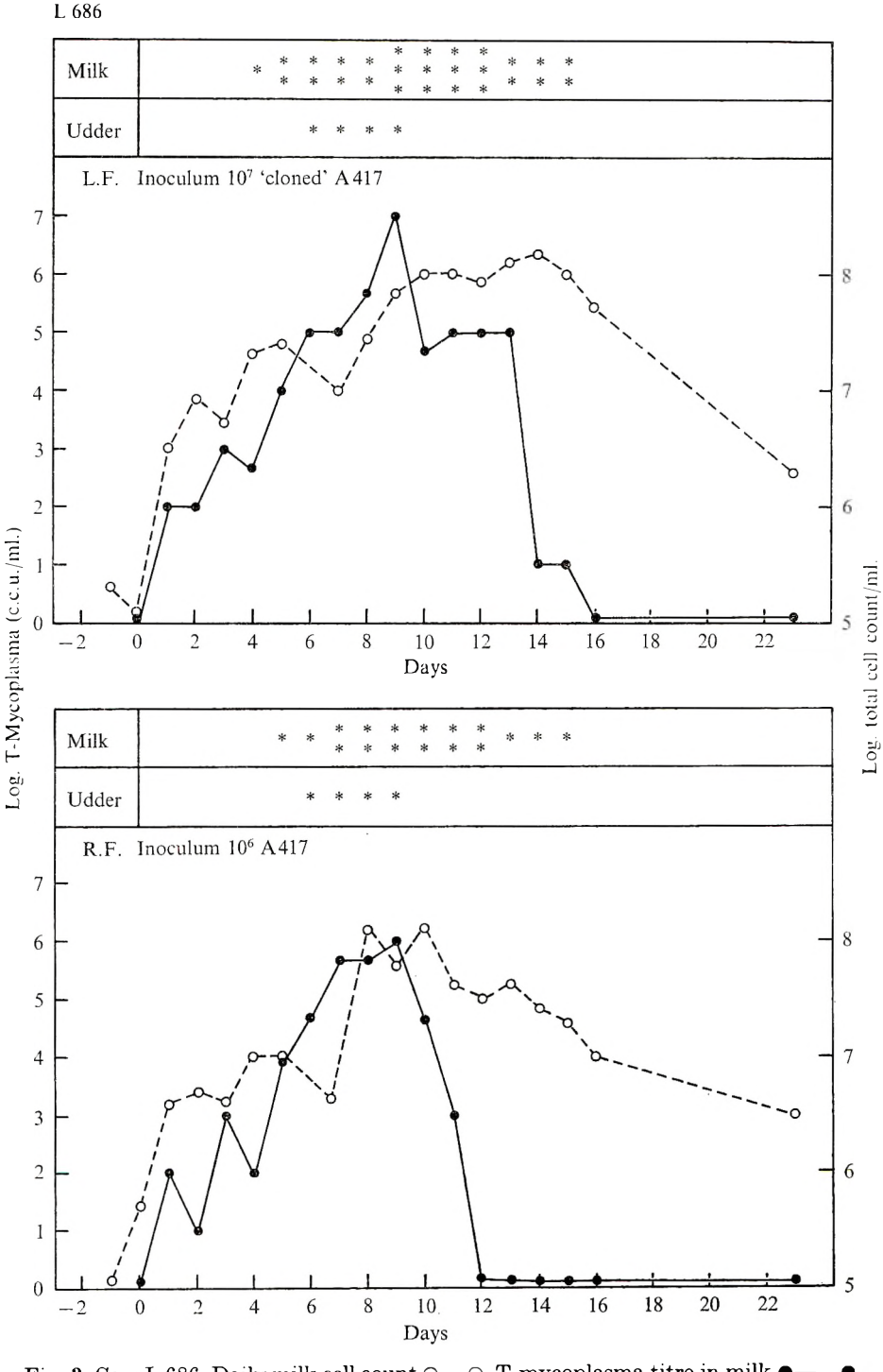

Fig. 3. Cow L 686. Daily milk cell count ○– –○, T-mycoplasma titre in milk ●——●, and milk and udder abnormalities. Inocula: L.F. quarter, 10^7 'cloned' A 417; R.F. quarter, 10^6 A 417. Milk abnormalities: * dark yellow, ** containing clots, *** separated – clear whey and floccular material. Udder abnormalities: * induration, ** reduced gland size and milk secretion, *** cessation of milk secretion.

inoculated quarters of cows L 686 and L 626 and in the whey from the LF quarter only of cow L 91. Whey from the three remaining quarters of cow L 91 still stimulated growth of T-mycoplasmas. The whey samples from both quarters of cows A 5 and L 629 had titres of < 10 and 20 respectively.

HRP was not detected in any of the pre-inoculation serum samples except for cow L 91. HRP was detected in the sera of only two of the cows after infection. Cow A 5 possessed HRP in serum taken on day 13, and cow L 629 possessed HRP on day 10 and slight HRP on day 20.

Histopathology

Histopathological examination of udder sections from cow M 153 showed marked infiltration of neutrophils into the alveolar lumina together with interstitial hyperaemia (Plate 1B). Udder sections from cow M 626 showed a similar picture but in addition the RH quarter showed evidence of interstitial infiltration and involution.

DISCUSSION

Certain mycoplasmas have been shown to be responsible for natural outbreaks of mastitis. These are *M. bovigenitalium* (Davidson & Stuart, 1960; Stuart *et al.* 1963), *M. agalactiae var. bovis* or *M. bovimastitidis* (Hale, Helmboldt, Plastridge & Stula, 1962; Jain, Jasper & Dellinger, 1969) and mycoplasma belonging to Leach's (Leach, 1967) serological group 7 (Connole, Laws & Hart, 1967). In addition there is one recorded natural case of mastitis due to *M. bovirhinis* (Langer & Carmichael, 1963). All these mycoplasmas can cause mastitis when experimentally inoculated into the udder. *Acholeplasma laidlawii* failed to produce experimental mastitis (Jain *et al.* 1969).

From our work it is evident that certain T-mycoplasmas isolated from pneumonic calf lungs can produce mastitis when inoculated into the bovine udder. From a total of 10 quarters, in 6 different cows, inoculated with these bovine T-mycoplasmas, 7 developed clinically observable changes in the quarter, milk changes and a greatly increased cell count, and in 3 of these quarters milk secretion ceased completely. Two quarters showed milk changes and greatly increased cell counts, and one did not react at all. Two other quarters, inoculated with human T-mycoplasmas, did not develop mastitis. Five quarters were inoculated with control materials in 4 different cows, 2 with uninoculated broth, 2 with non-viable T-mycoplasmas and 1 with a mixture of non-viable and viable T-mycoplasmas. Uninoculated broth produced only a minimal cell response on the day following inoculation, which rapidly reverted to normal. The non-viable T-mycoplasmas produced a more severe cell response on the day after inoculation; this decreased progressively to revert to normal after about 5 days. The low titre of viable organisms in one of the 'dead' mycoplasma controls had no apparent effect.

T-mycoplasmas were detected in milk from all quarters that were inoculated with the bovine T-mycoplasmas. From one quarter (that which did not develop any signs of mastitis) these organisms were only detected at a very low titre on the day after inoculation. In all the other infected quarters mycoplasmas were

detected daily until either the quarter went dry, the animal was killed or the infection was resolved. However, there was one exception, when T-mycoplasmas were not detected, this being from the LF quarter of cow L 91 on the 5th day after inoculation. There can be no doubt that the T-mycoplasmas actually multiplied in the quarters, considering the large volume of milk produced by the cows throughout the infection period and the consistently high titres of mycoplasmas recorded in the milk. The titre of mycoplasmas excreted in the milk increased to a peak that corresponded roughly to the maximum cell response and also the maximum milk and udder abnormalities. The titre of T-mycoplasmas and the total cell counts in the milk usually corresponded closely and indicated a close relationship between the mycoplasmas and cells. Following the inoculation of human T-mycoplasmas, organisms were detected only on the day following inoculation and this appeared to be due to survival of the inoculum. Except in one animal, T-mycoplasmas were not detected in the milk from quarters inoculated with control materials. In this one animal (L 629) the detection of T-mycoplasmas was not accompanied by a cellular response and we presume contamination of the milk samples occurred at milking. This failure to detect T-mycoplasmas in the control quarters would indicate a lack of spread of infection between quarters, in contrast to what occurs with other mastitis-producing mycoplasmas (Stuart *et al.* 1963; Hale *et al.* 1962; Jain *et al.* 1969).

There was some variation in response of these cows to inoculation of T-mycoplasmas unrelated to inoculum titre. Cows A 5, L 629 and M 153 reacted quickly and severely while the remainder (L 686, L 91 and M 626) reacted more slowly and on the whole less severely. It would appear that these latter three cows possessed some resistance to T-mycoplasma infection, the nature of which is unknown at present.

The failure of the human T-mycoplasmas to produce mastitis may be indicative of a species specificity, but only the REOW strain result can be considered at all significant as the M 126/68 strain was perhaps inoculated at too low a titre in a relatively resistant animal. It is, however, possible that the REOW strain had become attenuated or was an avirulent strain originally.

The evidence from cow M 626 may indicate that the A 417 strain of bovine T-mycoplasma is more pathogenic than the D 32 strain.

Infection of the udder with T-mycoplasmas did not stimulate high titre serum antibody levels. It is interesting to note that in the two cases, cows A 5 and L 629, where high titre MI antibody was not found in whey, the infected quarters eventually ceased to produce milk. In cows L 686, L 91 and M 626 whey samples gave high MI titres as a result of the infection and in the case of L 91 and L 686 this was associated with an ability of the animals to control and resolve the infection respectively. Animal M 626 was killed before the infection had run its course.

The milk and udder abnormalities together with the histopathological findings indicate that T-mycoplasmas produce an inflammatory reaction in the udder and furthermore the demonstration of HRP in the sera of two cows indicates that this may sometimes be of an acute nature.

We have no evidence at all that T-mycoplasmas are responsible for natural cases

of mastitis in cattle, although from the experimental results it is not inconceivable that they might be. From our point of view, however, the main conclusion to be drawn from this work is that the bovine udder appears to be a suitable model for studying the pathogenesis and immunity of bovine T-mycoplasma infections.

We wish to thank Miss J. Wren and Miss M. Admans for technical assistance.

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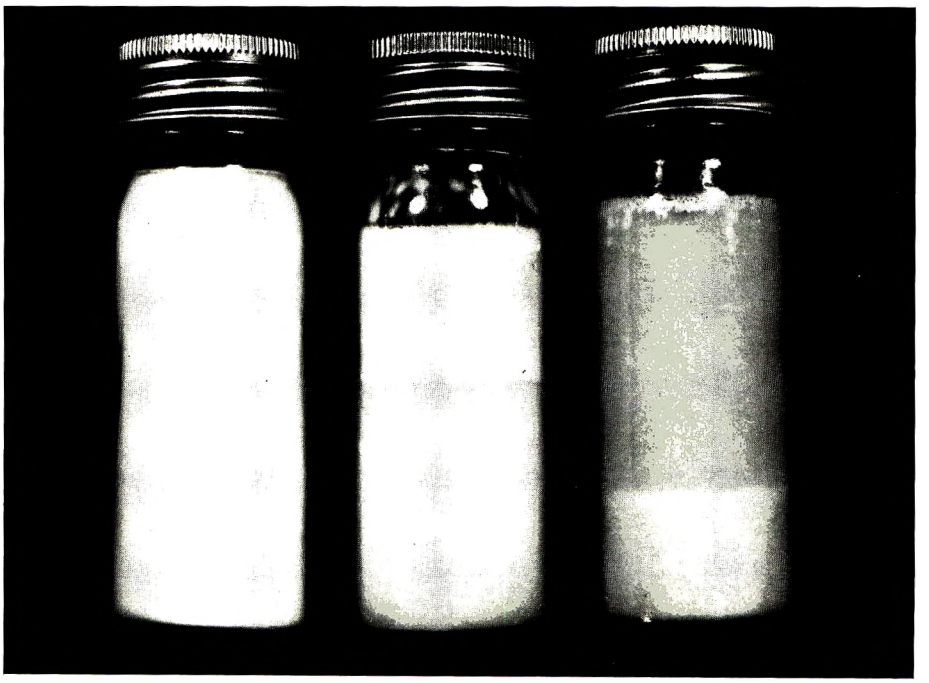

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EXPLANATION OF PLATE

(A) Changes in the appearance of milk from the L.F. quarter of cow L 629 following the intramammary inoculation of 10^7 uncloned A 417 T-mycoplasma culture. Left, day 0; centre, day 5; right, day 7.

(B) Cow M 153. L.F. quarter. Histological section showing neutrophil infiltration of the alveoli, and slight interstitial hyperaemia. H and E. × 147.

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Adenovirus, parainfluenza virus and respiratory syncytial virus antibodies in the sera of Jamaicans

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SUMMARY

Surveys for respiratory virus antibodies in the Jamaican population have shown that adenovirus, respiratory syncytial virus and parainfluenza types 1 and 3 virus antibodies are acquired early in life. The incidence of haemagglutination-inhibiting antibodies to parainfluenza viruses increases rapidly with age and almost all adults possess parainfluenza type 3 antibody, usually in high titre. Parainfluenza type 1 antibodies are only slightly less common. Complement-fixing antibodies to the adenovirus group were also observed to increase in incidence with age.

Complement-fixing antibody to respiratory syncytial virus was less common in Jamaican sera than antibody to the other respiratory viruses described here. The highest titres were observed in the youngest age-group.

INTRODUCTION

The aetiology and prevalence of a variety of respiratory viruses in the population of Jamaica has been examined by determining the pattern of virus isolations from persons clinically ill with respiratory infections (Jennings & Grant, 1967*a*) and by serological surveys for antibodies to influenza viruses in sera collected from Jamaican individuals of all ages (Jennings & Grant, 1967*b*; Jennings, 1968). The present paper is concerned with the incidence of adenovirus, respiratory syncytial virus and parainfluenza virus antibodies in Jamaica as determined by serological surveys.

Parainfluenza viruses, respiratory syncytial virus and adenoviruses have been associated with serious respiratory diseases of children. Parainfluenza viruses have been associated with croup (Lewis, Lehmann & Ferris, 1961) and respiratory syncytial virus with bronchiolitis in infants (Channock *et al.* 1961). Adenoviruses have been shown to cause severe and fatal pneumonia in young children (Chany *et al.* 1958). In adults all these viruses can cause mild, upper respiratory tract infections (Rhodes & van Rooyen, 1968).

Studies on the prevalence of antibodies to parainfluenza and respiratory syncytial viruses in different parts of the world have indicated that such antibodies are

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fairly widespread (Jensen, Peeler & Dulworth, 1962; Hambling, 1964; Suto *et al.* 1965) and are acquired early in life. Neutralizing antibodies to certain adenoviruses are also acquired early in life (Potter & Shedden, 1963).

Few studies on the incidence of respiratory viruses and their antibodies in tropical or subtropical areas have been carried out although surveys in Nigeria (Njoku-Obi & Ogunbi, 1966) and Panama (Monto & Johnson, 1967) have shown that antibodies to several respiratory viruses are not uncommon.

MATERIALS AND METHODS

Sera

The sources of the sera used have been described previously (Jennings & Grant, 1967*b*). They were from both sexes and all age-groups of the Jamaican population. Sera from 449 individuals were examined for haemagglutination-inhibiting (HI) antibodies to parainfluenza types 1 and 3 viruses, 510 sera tested for complement fixing (CF) antibodies to the adenovirus group and 558 sera tested for CF antibodies to respiratory syncytial (RS) virus. Most sera were from infants and children under 10 years of age.

Viruses and antigens

Parainfluenza type 1 antigen was prepared in human thyroid tissue cultures from a locally isolated strain, CV 837/64. Thyroid tissue monolayers were maintained using Eagle's basal medium (Eagle, 1955) with 2% calf serum and 0·5% lactalbumin hydrolysate. Antigen was prepared from monolayers showing strongly positive haemadsorption 12–14 days after inoculation. They were frozen and thawed three times, centrifuged at 2000 rev./min. for 60 min., the supernatant removed, dispensed and stored at $-70°$ C. The haemagglutinin titres of these fluids ranged from 1/32 to 1/128. Control antigen was prepared similarly using uninoculated thyroid cultures.

Parainfluenza type 3 antigen and control were obtained commercially from Flow Laboratories, Rockville, Maryland, U.S.A.

CF antigens from a locally isolated strain of type 3 adenovirus, RV 757 (Jennings & Grant, 1967*a*) and an RS strain, TRVL 1283, kindly supplied by Professor L. Spence from the Trinidad Regional Virus Laboratory were prepared in HEp-2 or HeLa cells according to methods described by Rose (1964).

Antisera

Standard HI antisera to parainfluenza types 1 and 3 viruses were obtained from Flow Laboratories.

Adenovirus group antiserum was prepared in rabbits by standard methods (Rose, 1964) using the locally isolated type 3 adenovirus strain, RV 757.

Antiserum for use in the RS virus CF test was obtained commercially from Microbiological Associates Inc., Bethesda, Maryland, U.S.A.

Serological procedures

The methods used for detecting HI antibodies to parainfluenza viruses have been described previously (Jennings & Grant, 1967*a*).

CF tests for RS virus and adenovirus antibodies were carried out using standard methods (Lief, Fabiyi & Henle, 1958) with the exception that fixation of complement was for 90 min. at 37° C. and incubation time following the addition of sensitized cells was for 30 min.

Sera for HI tests were treated with acid-washed kaolin and absorbed with guinea-pig erythrocytes before testing. Sera for CF tests were inactivated at 56° C. for 30 min. The microtechnique (Sever, 1962) was employed for all serological tests. In HI tests the lowest serum dilution examined was 1/20; in CF tests it was 1/4. To estimate geometric mean titres, sera negative at 1/20 in the HI test were assigned an arbitrary titre of 1/10.

RESULTS

Sera were divided into nine groups according to age of donor. The youngest group were infants under 1 year of age. Sera from individuals 1–10 years inclusive were divided into five age-groups each spanning 2 years. Sera from persons aged 11 years and over were divided into three age-groups: 11 to < 20 years, 20 to < 40 years and 40 years and over.

Haemagglutination-inhibiting antibodies to parainfluenza virus types 1 and 3

Table 1 shows that 257 sera, about 60 % of those examined for antibodies to parainfluenza type 1, were positive at or above 1/20, but up to the age of 5 years the percentage of positives was considerably lower. The incidence of HI antibody to this virus also declined in sera from persons of 40 years or over, whilst the greatest acquisition of antibody was observed in the 5 to < 7 year age-group.

HI antibodies to parainfluenza type 3 virus were very common in Jamaican sera (Table 2), and a total of 387 (86·2 %) were positive. Antibodies were not detected in infants under 1 year, but were rapidly acquired with age and all sera from 5- to < 7-year-old children were positive. In older age-groups, sera without parainfluenza type 3 antibodies were rare. The highest geometric mean antibody titre level was observed in the 5- to < 7-year age-group.

Antibodies to respiratory syncytial virus

CF antibodies to RS virus were found in 208 (37·2 %) of the sera tested (Table 3). Of the sera, 358 were from infants and children less than 11 years old and 130 (36·3 %) of these were positive.

The incidence of antibodies to RS virus was relatively low in the sera of infants and children up to 3 years of age, but thereafter the frequency increased to about 40 %, a frequency that was maintained with minor fluctuations into late adult life. However, the percentage of sera showing high titres decreased fairly steadily

Table 1. *Age distribution and geometric mean titres of haemagglutination-inhibiting antibody to parainfluenza type* 1 *virus in the sera of Jamaicans*

Age-group (years)	No. of sera tested	No. positive	Positive (%)	Geometric mean antibody titre
0 to < 1	21	1	4·8	13·3
1 to < 3	51	8	15·7	13·5
3 to < 5	47	16	34·0	18·5
5 to < 7	54	34	63·0	64·1
7 to < 9	64	52	81·3	49·4
9 to < 11	67	50	74·6	48·2
11 to < 20	64	51	79·7	35·5
20 to < 40	42	28	66·7	33·8
⩾ 40	39	17	43·6	17·4
Totals	449	257	57·2	

Table 2. *Age distribution and geometric mean titres of haemagglutination-inhibiting antibody to parainfluenza type* 3 *virus in the sera of Jamaicans*

Age-group (years)	No. of sera tested	No. positive	Positive (%)	Geometric mean antibody titre
0 to < 1	21	0	0	10·0
1 to < 3	51	23	45·1	38·0
3 to < 5	47	38	80·9	52·6
5 to < 7	54	54	100·0	193·7
7 to < 9	64	63	98·4	161·4
9 to < 11	67	67	100·0	127·5
11 to < 20	64	63	98·4	146·7
20 to < 40	42	41	97·6	139·3
⩾ 40	39	38	97·4	123·3
Totals	449	387	86·2	

Table 3. *Complement-fixing antibodies to respiratory syncytial virus in sera from the population of Jamaica*

Age-group (years)	No. tested	Positive		Sera with titre of 1/16 or above	
		No.	%	No.	%
0 to < 1	25	4	16·0	2	50·0
1 to < 3	66	17	25·8	5	29·4
3 to < 5	76	33	43·3	6	18·2
5 to < 7	58	20	34·5	3	15·0
7 to < 9	62	25	32·0	7	28·0
9 to < 11	71	31	43·7	2	6·5
11 to < 20	81	36	44·4	2	5·6
20 to < 40	61	21	34·4	1	4·8
⩾ 40	58	21	36·2	0	0
Totals	558	208	37·2	28	

Table 4. *Complement-fixing antibodies to the adenovirus group in sera from the population of Jamaica*

Age-group (years)	No. tested	Positive		Sera with titre of 1/16 or above	
		No.	%	No.	%
0 to < 1	20	3	15·0	0	0
1 to < 3	38	13	34·2	2	13·4
3 to < 5	64	38	59·4	10	26·3
5 to < 7	62	34	54·8	11	40·7
7 to < 9	76	45	59·2	18	40·0
9 to < 11	71	44	62·0	18	40·9
11 to < 20	78	50	64·1	9	18·0
20 to < 40	44	30	68·2	13	43·3
≥ 40	57	18	31·6	6	33·3
Totals	510	275	53·9	87	

as age increased and the titres, highest in the two youngest age-groups, were considerably lower in adults and adolescents.

Antibodies to the adenovirus group

A total of 510 sera were examined for antibodies to the adenovirus group CF antigen, and 275 (53·9 %) were positive at dilutions of 1/4 or higher.

The distribution of antibodies according to age (Table 4) shows that few infants possessed adenovirus CF antibodies. Such antibodies were present in the sera of more than half the children tested by 5 years, and although a slight decrease in frequency was noted in 5- to < 7-year-olds, adenovirus CF antibody became more prevalent as age increased up to 40 years. In older persons there was a decrease in antibody prevalence.

Table 4 also shows the percentage of positive sera with titres of 1/16 or above. Higher titres were encountered in the older age-groups, with most high-titred sera in the 20 to < 40 age-group.

DISCUSSION

The surveys for antibodies to parainfluenza types 1 and 3 viruses show they are widespread in the Jamaican population. Parainfluenza type 3 antibodies were detected in almost every serum tested from persons older than 4 years and over half also contained parainfluenza type 1 antibodies.

High incidence of parainfluenza antibodies has been reported in surveys in both tropical and temperate zones. In America, Jensen *et al.* (1962) found 80–90 % of children aged 1–4 years to have parainfluenza type 3 antibody in their sera and only two sera from 470 older individuals were negative in this respect. In the same survey, a third of the sera tested for parainfluenza type 1 antibodies in the 1–4 age-group were positive and 60–70 % positives were found in older children and adults. Antibodies to parainfluenza viruses are also common in sera from Europeans (Forsgren, Sterner & Wolontis, 1965) and in tropical countries (Monto & Johnson, 1967).

The incidence of parainfluenza type 1 and 3 antibodies in Jamaican sera is thus similar to that in other areas. They are acquired early in life and are very prevalent in the adult population.

The survey for RS virus antibodies in Jamaica shows the virus is active in the island, mainly in children. However, only 43 % of 76 sera from 3- to < 5-year-old Jamaican children were positive and in sera from children aged 5 to < 9 years antibody was even less frequent.

In England, the incidence of CF antibodies to RS virus is much higher. Hambling (1964) found 66 % of children aged 2–4 years to be positive and Moss, Adams & Tobin (1963) detected RS antibody in 93 % of sera from 14 children aged 3–5 years. In an industrial population in North America over 66 % of sera from children aged 6 years contained RS virus antibody (McClelland *et al.* 1961). Above 6 years of age RS virus antibodies are very common in the general population (Moss *et al.* 1963; Suto *et al.* 1965).

The findings reported here suggest that antibody to RS virus is relatively uncommon in the Jamaican population but agrees with findings elsewhere in showing that such antibody is acquired up to 5 years of age, indicating that this segment of the population may be particularly susceptible to infection by this virus.

RS virus epidemics occur over short periods of time and both neutralizing and CF antibodies have short survival times in humans (Suto *et al.* 1965). Thus, the time of collection of the sera would affect the prevalence of antibody to RS virus and the sera used in the present survey may have been obtained during an interepidemic period. On the other hand, it has been suggested that severe infections produced by RS virus may be more frequent in industrialized regions that in less urbanized areas (Holzel *et al.* 1965). Jamaica, besides being a tropical area is practically free from heavy industrialization, a factor which may have contributed to the results obtained.

The survey for adenovirus group CF antibodies in Jamaica shows such antibody to be acquired early in life, the incidence increasing from 15 % in infants under one year to over 50 % by 5 years of age. Similar findings have been reported in England (Potter & Shedden, 1963). Although no epidemiological information on the incidence of individual adenovirus types can be obtained using the CF test, it seems probable that the antibodies observed in young Jamaicans are produced in response to infections by types 1, 2, 3 and 5 adenoviruses as these types have been recovered from sick children in Jamaica (Jennings & Grant 1967*a*).

Adenovirus CF antibodies continue to be acquired with age by the Jamaican population, probably through infection by different adenovirus types, up to the age of 40, after which the prevalence of these antibodies is reduced.

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Antibody responses and resistance to challenge in volunteers vaccinated with live attenuated, detergent split and oil adjuvant A2/Hong Kong/68 (H_3N_2) influenza vaccines*

A report to the Medical Research Council Committee on Influenza and other Respiratory Virus Vaccines

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SUMMARY

Forty-nine subjects were vaccinated with either live attenuated, detergent split, or oil adjuvant A2/Hong Kong influenza vaccines, or a saline influenza B vaccine as control. Respiratory symptoms occurred more frequently in subjects who received the live vaccine but in total there was little difference between the symptoms in the four groups. Antibody titres in nasal washings and serum were measured by haemagglutination inhibition, neuraminidase inhibition and virus neutralization tests. The oil adjuvant vaccine stimulated larger antibody responses than the other procedures. Six weeks after vaccination the volunteers were challenged with partially attenuated live A2/Hong Kong influenza virus administered intranasally. The live attenuated and oil adjuvant vaccines provided the best protection against challenge.

INTRODUCTION

Many studies have been made of various influenza vaccines including unsplit inactivated virus in saline, detergent-split material, oil-adjuvant preparations of unsplit inactivated virus and live attenuated virus. Not one is agreed to provide a consistent high level of immunity against infection and there has been no study in which the efficacy of all four types have been compared. This is partly because of the difficulty of employing some of the methods used to determine the efficacy of vaccination and of interpreting the results of most of them. Most studies have measured circulating haemagglutination inhibiting (HI) antibody. However, it is known that vaccination produces also antineuraminidase (AN) antibody, and that

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both types of antibody appear in nasal secretion and serum and may be important in mediating resistance to infection (Slepushkin *et al.* 1971). Some workers emphasize the importance of neutralizing antibody. The relative importance of the site and type of antibody in protection against infection has not yet been established, although there is consistent evidence that the presence of circulating HI antibody is correlated with resistance to infection (Hobson, Beare & Gardner, 1972). Immunity induced by vaccination has also been examined by studies of the incidence of influenza in vaccinated subjects subsequently exposed to natural infection. Such studies have often been unsuccessful because the expected outbreak of influenza failed to occur. However, an artificial as opposed to a natural challenge procedure overcomes this difficulty and limits the number of subjects needed (e.g. Beare, Hobson, Reed & Tyrrell, 1968; 1969).

In order to acquire a better understanding of the immunogenicity of the various types of influenza vaccine in a partly immune population – typical of an interpandemic period – an intensive study was made in volunteers vaccinated with live attenuated, detergent split and oil-adjuvant influenza vaccines of the A2/Hong Kong serotype, who were subsequently challenged with live virus of the same serotype.

MATERIALS AND METHODS

A total of 49 healthy employees aged 21 to 65 years at the Beckenham Laboratories of the Wellcome Foundation volunteered for this study. Informed consent was obtained from all subjects. Subjects were allocated to one of four groups and the groups were matched as far as possible according to the history of influenza, vaccination against influenza in earlier years, age and sex (Table 1). No volunteer had been recently immunized against influenza, but five subjects suffered an influenza-like illness at or about the time that the first study specimens were collected. It was subsequently shown that four of these subjects were infected with influenza A, which was prevalent at the time the study began in December 1969. These subjects were accepted into the rest of the trial, but the results obtained were not included in the final analysis.

Subjects were vaccinated with one of three A2/Hong Kong/68 (or equivalent) influenza vaccines or a control influenza virus B vaccine. Six weeks after the vaccination all subjects were challenged with a partially attenuated A2/Hong Kong/1/68 influenza strain. Reactions to vaccination and challenge procedures were monitored for 10 days after administration.

Vaccines

Group A. Monovalent live attenuated influenza. A dose of $10^{5\cdot5}$ EID50 of A2/Hong Kong/1/68 (H_3N_2) was given intranasally as nose drops in a total volume of 1 ml. (0·5 ml. to each nostril). This strain was attenuated by 11 passages in leucosis-free eggs in the presence of equine serum (γ-inhibitor), at 33° C. The development and characteristics of this strain have been described in detail elsewhere (Beare & Bynoe, 1969).

Group B. Standard bivalent inactivated saline deoxycholate-split influenza

Table 1. *Composition of experimental groups*

	Live A	Split A+B	Oil adjuvant A	Unsplit B
No. of subjects	11	13 (9)	12	13
No. of females	3	3 (1)	4	4
No. of males	8	10 (8)	8	9
Average age females (years)	29	30	41	27
Average age males (years)	32	44	35	30
No. given influenza vaccine last year	7	6 (5)	7	7
No. who had recent influenza-like illness	2	2 (0)	3	4

( ) = number of subjects challenged.

vaccine was given subcutaneously in a 1 ml. volume. This vaccine contained, before detergent treatment, A2/Northern Territories/60/68 (H_3N_2), 8000 HA units, and B/Victoria/2/65, 3000 HA units and was provided by Wellcome Research Laboratories.

Group C. Monovalent oil-adjuvant double-emulsion vaccine was given intramuscularly in an 0·375 ml. volume. This vaccine contained A2/England/344/68 (H_3N_2) 3500 Ha units in a double emulsion of Drakeol and Arlacel. A2/Northern Territories/60/68 and A2/England/344/68 are both A2/Hong Kong/68 serotypes as indicated by the addition of (H_3N_2) as recommended recently by the World Health Organisation (1971).

Group D. Control group. A monovalent inactivated saline vaccine was given subcutaneously in a 1 ml. volume. This vaccine contained B/England/5/66, 7000 HA units, and together with vaccine for Group C was kindly provided by Evans Medical Limited.

Challenge virus

A dose of 10^5 EID 50 of monovalent live influenza A2/Hong Kong/1/68 was given intranasally as nose drops as for Group A. This strain was partly attenuated by six passages at temperatures down to 25° C. This and the vaccine for Group A were kindly provided by Dr A. S. Beare.

Specimens collected

Blood was collected at or shortly before the beginning of the experiment, 3 and 6 weeks after vaccination, and again 2 weeks after challenge – 8 weeks after vaccination.

Sets of nasal washings were collected on each of three successive days, one set before, one at 3 and one at 6 weeks after vaccination. A total of 20 ml. of phosphate-buffered saline was applied in small volumes, successively to each nasal cavity, with the subject in a sitting position. Five to 10 ml. of nasal effluent were usually

collected although recovery rates were somewhat variable. Specimens were initially stored at −20° C. then tested for the presence of blood (with Hemostix, Ames Limited). Negative specimens were dialysed against distilled water, pooled and freeze-dried, and then reconstituted in saline to one-tenth the volume of the original washing. Nasal washings for virus isolations were collected from the subjects who were vaccinated with live virus and from all subjects on the 1st, 2nd and 3rd days of challenge. These were mixed with an equal volume of nutrient broth and stored at −70° C. They were subsequently tested by inoculation of 0·2 ml. volumes into the allantoic cavity of 10-day-old chick embryos.

Antibody assays

Haemagglutination inhibition (HI) test. The virus used was an inhibitor-resistant strain of A2/Hong Kong/1/68. The sera were inactivated for 30 min at 56° C. but the nasal washings were not heated. Twofold dilutions were made in 0·2 ml. volumes in WHO plastic plates, using phosphate-buffered saline as diluent (World Health Organization, 1953). Four HA units of virus and 1 % human group O red cells were used. The serum-virus mixtures were held at room temperature for 30–60 min. before the addition of red cells. This method was compared with the use of cholera-filtrate treatment and inhibitor-sensitive virus and was simpler and gave more satisfactory results when antibody titres were low.

Neuraminidase-inhibition (AN) tests. For neuraminidase-inhibition tests the method used was essentially that described by Schild & Newman (1969) with the following modifications to increase the sensitivity of the test. (*a*) The concentration of neuraminidase (purified virus) was adjusted so that on incubation with excess substrate for 16 hr. at 37° C. the amount of *N*-acetyl/neuraminic acid released per 0·08 ml. of virus gave an OD_{549} reading of 0·4–0·5 OD units. (*b*) For the enzyme neutralization test virus and serum dilutions were incubated at room temperature for 3 hr. The virus used was a recombinant A/FPV/Dutch/27 (Havl)–A/Hong Kong/68 (N_2). The use of this recombinant eliminated the possibility of non-specific inhibition of neuraminidase activity by antibody to Hong Kong haemagglutinin (H_3).

Neutralization (N) tests. The virus was a calf-kidney-adapted strain of A2/HK/68. The sera were used after inactivation at 56° C. for 30 min; washings were not heated. Fourfold serial dilutions were mixed with an equal volume of a dilution of allantoic fluid containing an estimated 10 TCD50 of virus. The mixtures were held at room temperature for 20 min and 0·2 ml. was inoculated into each of two tubes of secondary calf kidney cells. Many tests were repeated because the dose of virus was too high or too low, but in general the results were reproducible. These tests were done last and specimens were only tested when a complete set was available.

In spite of the fact that groups were matched as far as possible on clinical grounds, some differences in base-line antibody titres were found between the groups (Tables 3–8). Thus serum HI antibody titres and nasal antineuraminidase titres before vaccination were somewhat lower in the control group and higher in the saline split vaccine treated group than in others.

Table 2. *Clinical reactions to vaccination*

	Indicated result in subjects given vaccine			
	Live A2/HK	Split A2/HK + B	Adjuvant A2/HK	Unsplit B
No. vaccinated	11	13	12	13
No. without symptoms	3	3	3	4
Constitutional symptoms (headache, fever, anorexia, pain in back and legs)	37*	27	25	16
Respiratory symptoms (nasal discharge, obstruction and sore throat)	37	21	18	11
Local symptoms (pain and redness at injection site)	—	21	10	34
Total symptoms	89	75	53	57
Total symptoms per subject	8·1	5·0	4·4	4·0
Total symptom points†	134	112	86	71

* Total number of symptoms of all degrees of severity reported by volunteers at any time during observation for 10 days after vaccination.

† The scores given above have been devised by allotting 3 points for severe symptoms, 2 for moderate and 1 for mild; severe symptoms were rare.

RESULTS

Clinical reactions to vaccination

Symptoms were recorded against a check-list each day for 10 days after the vaccination and challenge procedures. Injection sites were examined clinically 24 hr. after vaccination. The results, presented in Table 2, show that rather more constitutional and respiratory symptoms were encountered in those given live vaccine. The number of volunteers in each group was small and, as they were not in isolation, respiratory symptoms due to the vaccine could be confused with those of intercurrent respiratory infections. Since a sensitive method was used to record reactions to vaccination, these background symptoms were doubtless collected. Thus, although the number of symptoms recorded in the post-vaccination period in all groups is large, they were not all due to the vaccines and were higher than might be expected in a general vaccination programme. In fact, no volunteer complained about the reactions to vaccination. Symptoms at the injection site occurred only in the parenterally inoculated subjects and seemed to be less frequent in those given oil-adjuvant vaccine. In particular, on examination, local redness did not occur in any subject given oil-adjuvant vaccine, while it was seen in five subjects given control influenza B vaccine and in a few who received split vaccine.

Serological response to vaccination

The results of vaccination were assessed by HI, AN and N antibody responses in serum and nasal washings for each volunteer. The titres for each group are shown in Tables 3–8 and summarized in Table 9.

There was some rise in titre of circulating HI in the control group, presumably due to some undiagnosed or asymptomatic natural A 2 influenza. In the vaccinated

Table 3. *Distribution of serum HI titres before and after vaccination and challenge*

Vaccine	Live A2				Split A2+B				Oil-adjuvant A2				Unsplit B			
Serum specimen ...	i	ii	iii	iv	i	ii	iii	iv	i	ii	iii	iv	i	ii	iii	iv
Titres																
8	—	—	—	—	—	—	—	—	1	—	—	—	2	—	—	—
16, 24	1	—	—	—	2	—	—	—	2	—	—	—	1	1	2	—
32, 40, 48	3	1	3	—	3	1	3	—	1	—	—	—	2	2	2	1
64, 96	3	3	1	2	2	3	2	1	5	—	—	1	2	4	1	2
128, 192, 240	1	3	2	5	2	2	2	2	1	3	1	—	1	1	2	4
256, 384	1	1	—	1	1	1	2	3	1	1	—	1	2	2	1	—
⩾ 512	1	2	3	2	2	5	3	6	1	8	11	9	—	—	2	3
Totals	10	10	9	10	12	12	12	12	12	12	12	11	10	10	10	10
Geometric means	76·6	148·8	170·9	188·5	90·9	248·6	177·5	404·2	69·1	547·8	856·5	770·8	47·1	74·4	102·2	188·5

In these and following tables serum specimen i was taken before vaccination, ii at 3 weeks and iii at 6 weeks after vaccination. Specimen iv was taken after challenge. The table shows the number of specimens with a titre in the indicated range.

Table 4. *Distribution of nasal HI titres before and after vaccination*

Vaccine	Live A2			Split A2+B			Oil adjuvant A2			Unsplit B		
Nasal specimen ...	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
Titres												
2, 3	2	1	—	—	1	—	2	1	—	—	—	—
4, 6	4	2	1	3	1	—	—	1	—	2	3	1
8, 12	1	2	—	7	5	2	8	2	1	7	3	4
16, 24	3	4	5	2	3	4	2	4	3	—	3	4
32, 48	—	—	2	—	—	3	—	2	3	1	—	—
64, 96	—	1	2	—	—	—	—	2	3	—	1	—
> 128	—	—	—	—	—	—	—	—	2	—	—	—
Totals	10	10	10	12	10	9	12	12	12	10	10	9
Geometric means	7·5	12·6	22·9	8·4	8·7	20·7	8·7	19·8	47·3	10·6	12·6	11·0

Nasal specimen i was a pool of three samples collected before vaccination. Specimen ii was a similar pool collected 3 weeks and iii collected 6 weeks after vaccination.

Table 5. *Distribution of serum AN antibody titres before and after vaccination*

Vaccine ...	Live A		Split A+B		Oil-adjuvant A		Unsplit B	
Nasal specimen ...	i	iii	i	iii	i	iii	i	iii
Titre								
< 5	2	—	—	—	3	—	3	—
5	2	2	4	1	1	—	2	1
10, 15	2	1	5	5	1	1	3	6
20, 30	2	2	1	3	4	—	1	2
45, 50, 60	1	2	1	—	—	2	1	1
80, 120, 150	—	2	—	1	1	6	—	—
160	—	—	1	2	1	—	—	—
320	1	1	—	—	—	2	—	—
640	—	—	—	—	—	—	—	—
⩾ 1280	—	—	—	—	—	—	—	—
Totals	10	10	12	12	11	11	10	10
Geometric means	14	34	12	26	14	98	8	17

groups the mean serum antibody titres did not rise further after 3 weeks although in several instances nasal titres rose more slowly. Maximal titres were found in all groups by 6 weeks and on these further studies are concentrated. The statistical significance of each increase in titre is shown in Table 9. There were small but significant rises in serum AN after live and split vaccines but no significant changes in nasal AN, while the rises in nasal HI were greater and were highly significant after live vaccine. Neutralizing antibody rises were greater in serum after split than after live vaccine and the small rises of nasal neutralizing activity did not reach statistical significance even after oil-adjuvant vaccine. The most striking general conclusion was that there were large and highly significant rises of antibodies measured by all methods after oil-adjuvant vaccine, ranging from 2·29 for nasal AN to 17·84 for serum N.

Multiple antibody rises were seen in a number of volunteers. For example, of 25 volunteers given vaccine, eight showed fourfold or greater rises by two or three tests in the serum and four by the same criteria in nasal washings. On the other hand, of five subjects who gave a history of influenza about the time of vaccination, four showed a rise in nasal washings and five in serum. Furthermore there were instances in which a rise was detected by only one test – for example, in the serum of the same 25 volunteers 5 by HI, 4 by AN and 3 by N alone.

Challenge of volunteers

A partly attenuated virus was used to estimate the degree of immunity induced by vaccination. Subjects were inoculated intranasally approximately 6 weeks after they were vaccinated and the result was assessed by virus recovery, circulating HI antibody titrations and clinical response. There was in this study a clear relationship between infection and the occurrence of respiratory symptoms, for infection was detected in 14 of 21 subjects with symptoms and in four of 24 without ($P < 0{\cdot}01$). It can be seen in Table 10 that a majority of the control subjects

Table 6. *Distribution of nasal AN titres before and after vaccination*

Vaccine ...	Live A			Split A + B			Oil-adjuvant A			Unsplit B		
Nasal specimen ...	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
Titres												
< 2	7	6	5	5	6	5	7	4	3	8	8	7
2	—	—	—	—	—	—	—	—	—	—	—	—
5	—	1	—	—	—	—	2	3	3	1	—	1
10	1	2	2	3	4	3	1	2	—	—	—	—
15	—	—	—	1	—	—	—	2	2	—	—	—
20	—	—	1	—	—	—	—	—	—	—	—	—
25	1	1	1	—	—	1	—	1	1	—	1	1
50	—	—	—	—	—	—	—	—	1	—	—	—
Totals	9	10	9	9	10	9	10	12	10	9	9	9
Geometric means	1·8	2·6	3·3	2·9	2·5	3·1	1·7	4·5	5·7	1·2	1·4	1·7

Table 7. *Distribution of serum N titres before and after vaccination*

Vaccine ...	Live A		Split A+B		Oil adjuvant A		Unsplit B	
Nasal specimen ...	i	iii	i	iii	i	iii	i	iii
Titres								
4, 6	—	—	—	—	1	—	2	—
8	1	—	2	—	—	—	—	1
16	2	1	—	—	1	—	1	2
32	1	2	2	—	1	—	—	—
64, 96	—	—	1	—	—	—	1	—
128	1	1	3	7	1	—	1	1
256	—	—	—	1	—	—	1	2
512	1	1	—	—	2	1	1	1
⩾ 1024	1	2	—	—	—	5	1	1
Totals	7	7	8	8	6	6	8	8
Geometric means	70·7	141·3	41·5	139·6	76·9	1290·2	67·3	98·7

Table 8. *Distribution of nasal N titres before and after vaccination*

Vaccine ...	Live A		Split A+B		Oil-adjuvant A		Unsplit B	
Nasal specimen ...	i	iii	i	iii	i	iii	i	iii
Titres								
< 1	2	2	3	2	1	—	2	2
1	3	1	1	1	—	—	3	2
2	1	2	2	2	—	1	1	1
4	1	2	1	1	2	1	1	3
8	—	—	1	1	1	1	—	—
⩾ 16	—	—	—	1	2	3	1	—
Totals	7	7	8	8	6	6	8	8
Geometric means	< 1	< 1	< 1	1·5	4·3	8·0	1·0	1·0

Table 9. *Rises in antibody titres during the first* 6 *weeks after vaccination, expressed as a proportion of the initial value*

Vaccine	Live A	Split A+B	Oil adjuvant A	Unsplit B
Nasal HI	1·93***	1·36**	3·83***	0·05
Serum HI	0·89*	0·74	6·50***	1·02*
Nasal AN	0·80	0·06	2·27***	0·43
Serum AN	1·39*	0·97*	6·10***	1·05
Nasal N antibody	0·35	0·89	0·96	0·00
Serum N antibody	1·21	2·36**	17·84***	0·47

Each figure in the table is the geometric mean of the ratio of rise in titre to the initial titre for the subjects in the appropriate group. *, **, *** denote that the rises were significantly different from zero at 5 %, 1 % and 0·1 % levels, respectively.

The analyses of variance (using log titres) showed differences between the four groups in rises of titres of nasal and serum HI, and serum N antibodies (all at 1 % significance level) and of serum AN (at 5 % significance level); the heterogeneity is almost entirely due to the larger antibody rises in the subjects given oil adjuvant vaccine.

Any slight disparity between the results as shown above and in the previous tables is due to a difference in the approximations used.

Table 10. *Results of challenge*

	Wellcome Laboratories			Oil refinery	
Vaccine given	Antibody rise	Virus isolated	Clinical response	Antibody rise	Virus isolated
A. Live influenza A	2/10	1/10	3/10	—	—
B. Split A+B	3/9	6/9	7/9	3/38	5/33
C. Oil-adjuvant A	1/12	2/12	3/12	—	—
D. Unsplit B	3/10	7/10	7/10	9/24	6/23

Five volunteers at Wellcome Laboratories had natural influenza at the beginning of the study. Of the one receiving live influenza A vaccine and three receiving unsplit B vaccine none was infected or developed symptoms on challenge. One who received split A+B was not challenged. These have not been included in the above table.

and rather fewer of those given split vaccine were infected; on the other hand, most of those given either live or oil-adjuvant vaccine resisted challenge. With the small numbers in this study, the difference between the results in the split vaccine and control group are not significant. However, in a larger supplementary study, carried out at an oil refinery, it is clear that the split vaccine provided protection against a similar artificial challenge procedure. At Beckenham four subjects who had clinical influenza at the start of the trial followed by one or other vaccine were resistant when challenged.

Comparing these with the serological results, it is not surprising to find that the oil-adjuvant vaccine protected well, in view of the high titres of antibody it produced. However, it is surprising that the live influenza vaccine protected to a similar extent. The relation between antibody titres and resistance to infection was therefore studied in the whole group of volunteers.

The relationship between antibody titre and resistance to infection

The absolute and relative contributions of local antibody and circulating antibody to resistance to infection is still uncertain. To examine the evidence provided by the results of the serum titrations after vaccination and the outcome of the challenge, linear discriminant analysis was used, as in a previous study (Slepushkin *et al.* 1971).

The challenge result was quantified, no infection being taken as 0 and infection as 1, and regressed on each of the antibody titres, giving six linear predictors of the challenge result. A good predictor, or discriminant function, is one giving good separation between the two groups of subjects (i.e. those who contracted influenza and those who did not). As a measure of this separation, d/s, the ratio of the difference between the means of the predicted values for the two groups (d) to the estimated standard deviation within the groups (s), was calculated for each discriminant function; results are shown in Table 11, together with the number of observations on which the calculation was based. The value of d/s is approximately 4·0 for a discriminant function that correctly classifies 95 % of the subjects. Thus none of the antibody titres was particularly good at predicting the outcome of the challenge, but the values of d/s show that serum titres of HI, AN and N

Table 11. *Prediction of outcome of challenge from antibody titres*

	Circulating			Nasal			All six antibody titres
	HI	AN	N	HI	AN	N	
No. with antibody titre measures	45	44	31	43	32	31	45
d/s	0·97	0·97	1·01	0·67	0·68	0·32	1·18
No. correctly classified	32	31	21	26	24	17	37

antibody were all of approximately equal value, and better than nasal HI and AN Nasal N antibody was of no value as there was no significant difference between its titres for the two groups.

Similar results were obtained when estimates of the missing observations, calculated from those titres which had been measured, were used in the analyses. Using these estimated observations, multiple regression of the challenge result on all six titres gave a linear discriminant function for which d/s was found to be 1·18. The residual sum of squares was not significantly smaller than that for the linear regression on circulating N antibody titres alone, so that no combination of titres was a better predictor than the single titres mentioned above.

The predictors can also be roughly evaluated by classifying each subject as susceptible or resistant to infection, according to whether his predicted value is greater or less than 0·5. The predictions can be compared with the results of the challenge; the number of subjects correctly classified by each predictor is also shown in Table 11. A worthless linear predictor ($d/s = 0$) will classify about half the subjects correctly.

The results indicate that some factor other than those measured is involved in resistance to infection by artificial challenge. There were too few sera to examine the question of whether the predictive value of the antibodies was the same whether induced as a result of natural or vaccine infection or of either form of parenteral vaccination.

DISCUSSION

There is, as far as we know, no published record of a comparison of live attenuated, detergent-split and oil-adjuvant influenza vaccines in which reactions to vaccination, local and circulating HI, AN and N antibody responses and also resistance to challenge have been examined. The amount of laboratory work involved in this study limited the number of subjects, but the intensive monitoring of response to vaccination yielded results which would be difficult to achieve in a large-scale field trial. It is unfortunate that at the start of the trial natural influenza occurred in some subjects and it is impossible to be certain that no other subjects were infected. However, the clear differences between the vaccination groups and the results in the group given influenza B vaccine indicate that this did not disturb the trial results to an important degree. Large-scale field trials are necessary, for they provide a better assessment of the general acceptability and reactivity of vaccines than can the present type of investigation. Live vaccine strains which

produced readily detectable symptoms in volunteers at the Common Cold Unit produced no detectable symptoms when administered in offices and factories (unpublished data). It is difficult to compare the discomfort of one sort of symptom with another, but the respiratory symptoms produced by the live vaccine were numerically roughly equivalent to the local symptoms which followed the injected vaccines. However, this trial population was partially immune, and there might have been relatively more symptoms in those given live vaccine if a higher proportion had been susceptible.

In this study the protection afforded against artificial challenge forms the most important index of the efficacy of vaccination. It is clear that the live and oil-adjuvant vaccines gave the most satisfactory level of protection and, although the latter have been out of favour recently, they merit further consideration, and it would be worth confirming the degree of protection against natural infection in a field study.

The rises in titre of circulating AN after live and killed vaccines confirm those reported in previous studies (Slepushkin *et al.* 1971; Schild & Newman, 1969; Downie, 1970; Kasel *et al.* 1969). We were surprised to find such small increases in nasal N antibody titre even when good protection was produced, though our earlier studies had shown that circulating antibodies were more important than local in resistance to infection; this and the results of the statistical analysis make it clear that we cannot at the moment predict satisfactorily from antibody measurements the resistance to infection induced by vaccines. As in our early studies with influenza B, we cannot explain why the live vaccine protected better than saline-killed vaccine (Beare *et al.* 1968). Other immunological mechanisms must be involved and consideration is being given to cell-mediated immunity, and to IgE antibody attached to cells in the respiratory tract.

We would like to thank the volunteers for their willing and conscientious help and Mr R. Newman for technical assistance. We thank Dr A. Ward Gardner and Dr A. S. Beare for conducting the trial at the oil-refinery and making their results available to us.

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Isolation for the control of infection in skin wards

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SUMMARY

An isolation policy in a hospital for skin diseases is reported. Patients carrying penicillin- and tetracycline-resistant organisms were to be isolated in single rooms, though barrier nursing was not practised. The policy failed because the single beds rapidly became blocked with long-stay patients and because even in a single-bed unit patients acquired staphylococci within 3–7 days of admission. Patients with skin diseases often do not feel 'ill' and resent isolation.

INTRODUCTION

Studies on the transmission of micro-organisms, especially *Staphylococcus aureus*, in wards for patients with diseases of the skin have revealed that such patients are not only prolific sources of organisms but also are more susceptible to colonization and infection than are other patients (Biro *et al.* 1960; Biro, Gibbs & Leider, 1960; Selwyn, 1963, 1965; Wilson, White & Noble 1971; Nystrom & Molin, 1972). Patients with diseases of the skin may suffer sepsis as a result of acquiring organisms in hospital and they may also prove a menace to others (Ayliffe & Collins, 1967; Payne, 1967). Clinicians feel that skin patients acquiring penicillin and tetracycline resistant organisms more often have their progress retarded than do other patients (Noble, 1970). Finally, although many factors other than purely microbial ones must influence a patient's progress (PHLS, 1960), patients with, for example, psoriasis, who are colonized by *Staph. aureus* whilst in hospital have a longer stay in hospital than do those not so colonized (Wilson *et al.* 1971). Finally the works of Hurst & Grossman (1960) and Wentworth, Miller & Wentworth (1958) showed clearly that the discharge of patients carrying staphylococci that they had acquired in hospital may result in the appearance of lesions in members of the patients' families. Given the propensity of skin patients to disseminate their organisms this can also be assumed to occur in the families of patients with diseases of the skin.

During the studies reported by Wilson *et al.* (1971) St John's Hospital for Diseases of the Skin consisted of four open wards each housing 16 or 17 patients with two single-bed side-rooms only. The opportunity was taken during upgrading of these facilities to combine the two male wards and also the two female wards and to reconstruct the interior of one ward for each sex as single rooms although this meant a sacrifice of beds (Fig. 1). Each of the 12 single rooms in each unit is ventilated by extracting air at a rate of 150 ft.^{3}/min. and the central corridor is

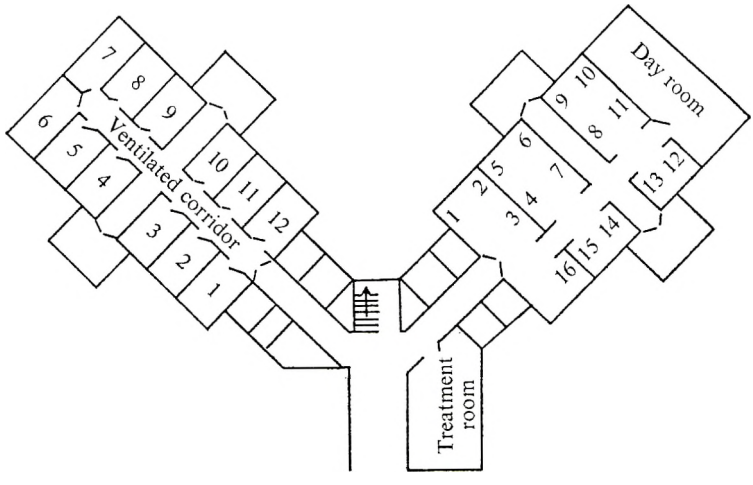

Fig. 1. Plan of ward to show single rooms and open beds.

fed with warmed air filtered to 5 μm. at 2000 ft.^{3}/min., the temperature of the incoming air being under the control of the ward sister. The open-ward sector of each new unit was reconstructed in enclaves of two or four beds to provide sound-baffling, and a day-room with colour television was completely divided off, where patients could talk, play card-games, etc., and also take their meals.

This seemed a reasonable situation in which to try to isolate patients with 'dangerous' organisms with a view to reducing the transmission of organisms between patients. This paper reports the investigation and its failure.

MATERIALS AND METHODS

As previously (see Wilson *et al.* 1971, for details), patients were swabbed in the nose, chest and groin on admission and twice a week thereafter.

In the absence of any known marker of 'dangerous' organisms that could readily be used in an epidemiological investigation, it was decided to isolate patients carrying organisms resistant to both penicillin and tetracycline. Patients were admitted to the single-bed sector and swabbed on, or soon after, admission. Written reports were taken to the ward sister each day and twice a week a complete statement of the carriage state of all patients was prepared for use on the ward. If a patient was found to be free of 'dangerous' organisms at the three control sites and any lesion sites he was moved to the open ward. If carrying 'dangerous' organisms he was left in his single room. Thus a patient free of any *Staphylococcus aureus*, *Pseudomonas* or *Proteus* could be transferred to the open ward within about 24 hr. of admission; reports on other patients took longer to prepare when antibiotic sensitivity patterns had to be determined. An exception to the rule was that cutaneous beta-haemolytic streptococci were regarded as 'dangerous' although penicillin-sensitive.

No changes were made in nursing procedures; it is recognized that 'isolation' or 'barrier' nursing requires more staff than open-ward nursing and change in nursing

procedures was not contemplated in this investigation. However, when the new nursing units were formed a ruling had been laid down that patients in single rooms (for whatever cause) should be nursed in those rooms and all treatments, etc., take place there. Patients in the open sector had their treatments carried out in cubicles in the newly reconstructed treatment room. This policy was of course adhered to in the study. Patients were asked to remain in their rooms with the door shut at all times.

In order to 'start clean', admission of all except emergency cases was stopped until the open sector of the ward unit was free of patients with penicillin- and tetracycline-resistant organisms. The study was carried out twice, first in February/April 1971 and then December 1971/January 1972, the wards being emptied as far as possible at Christmas as a normal policy. On the first occasion only the male unit took part but on the second occasion both male and female units took part in the study.

RESULTS

On each occasion the study had to be abandoned, after 2 months on the first occasion and after 3 weeks on the second, because no beds were available in the single-bed rooms, the open sector was empty and the waiting list was growing steadily.

The reasons for this are of interest for the light they shed on isolation policies.

(1) The current hospital staphylococcus is resistant to penicillin, tetracycline, erythromycin, streptomycin and neomycin and is thus classed as a 'dangerous' organism. This organism appears to be very readily acquired by the patients, who become carriers in the nose, chest and groin and doubtless elsewhere and are discharged and may be readmitted still carrying this strain.

(2) Some skin patients are in hospital for long periods; during 1966–8, when 1548 patients were studied, 24% stayed for more than 4 weeks and 6% for more than 8 weeks. It is these patients, the most severely affected, who are most at risk and are most likely to acquire hospital staphylococci. Clearly, when this occurs a single room is blocked for a considerable period.

(3) The majority of patients with diseases of the skin do not in themselves 'feel ill'. They resent therefore being denied access to other patients for social contact and resent being unable to use the day-room and view television. Unless senior nursing staff are constantly present they are therefore liable to leave their rooms and go visiting.

These factors combined to prevent the isolation policy from operating. During the 8 weeks of the first period when the scheme was in operation 58 patients were admitted to the 12 beds, though 3 of those were immediately blocked by long-stay patients for the duration of the trial. Twelve of the remaining 55 patients were admitted carrying a penicillin- and tetracycline-resistant organism, usually from a previous admission; a further three were not apparently carriers on admission, but were so within 3 days. Thirty-four patients were transferred to the open sector, where 19 subsequently exhibited a resistant organism, ten within 3 days of transfer, i.e. within about 5–7 days of admission. As a patient known for his ability

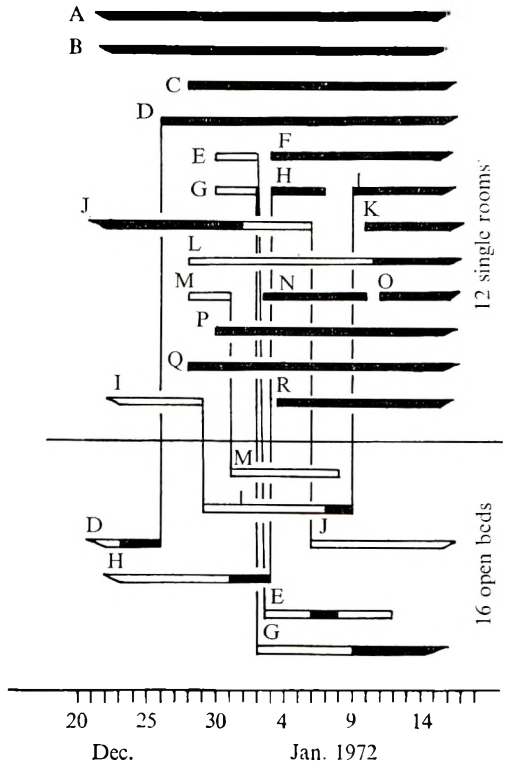

Fig. 2. Diagrammatic representation of male patients' stay in hospital. Bar indicates stay in hospital, black area indicates carriage of penicillin and tetracycline resistant organisms. Vertical lines show transfer of patient between ward sections.

to disperse organisms was one of the three long-stay patients referred to above, it is presumed that he was instrumental in causing at least some of the transmission.

The situation was thus reached in which all single beds were blocked and there were patients in the open sector who also carried resistant organisms. The investigation was abandoned.

During the second period the impasse was reached sooner than before. The result for the male ward is shown diagrammatically (Fig. 2). Four patients in hospital during the Christmas period already carried resistant staphylococci, as did two of the four admitted on 28 December and one of the three admitted on the 30th. All four admitted on 2–3 January, as well as two admitted subsequently, carried resistant staphylococci. There was some acquisition by patients in the open-ward section. The picture in the female ward was essentially the same.

DISCUSSION

Full and strict isolation can reduce the incidence of acquisition of nasal staphylococci to small proportions (Parker, John, Emond & Machacek, 1965). In the present investigation it was felt undesirable to insist on strict isolation as skin-patients do not often feel 'ill' and also often have a 'leper' complex regarding their skins – they

do not like to be thought 'dirty' or 'contaminated'. (It is still a comparatively common belief that skin diseases are somehow a product of dirt or uncleanliness.) Additionally no changes were to be made in the nursing procedures.

Twelve single beds were not sufficient to feed 16 beds in the open sector owing to the propensity of long-stay patients to become colonized and block the beds. This raises important problems in the design of hospital isolation facilities for any area in which severely affected skin patients are to be nursed. There is sufficient evidence to suggest that they should not be put in surgical wards owing to their ability to transmit their flora to other patients (Williams, 1966). It would seem more satisfactory in any subsequent hospital design to construct units of 4–6 beds each but to insist that patients remain in their rooms. In this way patients would not lack company yet would not be exposed to – or be able to donate their micro-organisms to – many other individuals.

We are grateful to the Consultant staff of St John's Hospital for Diseases of the Skin for permission to examine the patients bacteriologically, and for supporting this trial; to the nursing staff who tried to work the policy and to Mrs A. El Sherif for technical assistance.

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The effects of spraying on the amounts of airborne foot-and-mouth disease virus present in loose-boxes

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SUMMARY

The air of loose-boxes which had previously held pigs infected with foot-and-mouth disease was sampled for virus after various procedures. Removal of infected pigs led to a 12- to 16-fold reduction in the amount of virus after 5 min. and a 400-fold reduction after 60 min. After heavy spraying (1·2 mm. of water in 5 min.) the amount of virus was reduced 500-fold compared to 30-fold after light spraying (0·20 mm. of water in 5 min.). The partition of infectivity associated with particle size was measured. The partition found after light spraying was similar to that found 5 min. after the pigs had been removed. Heavy spraying brought about a reduction in the infectivity associated with the large particles ($> 6\ \mu$m.) but had no effect on particles less than $3\ \mu$m. A similar partition was found 60 min. after the pigs had been removed. The findings are discussed in relation to the spread of foot-and-mouth disease by the airborne route.

INTRODUCTION

When pigs infected with foot-and-mouth disease were held in a loose-box, large amounts of virus were recovered from the air, the highest percentage of infectivity being associated with particles greater than $6\ \mu$m. in diameter (Sellers & Parker, 1969). The present experiments were carried out in order to measure the concentration of virus and the association of infectivity with particle size after the air in the loose-box had been sprayed with water.

MATERIALS AND METHODS

Animals

Large White pigs, weight 30–40 kg., were housed in loose-boxes.

Viruses

Three strains of foot-and-mouth disease (FMD) virus were used: O_1 BFS 1860, A Pando and C Noville. They were stored at $-70°$ C. as suspensions of infected cattle-tongue epithelium.

Virus assay

Virus was assayed by inoculating serial dilutions into calf thyroid tissue culture tubes.

Plan of experiments

After the infected pigs had been removed, measurements were made of the airborne virus remaining in the loose-box under the following conditions:

(i) When the box had been empty for 5 min.

(ii) When the box had been empty for 60 min.

(iii) When the air in the box had been sprayed with a heavy spray for 5 min.

(iv) When the air in the box had been sprayed with a light spray for 5 min.

These amounts were compared with the amount of airborne virus collected when the box contained infected pigs.

Experimental procedure

The pigs were inoculated on both forefeet with virus diluted 10^{-1} in phosphate buffered saline. The animals were observed daily. At 48 and 72 hr. after inoculation, when generalization of lesions had occurred, spraying and air sampling were carried out in the following sequence:

(1) The air inlets and outlets of the loose-box which housed the pigs were blocked and the walls were drenched with water to provide a relative humidity of greater than 95%.

(2) The air in the loose-box was sampled for 25 min. with the pigs present (see 'Loose-box containing infected pigs' in tables).

(3) The pigs were removed.

(4) The box was left for 5 or 60 min.

(5) The air in the box was sampled for 25 min. ('Empty loose-box 5 min.' and 'Empty loose-box 60 min.' in tables).

(6) The pigs were taken back into the box and the box was kept closed for 30 min. to recharge the air with virus.

(7) The air in the loose-box was sampled for 25 min. with the pigs present ('Loose-box containing infected pigs' in tables).

(8) The pigs were removed.

(9) The air in the box was sprayed with a heavy or light spray for 5 min.

(10) The air in the box was sampled for 25 min. ('Empty loose-box after heavy spraying' and 'Empty loose-box after light spraying' in tables).

On alternate occasions steps 9 and 10 were carried out after step 3, and steps 4 and 5 after step 8. In preliminary experiments and previously (Sellers, Herniman & Donaldson, 1971) it was found that the amounts of virus collected when the pigs were present in the box during steps 2 and 7 did not differ by more than 0·3 log units. This means that the period of 30 min. when the pigs were returned to the box (step 6) was sufficient to raise the amount of virus to the level at the beginning (step 2). Subsequently, sampling during step 2 was omitted; instead the pigs remained in the box for 1 hr. before being removed.

Air sampling

The air in the loose-boxes was sampled with a large-volume sampler (Litton Model M, Litton Systems Inc., Minneapolis, U.S.A.) and with a multistage impinger (May, 1966).

Table 1. *Percentage distribution of droplet size during spraying*

	Size of droplet (mm.)					
	< 0·5	0·5 to < 1·0	1·0 to < 1·5	1·5 to < 2·0	2·0 to < 3·0	⩾ 3·0
Heavy spray						
In air	53	39	6	1·4	0·4	0·2
On ground	26	55	13	4·0	1·0	1·0
Light spray						
In air	63	34·5	2·3	0·2	0	0
On ground	36	57	6·0	1·0	0	0

Spraying

The heavy spray was produced by forcing tap-water under pressure (2·8 kg./cm.^{2}) through a double-headed spray with nozzles of 1·6 mm diameter (Eclipse Spray Co. Ltd., Warley, Smethwick).

A Killaspray pressure-sprayer (ASL Super 828, ASL, Birmingham) was used to provide the light spray. Two litres of tap water were put in the container, which was pressurized with 80 strokes of the pump.

During spraying the sprays were held aloft with the jets directed towards the top of the loose-box and moved to and fro so that spray fell everywhere. Falling spray was collected through a filter funnel into a conical flask. The depth of water collected was calculated by dividing the volume by the area of the filter-funnel aperture.

Measurement of the size and distribution of the spray droplets was done by collecting the spray on Whatman No. 1 filter papers previously treated with potassium permanganate and correlating the size of splashes with the drop diameter derived from the weight of water required to produce them (Anderson, 1948). To give the distribution in the air, the number of drops of a particular size was divided by the terminal velocity (Best, 1950).

RESULTS

Amount of water sprayed and size of droplet

After 5 min. of spraying with the heavy spray, water to a depth of between 0·80 and 1·75 mm. (mean 1·21 mm.) was collected; after light spraying, 0·16–0·29 mm. (mean 0·20 mm.) was collected.

The percentage of droplets within each size range is shown in Table 1. The majority of droplets in the air were < 0·5 mm. in diameter, with rather more in the light spray than in the heavy spray.

Amount of virus collected

In Table 2 it can be seen that, as has previously been found (Sellers *et al.* 1971) removal of infected pigs from a loose-box reduced the amount of airborne virus by 12-fold to 16-fold (1·1–1·2 log units) after 5 min. and by 400-fold (2·6 log units) after 60 min. After heavy spraying, the amount was reduced 500-fold (2·7 log units)

Table 2. *Amounts of virus recovered in the large sampler from loose-boxes after spraying*

Loose-box containing infected pigs			Empty loose-box after 5 min.			Empty loose-box after heavy spraying		
4·4*	4·7	5·3	4·2	3·8	4·4	2·4	1·5	3·4
5·3	6·1	6·7	4·3	4·1	5·3	2·1	3·3	3·8
Mean 5·4			Mean 4·3			Mean 2·7		
Loose-box containing infected pigs			Empty loose-box after 5 min.			Empty loose-box after light spraying		
5·3	5·5	7·1	4·3	4·7	5·3	3·9	4·4	5·3
Mean 6·0			Mean 4·8			Mean 4·5		
Loose-box containing infected pigs			Empty loose-box after 60 min.					
4·7	6·1	6·7	2·7	3·1	3·9			
Mean 5·8			Mean 3·2					

* Log ID 50 per collection.

Table 3. *Amounts of virus recovered in the different stages of a multi-stage impinger from loose-boxes after spraying*

	Loose-box containing infected pigs				Empty loose-box after 5 min.				Empty loose-box after heavy spraying			
Stage 1, > 6 μm.	3·2*	4·5	4·5	4·8	1·2	2·0	3·1	3·2	⩽ 1·0	1·2	1·2	1·5
	Mean 4·2				Mean 2·4				Mean ⩽ 1·2			
Stage 2, 3–6 μm.	3·2	3·5	4·2	4·2	1·5	2·2	1·8	3·4	⩽ 1·0	1·4	2·2	1·1
	Mean 3·8				Mean 2·2				Mean ⩽ 1·4			
Stage 3 < 3 μm.	3·9	2·6	3·2	4·6	1·5	2·6	3·2	3·2	1·5	2·4	2·6	2·2
	Mean 3·6				Mean 2·4				Mean 2·2			
	Loose-box containing infected pigs				Empty loose-box after 5 min.				Empty loose-box after light spraying			
Stage 1, > 6 μm.	3·4	3·9	4·8		2·2	3·0	3·4		2·0	2·2	2·2	
	Mean 4·0				Mean 2·9				Mean 2·1			
Stage 2, 3–6 μm.	2·6	3·9	4·4		1·9	2·8	3·0		2·0	2·2	2·2	
	Mean 3·6				Mean 2·6				Mean 2·1			
Stage 3, < 3 μm.	2·6	3·5	4·0		1·9	2·8	2·8		2·2	2·0	2·2	
	Mean 3·4				Mean 2·5				Mean 2·1			
	Loose-box containing infected pigs				Empty loose-box after 60 min.							
Stage 1, > 6 μm.	3·7	4·1	4·5		⩽ 1·0	1·5	1·9					
	Mean 4·1				Mean ⩽ 1·5							
Stage 2, 3–6 μm.	3·5	4·1	4·1		1·9	2·1	2·5					
	Mean 3·9				Mean 2·2							
Stage 3, < 3 μm.	2·8	3·0	3·2		2·1	2·3	3·3					
	Mean 3·0				Mean 2·6							

* Log ID 50 per collection.

compared to 30-fold (1·5 log units) after light spraying. The reduction after heavy spraying was of the same order as the reduction after leaving the box empty for 60 min.

The results of experiments to determine the partition of infectivity between the different stages of a multi-stage impinger are given in Table 3. The partition, when infected pigs were present (63 %, 27 %, 10 % – mean of ten experiments), was similar to that previously found (Sellers & Parker, 1969). When the box had been empty for 5 min. the partition (mean of seven experiments with empty loose-boxes) was 43 %, 27 %, 30 %. After light spraying the percentages were 33·3 % for each stage, and after heavy spraying 9 %, 14 % and 77 % respectively. When the box had been empty for 60 min. the percentages were 5 %, 27 %, and 68 %.

DISCUSSION

Spraying a loose-box with water after removal of infected pigs reduced the concentration of FMD virus in the air, the heavy spray causing a greater fall in titre than the light spray. There was a selective action; the heavy spray brought about the greatest reduction in titre of the infectivity associated with particles > 6 μm. but the infectivity associated with the small particles remained almost the same. In this respect the spraying intensified the loss of the larger particles from the air by sedimentation, which was taking place when the pigs were removed from the loose-box.

Spraying may simulate the action of rain. If so, rain would have no effect on the smaller particles and the larger particles would be washed out only by heavy rain. Such an effect would be predicted from previous work on wash-out of particles by rain, where the efficiency of capture by raindrops falls off very sharply for particles of 5 μm. or less (Chamberlain, 1967).

The site of initial infection appears in cattle to be the upper respiratory tract, especially in the pharyngeal area (Sutmöller, McVicar & Cottral, 1968; McVicar, Graves & Sutmöller, 1970; Burrows *et al.* 1971). The larger particles would be expected to be trapped here, the smaller particles being taken into the bronchi and alveoli (May, 1966). The fate of the large particles is therefore important in determining the outcome of airborne spread of foot-and-mouth disease.

The minimum infective dose for cattle by inhalation is 10^1 ID 50 and by ingestion 10^6 ID 50 (Sellers, 1971). Animals downwind could be infected in two ways; by breathing in the large particles present in the air or by breathing in droplets formed by the splash of heavy rain on impaction. Infection by ingestion of contaminated herbage would be less likely, even if under some circumstances there is a 350:1 ratio in favour of the dose available for ingestion (Chamberlain, 1970).

On the basis of these and previous findings (Sellers & Parker, 1969; Sellers, 1971), the following hypothesis for airborne spread of foot-and-mouth disease is put forward. Infected animals excrete virus into the air, pigs excreting the most, followed by cattle and sheep. The size of particle associated with infectivity ranges from > 6 to < 3 μm. If the relative humidity is less than 60 % (Barlow, 1972; Donaldson, 1972), inactivation takes place and spread would be only over short

distances, i.e. metres. If the relative humidity is higher than 60% and in the absence of sunshine and pollutants, the infective particles would be expected to survive for hours, especially the larger ones (Norris & Harper, 1970; Benbough & Hood, 1971). In calm, the larger particles would sediment; in wind they would be carried long or short distances, depending on the speed of the wind and the nature of the surface over which they travel. Under certain conditions (transport over sea or as lee waves over land – Tinline, 1970), the concentration of the larger particles apparently remains the same. Deposition of the larger particles would take place in heavy rain and they would either initiate infection by aerosols from the splash on impaction or be washed away. Light rain would have no effect on the larger particles, but in such conditions and in the absence of rain the larger particles would either sediment or be blown down by wind to a level where they would be inhaled by susceptible animals.

The technical assistance of Mrs I. S. Mackenzie is gratefully acknowledged.

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With 1 plate
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Simulated human skin scales

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SUMMARY

Human skin scales which have been shed naturally bear a flora of micro-organisms which is unknown until tested. To replace these scales in a study of the micro-environment of both the human body and of models a method has been devised of making synthetic scales which behave both physically and aerodynamically in a similar way to the natural material. The synthetic materials carry no natural flora and it is possible to include in them test markers of several kinds to assist in identification after dispersion.

INTRODUCTION

At the boundary between animal bodies and the air surrounding them there exists a layer of convected air which moves continually upward (Lewis *et al.* 1969). It has been shown (Clark, Cox & Lewis, 1971) that small particles may be carried upwards in this layer. It has also been shown (Davies & Noble, 1962) that micro-organisms are carried on naturally shed skin squames, and it is possible that aerial microbial contamination is caused at least in part by the movement of infected squames at first in the boundary layer, and later in the environment after the mixing of the boundary layer.

As part of the study of the micro-environment of the human body a supply of particles was required which could be introduced into the boundary layer, and which would carry test markers of several kinds, both animate and inanimate. Although natural skin squames can be collected from laundries, such particles carry a microflora which is unknown until tested and identified. We therefore decided to prepare synthetic squames having the same physical characteristics as the natural material, but which were free of natural micro-organisms and were non-antigenic.

Several natural polymers were tried, and were finally discarded in favour of a mixture of ethyl cellulose and stearic acid; this was found to have the nearest physical properties to those of human squames judged by particle size distribution, shape, density, aerodynamic behaviour, electrical charge and moisture regain capacity.

MATERIALS AND METHODS

Preparation of synthetic particles

Particles were initially prepared from gelatin, agarose, dextran T70 and bovine serum albumin, by preparing 10% (w/v) aqueous solutions of the polymers and spreading them in a thin film on siliconed glass sheets. When dry, the film was scraped off the glass and the particle size reduced by roller-ball milling for 3–5 hr. Alternatively a vibrating-ball mill was used for 15 min. The fraction of powdered material which passed through a 300 mesh sieve (i.e. all particles $< 53\ \mu$m.) was collected. To assist in the study of the aerodynamic properties of the particles while in flight, gelatin particles were made fluorescent by the addition of fluorescein isothiocyanate and rhodamine isothiocyanate (FITC and RITC, BDH).

Subsequently particles were made using ethyl cellulose, grade N50 (Hercules Powder Co. Ltd.), and finally ethyl cellulose mixed with stearic acid was used in the proportions by weight 50:50 and 60:40. These were made up as 10% (w/v) solutions in chloroform and the addition of 0·05% dimethyl POPOP (Packard, Zurich) to the solution made fluorescent particles. Thin films were spread on glass, dried and scraped off, as before.

Particle-size reduction was initially carried out using a vibrating-ball mill (Podmore), in which the scraped particles were wet-ground for 10 min. intervals, wet seived through a 325-mesh sieve (particle size $< 44\ \mu$m.), the eluate centrifuged to concentrate, and dried in a hot-air oven at 40° C. This process was tedious and gave particles of wide size range. Size reduction was finally affected using another method.

When a pressure of 20 tons/in.^{2} is applied to ice at $-25°$ C. a change in crystal structure occurs. A press (X press Biox, Nacka, Sweden) using this principle was employed to shear particles suspended in ice at $-25°$ C. Particles obtained by this method had a narrower size distribution than particles prepared previously.

After size reduction a variety of drying methods were tried: rotary evaporation, freeze-drying and finally filtration of the thawed suspension through sintered glass filter porosity no. 3, followed by desiccation of particles retained on the filter.

Evaluation of particles

Size and shape

Particle-size distribution and shape were determined microscopically using a calibrated eyepiece. A few skin scales were obtained for comparison by lightly scraping the skin with a scalpel from arms and legs of several individuals. Measurements were also made by means of fluorescent incident differential interference contrast microscopy of the scales on the surface of human and 2-day-old rat skin.

Density measurements

Density of synthetic particles was determined by calculation and that of skin scales taken as the equivalent density of alcohol in which the scales remained suspended in dynamic equilibrium.

Table 1

s.g. H_2SO_4	% H_2SO_4	Relative humidity	Vapour pressure
	$CaCl_2$ desiccator	0	0
1·50	61·0	18·8	3·3
1·40	51·0	37·1	6·5
1·30	40·0	58·3	10·1
1·20	28·0	80·5	14·0
1·00	1·0	100·0	17·4

Where 1·83 g./ml. = 100% H_2SO_4.

Aerodynamic behaviour

Aerodynamic behaviour of the particles was compared using a vertical laminar air-flow channel (Clark, Cox & Lewis, 1970) with constant upward air velocity of 20 cm./sec. Particles injected into the upward moving layer of air from a capillary tube by tapping, either impinged on the side walls or on a filter at the head of the channel. Particles deposited at graduated increments up the front glass wall of the channel were counted and a particle-size distribution at three equidistant positions determined.

Electrophoresis and surface charge

Electrostatic charges on different particles were found to affect their aerodynamic behaviour. Measurement of electrostatic charges of particles in air proved to be difficult, hence intrinsic surface charge on particles was compared by particle electrophoresis in a conducting liquid (Brinton & Lauffer, 1959). Particle electrophoresis was carried out in a small glass electrophoresis chamber filled with a suspension of particles in barbiturate buffer (ionic strength = 0·01, pH 8·5). Movement of particles was observed microscopically and the time taken for particles to travel a fixed distance (using a calibrated eyepiece grid, 1 division = 180 μm.^{2}) was determined over a range of voltages (0–100 V.).

Moisture regain capacity

Determination of moisture change of ethyl cellulose/stearic acid (60:40) particles and human skin scales over a range of relative humidities at room temperature (21° C.) was carried out in equilibrated sealed humidity chambers, using differing sulphuric acid concentrations to give known relative humidities (Table 1). Moisture change was taken as the difference in weight of the particles before and after equilibration for 2 weeks in the sealed chambers.

RESULTS

The large numbers of particles counted for particle-size distribution gave a normal Gaussian distribution, with the mean sizes (length × width) shown in Table 2. The figures for scraped skin scales (Table 2) refer to the scales scraped from four individuals, the back of the hand being used in each case; the fifth value refers to scraped scales from legs used in the aerodynamic evaluation.

Table 3 shows specific gravity of natural and synthetic particles.

Table 2. *Mean sizes (μm.) of natural skin scales and synthetic particles*

Human skin scales			Synthetic particles	
Scraped	*in situ* on skin	Rat scales: *in situ* on skin	Ethyl cellulose (vibrating ball mill)	Ethyl cellulose/ stearic acid (X-press)
34·0 × 22·6	23·0 × 17·1	38·1 × 25·0	20·5 × 14·0	32·4 × 32·6
35·1 × 22·8				
37·3 × 29·3				
28·6 × 19·2				
43·2 × 24·8				

Table 3. *Specific gravity of natural and synthetic particles*

Scraped skin scales	0·95
Gelatin	1·27
Ethyl cellulose	1·15
Ethyl cellulose/stearic acid	1·01

Table 4. *Distribution of natural and synthetic particles in laminar-air flow channel*

Particle	Weight used (mg.)	Position along channel from point of injection (Y)									
		28·6	35·0	41·2	47·7	54·0	60·3	66·6	72·9	78·0	83·1
Scraped skin	2·15	61	58	69	70	60	40	41	62	73	74
Gelatin	20·90	15	14	26	32	39	30	24	40	36	23
Ethyl cellulose	20·60	68	72	59	62	54	55	33	36	49	75
Ethyl cellulose/stearic acid, 60:40	4·10	55	61	41	43	55	53	46	64	40	45

Y = length of channel travelled (cm.).

Table 4 shows weights of particles injected into the laminar air-flow channel and the numbers of particles deposited on the front wall.

Agglomerates and particles > 40 μm. fell out of the channel as a low upward velocity was used. Fig. 1 shows the channel wall deposition of the particles mentioned in Table 4. Although low weights of skin scales and ethyl cellulose/stearic acid particles were used, similar counts to those of ethyl cellulose were obtained, indicating that the size ranges of the two former types of particle were narrower than that of ethyl cellulose.

Figs. 2–5 give a size distribution of the four different types of particles counted above at three positions along the channel wall, where Y equals 35·0, 54·0, 72·9 cm. respectively from the point of injection. In each case, particles were evenly distributed up the channel face, a complete size range being counted at each position.

The results of particle electrophoresis are shown in Fig. 6. All particles were negatively charged, all moving in the same direction, towards the anode.

Fig. 7 shows percentage moisture change of ethyl cellulose/stearic acid particles and skin scales with humidity, after 2 weeks equilibration.

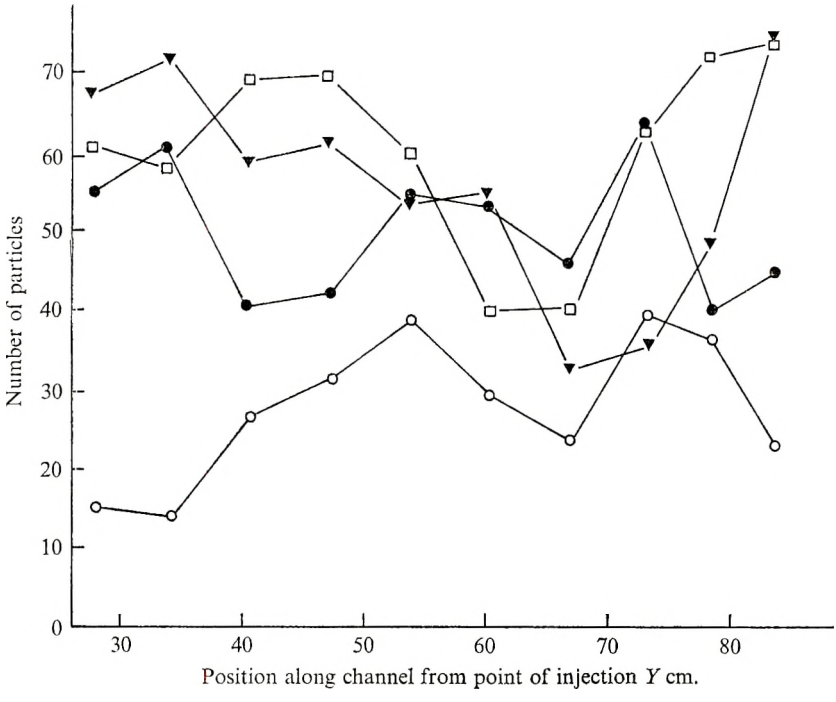

Fig. 1. Channel wall deposition of (○) gelatin, (▼) ethyl cellulose, (●) ethyl cellulose/stearic acid particles, and (□) skin scales.

DISCUSSION

Manufacture of the synthetic particles by ball milling was discontinued as the process was too lengthy and gave too wide a particle size distribution. The time taken to produce discrete particles in the range 15–50 μm. was greatly reduced using the X-press. Agglomerates readily broke down into discrete particles after desiccation.

Initially natural polymers were chosen to resemble keratinized skin squames chemically, however, their physical properties proved them to be unsuitable. Particles of bovine serum albumin and dextran were extremely friable and developed such high electrostatic charges as to make them unsuitable. A further defect was that owing to their antigenic nature they were likely to present a hazard to workers in subsequent air flow experiments. Gelatin, although the most suitable natural polymer since the size and shape of milled particles closely resembled those of skin scales, was too dense and its hygroscopic nature caused particles to become even more dense with a tendency to agglomerate, as shown by the unusual flight properties (compared with other particles) in the channel.

Inert non-antigenic materials were then used, but pure ethyl cellulose particles made by milling had a wide size distribution and were too dense compared to skin scales. Addition of the free fatty acid, stearic acid, to ethyl cellulose reduced its density to 1·01, and the waxy nature of the mixed particles simulated the physical characteristics of the lipid-keratin composition of skin squames. Particles made with equal proportions of ethyl cellulose and stearic acid were unsuitable as they

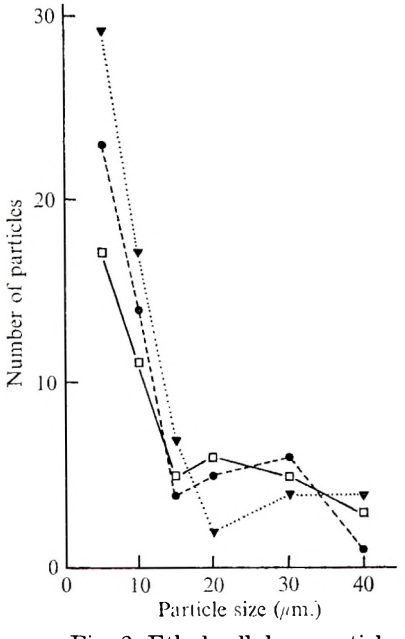

Fig. 2. Ethyl cellulose particles.

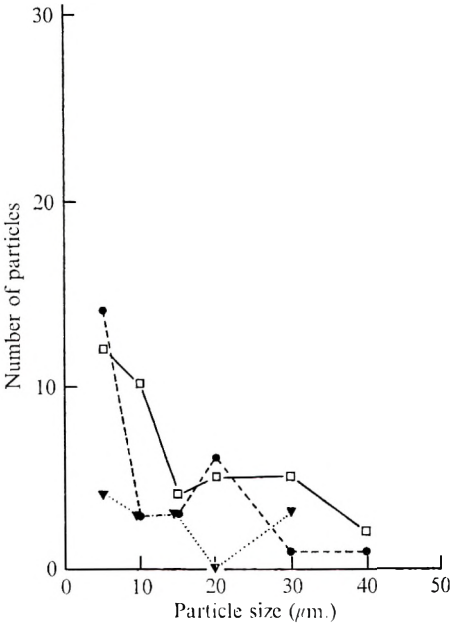

Fig. 3. Gelatin particles.

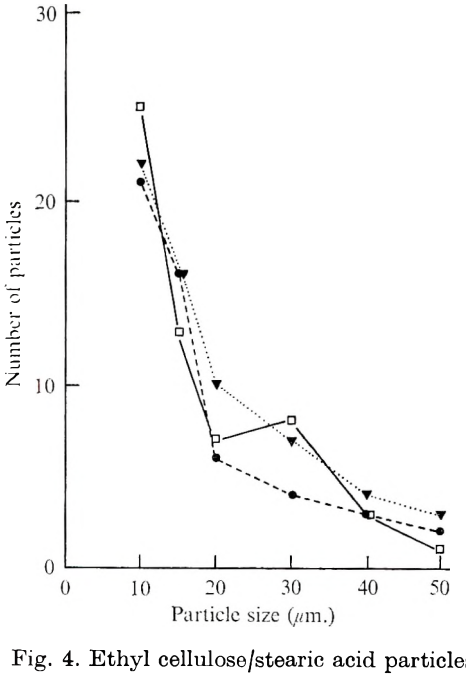

Fig. 4. Ethyl cellulose/stearic acid particles.

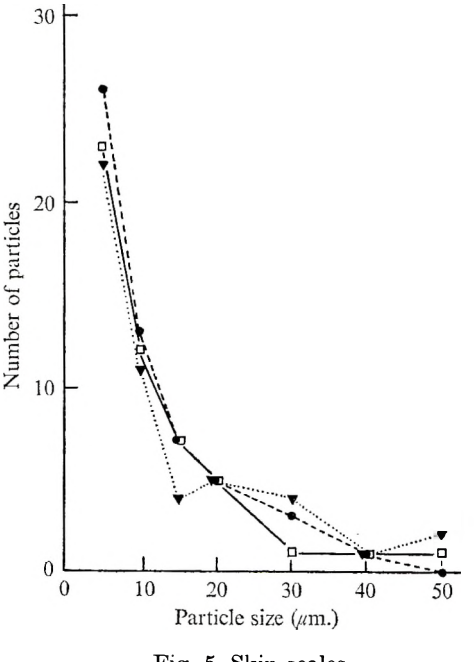

Fig. 5. Skin scales.

Particle-size distribution at channel positions Y = 35·0 cm. (▼); Y = 54·0 cm. (●); Y = 72·9 cm. (□).

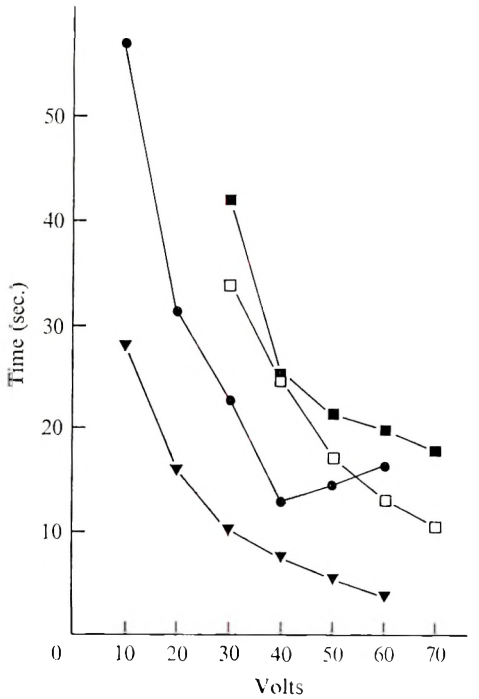

Fig. 6

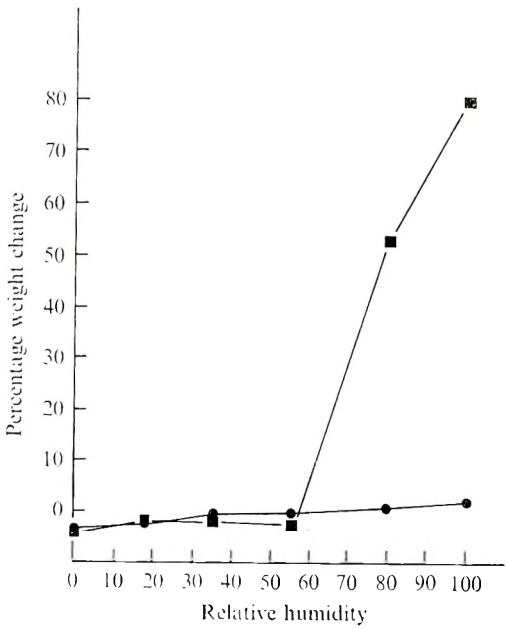

Fig. 7

Fig. 6. Particle electrophoresis of ethyl cellulose (▼), ethyl cellulose/stearic acid (●), scraped-skin scales (□) and naturally shed skin scales (■).

Fig. 7. Moisture regain of ethyl cellulose/stearic acid (●) and skin scales (■).

proved to be too friable, with a tendency to agglomerate readily owing to their extreme waxiness. However, with a lower proportion of stearic acid (ethyl cellulose:stearic acid, 60:40) particles remained coherent and discrete.

Particle electrophoresis showed that the intrinsic charge of ethyl cellulose/stearic acid (60:40) particles was closest to that of skin; however, the difference between all three types of particles was not significant.

From the above data, ethyl cellulose/stearic acid (60:40) particles were those most closely resembling normal skin scales in their physical properties (see Plate 1, Figs. 1, 2).

In the moisture regain experiments comparison of moisture uptake over the range RH (0–60) showed good correlation between ethyl cellulose/stearic acid particles and skin scales. However, above RH 60, there was a difference between the two kinds of particle, moisture uptake by skin scales far exceeding that of the synthetic particles. Increasing relative humidity causes physical uptake of moisture by the scraped skin scales, and although the effect of increasing RH on skin *in vivo* has been attributed to an increase in insensible perspiration (Mole, 1948) uptake of moisture from the atmosphere may also occur, forming a dynamic equilibrium between diffusion of water vapour across the skin. Ambient room humidity is normally in the range of 40–60% RH; and it is of interest to speculate that physiological degree of comfort may be attributed to the sharp transition of moisture regain of the keratinized layer of skin.

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EXPLANATION OF PLATE

Fig. 1. Particles of ethyl cellulose/stearic acid.

Fig. 2. Human skin scales. Scale: 1 division = 10 μm.

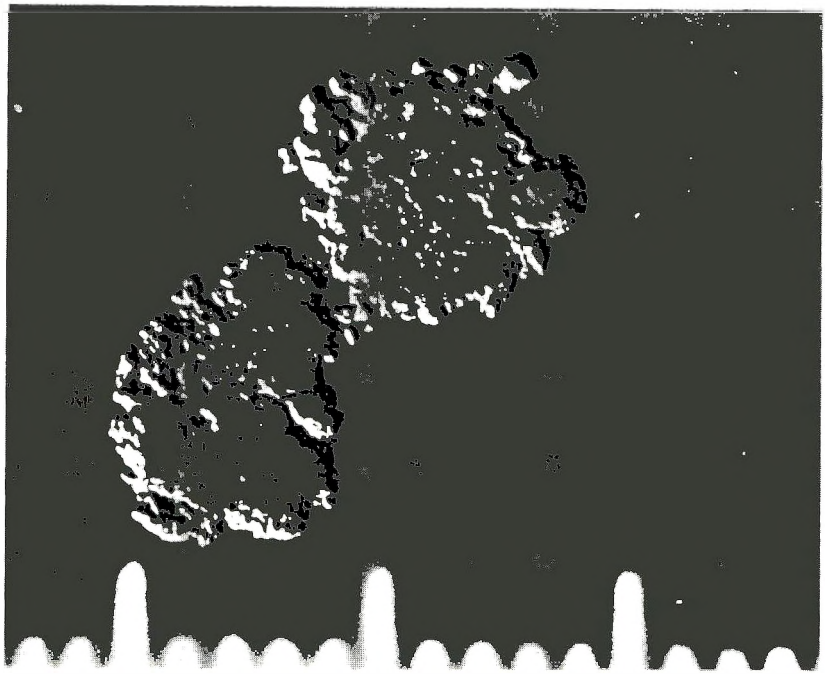

Fig. 1

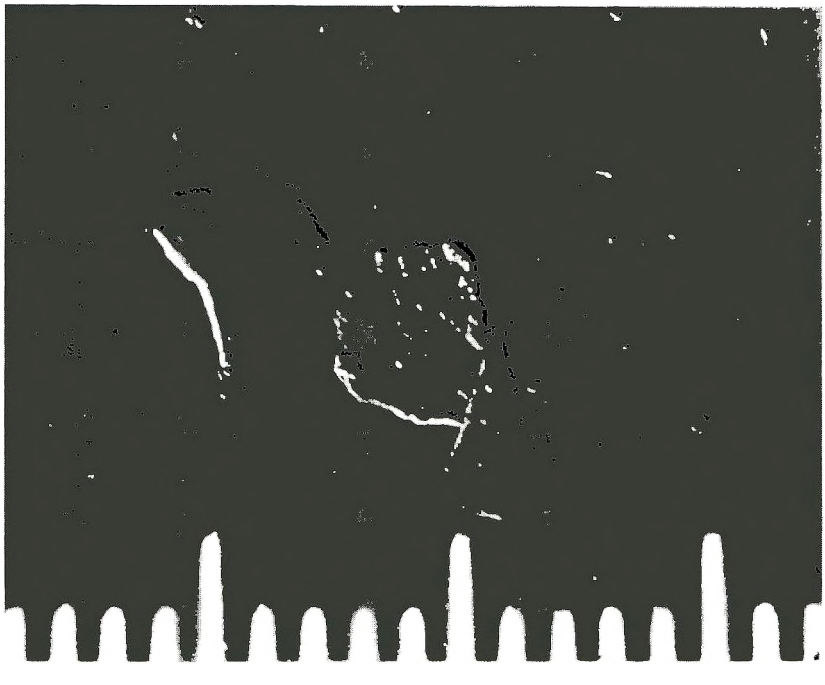

Fig. 2

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Observations on procedures for thawing and spit-roasting frozen dressed chickens, and post-cooking care and storage: with particular reference to food-poisoning bacteria

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SUMMARY

A comparison was made of four methods of thawing frozen chickens and an average thaw time for each method was determined.

Fully and partially thawed chickens, inoculated with salmonellas, *Clostridium welchii* and *Staphylococcus aureus* were cooked in a spit-roasting oven at different temperatures for different lengths of time. The chickens were examined freshly cooked and after storage under various conditions.

Spit roasting fully thawed chickens until the outer skin was golden brown was sufficient heat-treatment to kill salmonellas and *Staph. aureus* but *Cl. welchii* could survive. Salmonellas could also survive if the chickens were not fully thawed before cooking.

Incorrect storage after cooking was shown to encourage the growth of pathogens.

The incidence of intestinal pathogens in frozen dressed chickens and environmental hazards in spit-roasting establishments were also studied. Of raw chickens examined 35% contained salmonellas (9 serotypes), 63% contained *Cl. welchii* and 63% *Staph. aureus*.

INTRODUCTION

Over the past years there has been a change in production methods for certain livestock, notably poultry, intended for domestic consumption.

The systems of intensive rearing have brought about an increased consumption of poultry. Surveillance studies were directed towards home-produced as well as imported foods in an effort to find the source of salmonella serotypes causing gastroenteritis in the human population.

Salmonellas have been found in a high percentage of dressed carcasses both frozen and chilled examined on a large scale in Britain (Hobbs, 1971; Crabb & Walker, 1971), Ireland (Timoney, Kelly, Hannan & Reeves, 1970), United States of America (Wilder & MacCready, 1966; Surkiewicz, Johnston, Moran & Krumm, 1969; Morris, McMurray, Galton & Wells, 1969; Morris & Wells, 1970), Israel (Seligmann & Lapinsky, 1970) and Italy (Pascucci *et al.* 1970).

Attention was drawn to the hazards of contaminated poultry by a series of outbreaks due to *Salmonella virchow* from chickens stored after cooking (Semple,

Turner & Lowry, 1968). It was suggested that inadequate thawing and unhygienic conditions contributed to a massive build-up of salmonella contamination in the shop and in the chicken portions sold. In tracing the source of this outbreak a chicken-packing station and associated rearing farms were investigated (Pennington, Brooksbank, Poole & Seymour, 1968). *S. virchow* was found in samples including litter, cloacal swabs, plucked and unplucked chickens and feedstuffs from 9 of 14 farms as well as from eviscerated chickens, giblets, water from a chill tank and sewer swabs at the processing plant. Many salmonella serotypes are carried by poultry without evidence of illness so spread of the organism cannot be prevented by removal of sick birds before processing. However, inspection of edible offal from chickens on the processing line may reveal evidence of infection, for example spots on the liver (H. E. Marthedal, personal communication).

The infection rate of the live birds and the serotypes found will vary from farm to farm, depending on a number of factors including infection on the breeding farm (J. Lee, personal communication; Jackson, Lindsay & Shiel, 1971) and contamination of feedstuffs. It is likely that spread from carcass to carcass will take place during processing. The correct use of chlorinated water will reduce the spoilage flora and may reduce the incidence of salmonellas but the organisms will still remain inside the carcass and deep in the skin – sucked inside the feather follicles (Dixon & Pooley, 1961; Barnes, 1965). The inclusion of packets of edible offal inside the carcass may add to the spread of serotypes.

This report describes an investigation initiated by a request from the British Poultry Meat Association Ltd. It was desired to find out methods of thawing and cooking (spit-roasting) frozen dressed chickens so that consumers would be protected against the hazard of food poisoning.

Frozen dressed and wrapped poultry with weights ranging from 2 lb. 4 oz. to 2 lb. 14 oz. with enclosed giblets were supplied by four English packing stations. The chickens were stored frozen at $-10°$ C. (14° F.) until required.

The investigation included experiments on thawing, cooking and storage. The incidence of intestinal pathogens in frozen dressed chickens and environmental hazards in spit-roasting establishments were also studied.

THAWING

Methods

Experiments were carried out based on the recommended methods of thawing frozen chickens, as printed on wrappers or included on leaflets by the various poultry-packing companies.

Temperature recordings were made throughout the thawing periods by inserting chrome alumel thermocouples deep into the cavity of each bird, into the breast meat and into the meat between the fleshy portion of the leg and the side of the breast to a depth of 2–2·5 cm. The thermocouples were connected to a Comark electronic thermometer (Comark Electronics Ltd., Littlehampton, Sussex) calibrated in ° C. with a recording range of -100 to $+300°$ C. with an accuracy of $\pm 0·5°$ C. Ten thermocouples could be attached to the thermometer at any one

time. Ambient temperatures were recorded throughout each experiment. The thawing period was taken from the time the chickens were placed in the required situation, with the least possible delay after removal from the deep freeze, to the time when all the thermocouples registered 0° C.

Holding at room temperature at 15–21° *C.* (59–69·8° *F.*)

Ten chickens between 2 lb. 4 oz. and 2 lb. 2 oz. in weight were thawed at room temperature 15–21° C. individually on stainless-steel or enamel trays; five chickens were thawed within and five without their polythene bags. Temperature recordings were made at hourly intervals up to 8 hr. In a second experiment chickens were left to thaw at room temperature for 24 hr., which is longer than the recommended period.

Storage in a domestic refrigerator at 4° *C.* (39·2° *F.*) *overnight*

Ten chickens, 2 lb. 8 oz. to 2 lb. 14 oz. in weight, were thawed individually on trays placed on a shelf in a refrigerated room, at approximately 4° C.; five chickens were thawed within and five without their polythene bags. Temperature recordings were made at hourly intervals except during the overnight period.

Immersion in cold running water at 16–21° *C.* (60·8–69·8° *F*)

Five chickens, 2 lb. 4 oz. in weight and in polythene bags, were placed on a shallow enamel tray in the sink under a stream of cold water directed on to the centre of the breast bone so that the water flowed fairly evenly over the whole bird. Temperature recordings were made at half-hourly intervals of both air and water and also at the usual points in the chicken.

Holding at room temperature with removal of giblets as soon as possible

Ten chickens, 2 lb. 4 oz. to 2 lb. 12 oz. in weight, five within and five without the bag, were left to thaw at room temperature and recordings were made every 30 min. The giblets were removed when the cavity was sufficiently thawed but before the whole carcass was thawed out. Care was taken with the thermocouples in the cavity, and if disturbed they were replaced immediately near the original position.

In each experiment samples of chicken were taken at the beginning and end of the period of time required for thawing. The samples included portions of the breast and back skin, portions of mixed giblets and drip and cavity fluids. Tests included general and coliform counts and enrichment cultures for pathogenic organisms. Skin from the wing and leg area and part of the cavity wall were also examined by enrichment cultures for salmonellas only.

Selenite F and tetrathionate (Rolfe, 1946) fluids incubated at 37° C. for up to 72 hr. were used for enrichment for salmonellas. Subcultures were made on deoxycholate sucrose and bismuth sulphite agars. Suspicious colonies were picked, identified and serotyped. Two bottles of veal cooked-meat medium, one heated at 60° C. for 15 min. and the other left unheated after inoculation and incubated overnight, were used for the isolation of *Clostridium welchii*; both bottles were

Table 1. *Mean thawing times/lb. weight chicken*

Method of thawing	Mean thawing time (hr./lb. weight of chicken)	
	In bag	Out of bag
Room temperature	2·9	2·0
Domestic refrigerator	8·1	7·9
Cold running water	1·2	Not tested
Room temperature with removal of giblets as soon as possible	2·2	1·7

subcultured on neomycin blood agar (Sutton & Hobbs, 1968) incubated anaerobically. Colonies thought to be *Cl. welchii* were picked, identified by the Nagler reaction and serotyped.

Cooked-meat medium containing 10% NaCl incubated at 37° C. overnight and subcultured on phenolphthalein phosphate plus polymyxin agar was used for the selection of *Staphylococcus aureus*. Colonies thought to be *Staph. aureus* were tested for coagulase production, phage-typed and in some instances examined for enterotoxin production.

Results

The results are given in Table 1. They showed that removal of the polythene bag shortened the thaw time. At room temperature the average thaw time for chickens with bags compared with chickens without bags was almost 1 hr./lb. shorter. With a lower temperature of storage, in the refrigerator, there was little difference in the thaw time of chickens with and without the bags. When chickens were thawed in the refrigerator without a bag the skin showed signs of dehydration, and marks of freezer burn became more obvious. The most rapid thaw time was achieved by holding the bagged carcasses in cold running water. The average time for 5 chickens was 1·2 hr./lb. Removal of the giblets while chickens were thawing in bags at room temperature reduced the thaw time 0·7 hr./lb. but only 0·3 hr./lb. when the bag was first removed.

The means of the total aerobic colony counts (surface drop count on blood agar) on breast and back skin before and after thawing by the four methods are given in Table 2. The mean counts on other samples taken from the chicken after thawing were approximately the same except after prolonged thawing at room temperature, when the counts were higher.

In general, higher counts were obtained from the back skin than from skin taken from the breast region. The chickens were thawed breast upwards, so that fluid drained towards the back of the bird on to the metal tray and at the end of the thawing period the chicken carcass was resting in a pool of fluid which would encourage bacterial growth. Apart from method 1(*b*) there was no significant increase in the number of organisms found on the skin during the thawing periods. When chickens were allowed to thaw at room temperature for longer than the recommended period of time the number of bacteria per g. of back skin increased.

Table 7. *Growth of* Salmonella typhimurium *from fully thawed chickens inoculated at different sites with ca.* 750 *organisms and cooked at* 250° *F. (Expt.* 5)

Cooking time (min.)	Storage	Salmonellas/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	–	< 100	–	< 100
20	None	< 100	–	< 100	–
	O/N at RT	3,000	130,000	250	> 1,000,000
	O/N at 39·2° F. followed by 4 hr. at 116·6° F.	< 100	–	100	< 100

After 40 and 75 min. cooking there was no growth of *Salmonella* from any site either direct or after storage at RT or at 116·6° F.

For notes see Table 3.

Table 8. *Growth of* Salmonella typhimurium *from fully thawed chickens inoculated at different sites with ca.* 880 *organisms and cooked at* 400° *F. (Expt.* 6)

Cooking time (min.)	Storage	Salmonellas/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	< 100	< 100	< 100	< 100
20	None	–	–	–	< 100
	O/N at RT	15,000	< 500	< 500	UC
	4 hr. at 131° F.	–	–	–	–

After 40 and 60 min. cooking there was no growth of *Salmonella* from any site, either direct or after storage at RT or at 131° F.

For notes see Table 3.

S. virchow, is not known to be particularly heat resistant; it is more likely that the chicken was recontaminated accidentally after removal from the spit-roasting oven.

In Expt. 3, but not in Expts. 1 and 2, salmonellas were recovered from the chicken examined directly after 40 min. cooking but not from the chickens stored overnight. In all three experiments salmonellas were detected directly after 20 min. cooking at 149° C. (300° F.) and after storage overnight.

When chickens were cooked at 107·2° C. (225° F.) (Expt. 4) and at 121° C. (250° F.) (Expt. 5) salmonellas were not recovered after 40 and 75 min. cooking whether the birds were examined immediately or after storage overnight (Tables 6, 7). When the cooking temperature was increased to 204° C. (400° F.) (Expt. 6) salmonellas were again only detected after cooking for 20 min. but not after 40 and 60 min. (Table 8). Chickens cooked for only 20 and 40 min. looked obviously undercooked.

When the experiments were repeated with chickens given mixed inocula and cooked at 204° C. (400° F.), 149° C. (300° F.) and 107·2° C. (225° F.) (Expts. 7–9) salmonellas were recovered from carcasses cooked for 20 min. but not from those cooked for 40 min. or 60–75 min. unless the cooking temperature was low, 107·2° C. (225° F.), when they survived 20 min. and 40 min. (Tables 9*a*, 10*a*, 11*a*).

Table 9(*a*). *Growth of* Salmonella typhimurium *from fully thawed chickens inoculated at different sites with ca.* 3250 *organisms and cooked at* 400° *F. (Expt.* 7)

Cooking time (min.)	Storage	Salmonellas/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	+	+	+
20	None	+	−	+	+
	O/N at RT	UC	UC	UC	UC
	O/N at 131° F.	−	−	−	−

After 40 and 60 min. cooking there was no growth of *Salmonella* from any site, either direct or after storage at RT or at 131° F.
For notes see Table 3.

Table 9(*b*). *Growth of* Clostridium welchii *from fully thawed chickens inoculated at different sites with ca.* 10,000 *organisms and cooked at* 400° *F. (Expt.* 7)

Cooking time (min.)	Storage	*Cl. welchii*/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	+	+	+
20	None	+	−	+	+
	O/N at RT	> 1,000,000	700,000	700,000	5,000,000
	O/N at 131° F.	−	−	< 500	−
40	None	−	−	−	−
	O/N at RT	< 500	−	< 500	15,000
	O/N at 131° F.	130,000	−	2,500,000	100,000
60	None	−	−	−	−
	O/N at RT	−	−	−	−
	O/N at 131° F.	−	−	−	< 500

For notes see Table 3.

Table 9(*c*). *Growth of* Staphylococcus aureus *from fully thawed chickens inoculated at different sites with approximately* 1000 *organisms and cooked at* 400° *F. (Expt.* 7)

Cooking time (min.)	Storage	Staphylococci/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	−	−	−
20	None	−	−	−	−
	O/N at RT	−	−	−	−
	O/N at 131° F.	−	−	−	−

After 40 and 60 min. cooking there was no growth of *Staphylococcus aureus* from any site either direct or after storage at RT or at 131° F.
For notes see Table 3.

Results from the last group of cooking experiments (Expts. 10–12) when partially thawed birds were used are given in Tables 12(*a*), 13(*a*), 14(*a*). The results showed that the salmonellas survived for longer than when fully thawed birds were used, and the survival time increased as the cooking temperature decreased.

Table 10(*a*). *Growth of* Salmonella typhimurium *from fully thawed chickens inoculated at different sites with approximately* 1650 *organisms and cooked at* 300° *F.* (*Expt.* 8)

Cooking time (min.)	Storage	Salmonellas/g. of chickens from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	+	−	+
20	None	+	−	−	+
	O/N at RT	UC	UC	UC	UC
	O/N at 143·6° F.	−	−	−	−

After 40 and 60 min. cooking there was no growth of *Salmonella* from any site, either direct or after storage at RT or at 143·6° F.

For notes see Table 3.

Table 10(*b*). *Growth of* Clostridium welchii *from fully thawed chickens inoculated at different sites with approximately* 1,650 *organisms and cooked at* 300° *F.* (*Expt.* 8)

Cooking time (min.)	Storage	*Cl. welchii*/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	+	+	+
20	None	+	−	−	+
	O/N at RT	1,500,000	10,000	3,500,000	2,000,000
	O/N at 143·6° F.	4,000,000	> 10,000,000	35,000	10,000,000
40	None	+	+	−	+
	O/N at RT	< 500	−	< 500	2,000
	O/N at 143·6 °F.	45,000	< 500	400,000	> 10,000,000
60	None	−	−	−	−
	O/N at RT	< 500	< 500	< 500	< 500
	O/N at 143·6° F.	1,100,000	−	> 10,000,000	> 10,000,000

For notes see Table 3.

Table 10(*c*). *Growth of* Staphylococcus aureus *from fully thawed chickens inoculated at different sites with approximately* 1,650 *organisms and cooked at* 300° *F.* (*Expt.* 8)

Cooking time (min.)	Storage	Staphylococci/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	−	−	−
20	None	−	−	−	−
	O/N at RT	−	−	−	−
	O/N at 143·6° F.	−	−	−	−

After 40 and 60 min. cooking there was no growth of *Staphylococcus aureus* from any site either direct or after storage at RT or at 143·6° F.

For notes see Table 3.

Table 11(*a*). *Growth of* Salmonella typhimurium *from fully thawed chickens inoculated at different sites with ca.* 2,500 *organisms and cooked at* 225° *F. (Expt.* 9)

Cooking time (min.)	Storage	Salmonellas/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	+	−	+
20	None	+	+	+	+
	O/N at RT	500	38,000	15,000	950,000
	O/N +	85,000	300,000	330,000	> 10,000,000
40	None	−	−	−	+
	O/N at RT	250	< 500	750	20,000
	O/N +	−	−	25,000	5,000
75	None	−	−	−	−
	O/N at RT	−	−	−	−
	O/N +	−	−	−	−

O/N + = 3–6 hr. at 122° F. remainder of overnight period in slowly cooling cabinet.
For other notes see Table 3.

Table 11(*b*). *Growth of* Clostridium welchii *from fully thawed chickens inoculated at different sites with ca.* 17,500 *organisms and cooked at* 225° *F. (Expt.* 9)

Cooking time (min.)	Storage	*Clostridium welchii*/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	−	+	+
20	None	+	+	+	+
	O/N at RT	330,000	3,500,000	600,000	150,000
	O/N +	> 10,000,000	> 10,000,000	> 10,000,000	> 10,000,000
40	None)	+	−	−	+
	O/N at RT	6,500,000	< 500	300,000	200,000
	O/N +	5,250,000	15,000	> 10,000,000	> 10,000,000
75	None	−	−	−	−
	O/N at RT	−	−	−	1,300*
	O/N +	−	−	−	7,500

O/N + = 3–6 hr. at 122° F. remainder of overnight period in slowly cooling cabinet.
* β-haemolytic strain not typable.
For other notes see Table 3.

When the partially thawed birds were cooked at 121° C. (250° F.) (Table 14(*a*)) salmonellas could be isolated after 40 min. but not after 60 min. when the cooked chickens were examined immediately; a few organisms were, however, still present after 60 min. cooking as shown by isolation from enrichment cultures and direct counts when the birds were stored overnight at room temperature. When partially thawed chickens were cooked the final temperatures were appreciably lower than those recorded when fully thawed chickens were cooked for the same length of time. In the last experiment (Expt. 12) the temperatures after cooking were below 60° C. (140° F.) so survival of salmonellas was likely. The chickens looked unappetizing after cooking and the outer surfaces looked pale and undercooked.

Table 11(*c*). *Growth of* Staphylococcus aureus *from fully thawed chickens inoculated at different sites with ca.* 2250 *organisms and cooked at* 225° *F.* (*Expt.* 9)

Cooking time (min.)	Storage	Staphylococci/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	+	+	+
20	None	−	−	+	+
	O/N at RT	< 500	< 500	< 500	< 500
	O/N +	−	−	6,500	−
40	None	−	−	−	−
	O/N at RT	−	−	−	−
	O/N +	−	−	−	−
75	None	−	−	−	−
	O/N at RT	−	−	−	−
	O/N +	−	−	−	−

O/N + = 3–6 hr. at 122° F. remainder of overnight period in slowly cooling cabinet.
For other notes see Table 3.

Table 12(*a*). *Growth of* Salmonella typhimurium *from partially thawed chickens inoculated at different sites with ca.* 15,000 *organisms and cooked at* 400° *F.* (*Expt.* 10)

Cooking time (min.)	Storage	Salmonellas/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	< 100	< 100	< 100	< 100
20	None	< 100	< 100	−	< 100
	O/N at RT	250	15,000	< 500	100,000
	O/N at 145·4° F	−	−	< 500	1,000
40	None	−	−	−	−
	O/N at RT	−	−	−	250
	O/N at 145·4° F.	−	−	−	−
60	None	−	−	−	−
	O/N at RT	−	−	−	−
	O/N at 145·4° F.	−	−	−	−

For notes see Table 3.

Survival of clostridial spores

The results of examination of cooked chickens inoculated with spores of *Cl. welchii* showed that the organism could not be found at the end of the longest cooking period when the chickens were examined immediately after cooking at all temperaturesused (Tables 9–14, (*b*) only). Nevertheless some spores survived, and after storage at room temperature overnight *Cl. welchii* was isolated in appreciable numbers from chickens cooked for 20 and 40 min. and in smaller numbers from chickens cooked for 60 and 75 min.

Table 12(*b*). *Growth of* Clostridium welchii *from partially thawed chickens inoculated at different sites with ca.* 250 *organisms and cooked at* 400° *F. (Expt.* 10)

Cooking time (min.)	Storage	*Cl. welchii*/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	–	–	–	–
20	None	–	–	–	–
	O/N at RT	750	30,000	< 100	33,000
	O/N at 145·4° F.	250,000	250	250,000	1,500,000
40	None	–	–	–	–
	O/N at RT	–	–	150	250
	O/N at 145·4° F.	–	–	–	–
60	None	–	–	–	–
	O/N at RT	–	–	–	–
	O/N at 145·4° F.	–	–	–	–

For notes see Table 3.

Table 12(*c*). *Growth of* Staphylococcus aureus *from partially thawed chickens inoculated at different sites with ca.* 625 *organisms and cooked at* 400° *F. (Expt.* 10)

Cooking time (min.)	Storage	Staphylococci/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	–	–	< 100	–
20	None	–	–	–	–
	O/N at RT	–	< 500	–	–
	O/N at 145·4° F.	–	–	–	–
40	None	–	–	–	–
	O/N at RT	–	–	–	< 500
	O/N at 145·4° F.	–	–	–	–
60	None	–	–	–	–
	O/N at RT	–	–	–	–
	O/N at 145·4° F.	–	–	–	–

For notes see Table 3.

Survival of staphylococci

A few isolations of *Staph. aureus* were made from chickens cooked for 20 min. only; the inoculated strain CI/1968/7895 was not recovered after cooking for longer periods (Tables 9–14, (*c*) only). Occasionally other strains of *Staph. aureus*, those occurring naturally in the chickens, could be isolated after 20 min. cooking and in one instance after 40 min. cooking at 149° C. (300° F.) (Table 10*c*). However, it appears that there is little or no danger from the growth of *Staph. aureus* in undercooked birds as the organism seems to die out quickly. Cross-contamination to and more particularly handling of cooked birds are far greater hazards.

Table 13 (*a*). *Growth of* Salmonella typhimurium *from partially thawed chickens inoculated at different sites with ca.* 1,750 *organisms and cooked at* 300° *F.* (*Expt.* 11)

Cooking time (min.)	Storage	Salmonellas/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	+	+	+
20	None	+	+	+	+
	O/N at RT	50,000	> 100,000	200,000	+ + +
	O/N at 131° F.	–	–	–	< 500
40	None	+	–	–	+
	O/N at RT	100,000	3,000,000	> 10,000	> 10,000,000
	O/N at 131° F.	–	–	–	< 500
60	None	–	–	–	–
	O/N at RT	–	–	–	–
	O/N at 131° F.	–	–	–	–

+ + + = Not countable because of running together of colonies. Count very high.
For other notes see Table 3.

Table 13 (*b*). *Growth of* Clostridium welchii *from partially thawed chickens inoculated at different sites with ca.* 20,000 *organisms and cooked at* 300° *F.* (*Expt.* 11)

Cooking time (min.)	Storage	*Cl. welchii*/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	+	–	+	+
20	None	+	+	–	+
	O/N at RT	2,000,000	1,300,000	100,000	5,500,000
	O/N at 131° F.	–	1,300	500	7,500
40	None	–	–	–	+
	O/N at RT	5,000,000	3,500,000	+ + +	> 10,000,000
	O/N at 131° F.	–	–	–	–
60	None	–	–	–	–
	O/N at RT	–	–	250	–
	O/N at 131° F.	–	–	–	–

+ + + = Not countable because of running together of colonies. Count very high.
For other notes see Table 3.

Effect of storage after cooking

The experiments also showed the importance of storage after cooking. In Expts. 1, 2, 4 and 5 inoculated undercooked chickens were stored overnight at 4° C. (39·2° F.) and then placed in an incubator or warm cabinet at 43° C. (109·4° F.), 47° C. (116·6° F.) and 55° C. (131° F.) for 4–5 hr. This would be comparable to placing chickens left over from a previous day's cooking straight from the refrigerator into a warm cabinet. At 43° C. (109·4° F.) the salmonellas increased in numbers (Table 3, 4), at 47° C. (116·6° F.) the salmonellas survived but could only be detected from enrichment cultures (Table 7) while at 55° C. (131° F.) no salmonellas could be detected at the end of the storage period (Table 6).

Undercooked chickens which had received a mixed inoculum were stored over-

Table 13(*c*). *Growth of* Staphylococcus aureus *from partially thawed chickens inoculated at different sites with ca.* 2250 *organisms and cooked at* 300° *F. (Expt.* 11)

Cooking time (min.)	Storage	Staphylococci/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	–	–	+	–
20	None	–	–	–	–
	O/N at RT	+	–	> 500*	UC*
	O/N at 131° F.	–	–	–	–
40	None	–	–	–	+*
	O/N at RT	–	–	–	< 500*
	O/N at 131° F.	–	–	–	–
60	None	–	–	–	–
	O/N at RT	–	–	–	–
	O/N at 131° F.	–	–	–	–

* = *Staph. aureus* naturally occurring in chickens.
For other notes see Table 3.

Table 14(*a*). *Growth of* Salmonella typhimurium *from partially thawed chickens inoculated at different sites with ca.* 875 *organisms and cooked at* 250° *F. (Expt.* 12)

Cooking time (min.)	Storage	Salmonellas/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	< 100	< 100	< 100	200
20	None	< 100	< 100	< 100	200
	O/N at RT	30,000	2,500,000	1,000,000	200,000,000
40	None	< 100	–	–	< 100
	O/N at RT	3,300	43,000,000	400,000	250,000,000
60	None	–	–	–	–
	O/N at RT	–	–	200	1,000

For notes see Table 3.

Table 14(*b*). *Growth of* Clostridium welchii *from partially thawed chickens inoculated at different sites with ca.* 1,500 *organisms and cooked at* 250° *F. (Expt.* 12)

Cooking time (min.)	Storage	*Cl. welchii*/g. of chicken from sites (*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	–	–	–	< 100
20	None	–	–	–	–
	O/N at RT	1,300,000	2,300,000	250,000	2,500,000
40	None	–	–	–	–
	O/N at RT	500,000	15,000,000	35,000	28,000,000
60	None	–	–	–	–
	O/N at RT	–	10,000	200,000	750

For notes see Table 3.

Table 14(*c*). *Growth of* Staphylococcus aureus *from partially thawed chickens inoculated at different sites with ca.* 340 *organisms and cooked at* 250° *F.* (*Expt.* 12)

Cooking time (min.)	Storage	Staphylococci/g. of chicken from sites			
		(*a*)	(*b*)	(*c*)	(*d*)
Uncooked	None	–	–	< 100*	< 100*
20	None	–	–	–	+*
	O/N at RT	1,000*	1,300	30,000	–
40	None	–	–	–	–
	O/N at RT	< 500	–	< 500	< 500
60	None	–	–	–	–
	O/N at RT	–	–	–	–

* *Staph. aureus* naturally occurring in chicken.
For other notes see Table 3.

night (18–21 hr.) in a warm cabinet at 55° C. (131° F.), 62° C. (143·6° F.) and 63° C. (145·4° F.). The chickens were also stored at 50° C. (122° F.), but the warm cabinet was accidentally disconnected and they were only at this temperature for 3–6 hr. and for the remainder of the overnight period they were slowly cooling. Under these conditions there was a large increase in numbers of surviving salmonellas and *Cl. welchii* but only a slight increase in numbers of *Staph. aureus* (Tables 11*a–c*). This illustrates the danger of switching off a warm cabinet still containing cooked chickens and leaving them until the next day.

When chickens were stored after cooking at 55° C. (131° F.) salmonellas were not detected in one experiment (Table 9*a*), but they were detected in small numbers after 20 and 40 min. cooking at 149° C. (300° F.) in the cavity of the chicken in another experiment (Table 13*a*). In both instances there was an increase in the numbers of *Cl. welchii* (Tables 9*b*, 13*b*).

Salmonellas were not found in birds stored at 62° C. (143·6° F.) overnight but were recovered after storage at 63° C. (145·4° F.) in low numbers from the cavity and from the area between the leg and the body of the birds cooked for 20 min. (Tables 10*a*, 12*a*). The discrepancy may have been due to fluctuations in temperature of the warm storage cabinet which was also used to dry apparatus. *Cl. welchii* survived and increased in numbers in chickens stored at both these temperatures. (Tables 10*b*, 12*b*).

There is, therefore, a potential danger from the storage of cooked chickens in a warm cabinet when the temperature is not high enough to prevent growth of *Cl. welchii*; in these experiments 63° C. (145·4° F.) was the highest temperature used for storage overnight. It is suggested that storage cabinets should be either chilled or heated to 65° C. (149° F.).

Table 15. *Growth of intestinal pathogens in cooked chicken breast meat at different temperatures*

Storage temperature		Viable counts (thousands) per gram after				
° C.	° F.	0 hr.	6 hr.	24 hr.	30 hr.	48 hr.
		Salmonella typhimurium 425/g.				
4	39	1·2	1·3	0·5	0·3	1·4
22	72	.	1·8	60,000	400,000	500,000
30	86	.	10	3,500,000	500,000	3,500,000
43	109	.	280	450,000	200,000	30,000
55	131	.	0·25	< 0·5	< 0·5	< 0·5
		Clostridium welchii 2,250/g.				
4	39	0·7	1·1	0·2	0·7	1·4
22	72	.	< 0·5	230	10,000	45,000
30	86	.	35	1,500	18,000	1,500,000
43	109	.	1,500	200,000	200,000	30,000
55	131	.	1,500	< 0·5	< 0·5	< 0·5
		Staphylococcus aureus 325/g.				
4	39	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
22	72	.	< 0·5	50	550	200
30	86	.	< 0·5	2,500	1,500	2,000
43	109	.	< 0·5	2,500	2,500	1,000
55	131	.	< 0·5	< 0·5	< 0·5	< 0·5
		Total aerobic count				
4	39	6·0	3·8	2·9	5·0	55
22	72	.	10	150,000	2,000,000	4,000,000
30	86	.	30	4,000,000	2,500,000	15,000,000
43	109	.	3,500	3,000,000	4,500,000	2,000,000
55	131	.	2·8	0·25	0·5	15,000

GROWTH OF FOOD POISONING ORGANISMS IN COLD COOKED CHICKEN MEAT

Several experiments were carried out in which cooked chicken portions were inoculated with a mixture of *S. typhimurium* phage type 2c(14), spores of *Cl. welchii* type ix and *Staph. aureus* and left at various temperatures.

The results showed that bacteria known to cause food poisoning grow well in chicken meat (Table 15).

Growth was best at warm atmospheric temperatures when there may be a dangerous increase in the number of bacteria. The experiments were commenced with cold cooked chickens but there would be a greater increase in numbers if the cooked chickens were warm from the oven or warming cabinet.

The results emphasize the need for correct storage after cooking. When the chickens were stored in the refrigerator at approximately 4° C. (39·2° F.) the organisms did not die but the count remained stationary, although there was a tendency for numbers to increase after 48 hr. When stored at 22°, 30° and 43° C. (71·6°, 86·0° and 109·4° F.) numbers increased up to high levels thought to be significant in food poisoning. When stored at 55° C. (131° F.) there was an initial

Table 16. *Incidence of salmonellas in frozen chickens from four English packing stations* (1970–71)

Producer	Birds examined	Birds positive	Positive (%)	Serotypes isolated
A	53	33*	62	*S. senftenberg* (30) *S.* 4,12:d:– (4)
B	56	7	13	*S. senftenberg* (4) *S. montevideo* (1) *S. agona* (2)
C	53	30†	57	
Code A	37	14	38	*S. livingstone* (9) *S. typhimurium*, phage type 1 (8) *S. enteritidis*, phage type 8 (1)
Code P	16	16	100	*S. bredeney* (15) *S. livingstone* (3) *S. anatum* (1)
D	39	1	3	*S. bredeney*
Total	201	71	35	9 serotypes

* One chicken contained two serotypes.
† Seven chickens contained two serotypes.

increase in numbers of *Cl. welchii*, probably while the cold chicken was warming up; thereafter the organism died out. Salmonellas survived 6 hr. at 55° C. (131° F.) but the numbers dropped almost tenfold, and the organisms could not be recovered later. It is important to note that although storage at 55° C. (131° F.) is ultimately lethal to many organisms, multiplication may occur before chickens reach this temperature. It is therefore unsafe to put cold cooked chickens into a warming cabinet even if the temperature setting is high. Chickens left over from a previous cooked batch should be cooled rapidly, refrigerated and sold cold. The temperature of cabinets for storage of hot freshly cooked chickens should not be lower than 65° C. (149° F.).

INTESTINAL PATHOGENS IN FROZEN DRESSED CHICKENS

All the chickens used in the investigation were examined for the presence of the food poisoning organisms *Salmonella*, *Cl. welchii* and *Staph. aureus*.

Salmonellas were isolated from 71 of 201 (35%) of raw carcasses from four English packing stations. The lowest rates of contamination were 3% of 39 birds and 13% of 56 birds. The highest rates of contamination were 62% and 100% of 53 and 16 birds respectively. There were nine different salmonella serotypes all of which are known to cause human salmonellosis. The results are shown in Table 16.

Cl. welchii were isolated from 115 of 183 (63%) of chicken samples and the highest rate of contamination was 92% of 37 carcasses. The results with serotypes are given in Table 17.

Spores of this organism can survive heat-treatment, boiling or even light roasting. They are encouraged to germinate by cooking and the bacilli subsequently multiply

Table 17. *Incidence of* Clostridium welchii *in frozen chickens from four English packing stations* (1970–71)

Producer	Birds examined	Birds positive	Positive (%)	Serotypes isolated
A	42	25*	60	Type 18 (23) Not typable (5)
B	56	36†	64	Type 3 (29) Type 6 (1) Not typable (7)
C Code A	53 (37)	48‡ (34)	91 (92)	Type 3 (4) Type 4 (1) Not typable (32)
C Code P	53 (16)	48‡ (14)	91 (88)	Type 5 (6) Type 4 (1) Type 11 (1) Not typable (8)
D	32	6	19	Not typable (6)
Total	183	115	63	7 serotypes 56 untypable strains

* One chicken contained 3 serotypes and one chicken 2 serotypes.
† One chicken contained 2 serotypes.
‡ Five chickens contained 2 serotypes.

Table 18. *Incidence of* Staphylococcus aureus *in frozen chickens from four English packing stations* (1970–71)

Producer	Birds examined	Birds positive	Positive (%)	Predominant phage types
A	40	35	88	75 (10)
B	47	29	62	Not typable (20)
C Code A	53 (37)	19 (10)	36 (27)	No predominant phage type
C Code P	53 (16)	19 (9)	36 (56)	53/75 (4) 75 (4)
D	32	25	78	53 (11)
Total	172	108	63	

rapidly in slowly cooling masses of meat and poultry, so that rapid cooling and cold storage after cooking is very important if the chicken is not to be eaten freshly cooked and hot.

Staph. aureus was isolated from 108 of 172 samples of raw chicken. The highest rates of contamination were 78% and 88% of 32 and 40 carcasses respectively. The results, showing isolation rates and predominant phage types, are given in Table 18. Seventy strains of *Staph. aureus* were tested for enterotoxin production, one strain produced enterotoxin B and two strains produced enterotoxin C.

The danger of cross-contamination from raw to cooked poultry and the necessity for care in storage after cooking is relevant to contamination of poultry with all three food poisoning organisms.

Table 19. *Organisms isolated from 38 samples of cooked chicken from supermarkets, fish bars and delicatessens*

	Distribution of counts and pathogens				
	$< 5 \times 10^2$/g.	5×10^2–10^3/g.	10^3–10^4/g	$> 10^4$/g.	Total
TVC at 37° C./g.	11	6	12	9	38
Salmonella	0	0	0	0	0
Cl. welchii	3	0	6	1	10
Staph. aureus	3	2	5	0	10
Coliform bacilli (faecal)	0	0	5 (1)	6	8 (1)

TVC = total viable count.

ENVIRONMENTAL STUDIES AND EXAMINATION OF SPIT-ROASTED CHICKENS FROM RETAIL PREMISES

A survey of spit-roasting establishments showed faults in the handling procedures of raw and cooked poultry. One employee was usually responsible for both preparation of raw birds for the spit and delivery of cooked birds, whole or in portions, to the shop. The same boards or other surfaces, knives and other utensils and the same hands were used to carry raw birds to, and cooked birds from the spit.

In one of the five stores investigated, *S. typhimurium* was isolated from scrapings taken from a chopping board used both for the preparation of raw poultry carcasses for the spit and for cutting the cooked birds into portions for sale. It was observed that the cooked portions were exposed for sale unrefrigerated.

Results of examination of many samples of cooked chicken showed that heat-sensitive organisms such as *Staph. aureus* and coliform bacilli were present; it is likely that they came from hands, utensils and surfaces contaminated by the raw materials. Some heat-resistant organisms may have survived cooking or they may have reached the carcasses after cooking. Salmonellas were not found on the cooked chicken samples. The results are summarized in Table 19.

Handling and storage after cooking was regarded as a most important factor in the safety and keeping quality of cooked poultry.

CONCLUSIONS

Thawing

The recommended thaw times for 2–3 lb. chickens by the four methods tested are as follows:

(1) *Room temperature* 15–21° *C.* (59–70° *F.*). Left in polythene bag approx. 3 hr./lb. Polythene bag removed approx. 2 hr./lb.

(2) *Domestic refrigerator* 4° *C.* (39·2° *F.*). Left in polythene bag approx. 8 hr./lb. Polythene bag removed approx. 8 hr./lb. (the latter is not recommended as the skin becomes dry and patchy and spoils the appearance of the chicken).

(3) *Under cold running water* 16–21° *C.* (60·8–69·8° *F.*). Left in polythene bag approx. 1¼ hr./lb.

(4) *Room temperature removing the giblets as soon as possible.* Left in polythene bag approx. 2¼ hr./lb. Polythene bag removed approx. 1¾ hr./lb.

The thaw times were converted to hr./lb. weight of chicken to take into account the small differences in weights of the birds used. Experiments were carried out with chickens of one weight group only (2–3 lb.). Klose, Lineweaver & Palmer (1969) thawing turkeys of different weight classes at ambient air temperatures showed that the time of thawing was not proportional to the weight of the bird. At 12·8° C. (55° F.) turkeys weighing 4–6 lb. thawed in 10 hr. while 20–22 lb. birds required only 23 hr. to thaw.

Cooking

From the results of the cooking experiments, recommended cooking times and temperatures for cooking on the spit can be given as follows (preheated oven):

° F.	° C.	min./lb.
300	149	25–30
400	204	20–25

The results of this study show that spit-roasting fully thawed chickens until the outer skin is golden brown (i.e. they look 'done') gives adequate heat-treatment to kill salmonellas and staphylococci, but heat-resistant strains of *Cl. welchii* may survive recommended procedures. Hussemann & Wallace (1951) using conventional cooking methods found that chickens containing large numbers of *S. typhimurium* when broiled for 41 min. or roasted at 163° C. (325° F.) for 35 min./lb. still contained viable salmonellas at the end of the cooking period. But Mabee & Mountney (1970) could not recover *S. senftenberg* 775 W from chicken portions which were deep-fried at atmospheric or 15 lb. pressure for 11 min.

If chickens are not fully thawed, then the internal temperatures reached when the outside looked cooked may not be high enough to kill salmonellas; the lower the cooking temperature the greater the chance of survival.

A high proportion of raw frozen chickens may contain salmonellas but probably low numbers only. If the organisms survive cooking there would be little hazard of food poisoning if the poultry meat is eaten hot and freshly cooked, but when the salmonellas are allowed to grow in warm cooked flesh (e.g. overnight in a warm kitchen) they may increase to numbers able to cause clinical disease.

The results suggest that care in thawing and cooking might lessen the danger from food poisoning but the greatest emphasis should be placed on correct procedure *after* cooking.

Storage

If warm cabinets are used to store freshly cooked chickens they must be hot enough to prevent the survival and multiplication of salmonellas and *Cl. welchii*. The cooked chickens should not be allowed to cool before being placed in the hot cabinet. Cooked poultry required to be eaten cold should be cooled rapidly and refrigerated (or kept as cold as possible) for a limited period of time; it should be sliced when cold not hot.

Cross-contamination

Cooked chicken meat is a good growth medium for salmonellas, *Cl. welchii* and *Staph. aureus*. Any or all of these organisms are present in a large proportion of raw birds and they must be prevented from reaching cooked birds. Precautions to

(1) Keep separate from other foods

(2) Thawing (chickens 2–3 lb.)

	Left in polythene bag	Polythene bag removed
Room temperature 15–21° C. (59–70° F.)	*ca.* 3 hr./lb.	*ca.* 2 hr./lb.
Refrigerator 4° C. (39° F.)	*ca.* 8 hr./lb.	*ca.* 8 hr./lb.
Cold running water	*ca.* 1¼ hr./lb.	—
Room temperature removing giblets as soon as possible	*ca.* 2¼ hr./lb.	*ca.* 1¾ hr./lb.

(3) Remove giblets and wash hands

DO NOT WASH AND DO NOT WIPE

(4) Cook stuffing separately (for conventional roasting methods).

(5) Cooking on spit (chicken 2–3 lb.):
Preheat oven 149° C. (300° F.) 25–30 min./lb.
204° C. (400° F.) 20–25 min./lb.

(6) Wash hands and clean surfaces and equipment before taking cooked poultry out of oven. Avoid use of all-purpose cloths, use disposable paper 'kitchen towels'.

(7) EAT FRESHLY COOKED OR COOL QUICKLY AND STORE COLD.

(8) Avoid handling cooked product.

Fig. 1. Recommended label instructions for frozen chickens

prevent cross-contamination should include the following: (*a*) separate areas, surfaces, utensils and even personnel for handling raw and cooked chickens; (*b*) all surfaces and implements should be thoroughly cleaned as soon as the chickens are placed in the oven; and (*c*) hands must be washed between handling raw and cooked chickens.

Whole cooked chickens should be dissected using meat secateurs or similar implements. Carving should be carried out immediately before service to the table. *Staph. aureus* is found on the skin of many people as well as in cuts, boils and other septic lesions and is frequently transferred to cooked food by hands.

While a high proportion of raw chickens are contaminated with salmonellas the danger of salmonella food poisoning cannot be ruled out. While advice on efficient methods of thawing and cooking is a useful precautionary measure, more emphasis must be given to the danger of cross-contamination from raw to cooked birds by hands, surfaces, equipment and utensils and low temperature storage of cooked birds. Recommended label instructions are given in Fig. 1.

It is felt that efforts should be directed towards the provision of salmonella-free flocks, so that dressed poultry may be free from salmonellas before retail sale.

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An epidemiological study of strains of *Shigella sonnei* from two related outbreaks

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SUMMARY

During the winter of 1967–8 Sonne dysentery affected neighbouring North London primary schools. This was not simply due to cross-infection between the two schools, for two different, unusual strains of *Sh. sonnei* were distinguished. One was a novel kanamycin-resistant colicine type 7 strain, and as far as we know this was the first school outbreak due to such a strain to be documented. The other strain was kanamycin-sensitive and of colicine type 0 with a rare specific requirement for aspartic acid. There was some evidence to suggest that the kanamycin-resistant strain was more infective for adults and possibly more pathogenic than the kanamycin-sensitive.

Studies on the transfer of drug resistance and of colicinogeny revealed that the factor determining colicine type 7 was carried on a transmissible plasmid, a new observation. Various drug resistances were also transmissible experimentally, and some were spontaneously unstable. Non-transferable ampicillin-resistance in the colicine type 7 strain and aspartic acid dependence in the colicine type 0 strain enabled all but one of the isolates to be classified into two distinct lines. No common ancestor was found and it was concluded that although occurring together they must have arisen from separate sources.

INTRODUCTION

Strains of *Sh. sonnei* isolated from cases of dysentery at neighbouring primary schools differed initially in drug resistance and colicine type. This was unexpected since many families had children attending both schools. One of the strains was sensitive to kanamycin, while the other was kanamycin-resistant. Neomycin and kanamycin resistance was then rare (Davies, Farrant & Tomlinson, 1968*a*; Thomas & Datta, 1969). We report below our study of the epidemiology and bacteriology of the double outbreak.

EPIDEMIOLOGY

During November 1967 dysentery affected boys at a North London primary school. A kanamycin-resistant strain of *Shigella sonnei* of colicine type 7 was isolated from the cases. The building was old and washbasins were remote from

dirty and awkward playground lavatories. Strains of *Sh. sonnei* cultured from three sites in these toilets were kanamycin-sensitive and colicine type 0. Supervised hand-washing and dipping in 2% Hycolin after using the toilets and before eating dinner was arranged, and paper towels provided. The lavatories were ultimately replaced. Children who had been ill or absent during the autumn term were excluded from school and only readmitted after negative faeces cultures had been reported. Nevertheless, further cases arose in January. However, nearly all of these were kanamycin-sensitive infections of colicine type 0, and were secondary to infected contacts at a neighbouring mixed primary school. Altogether 28 cases of dysentery and 8 symptomless excretors were found during 3 months among the 200 pupils of the boys' school, 28 of these 36 infections were with kanamycin-resistant, colicine type 7 *Sh. sonnei*.

Early in the course of the boys' school outbreak a few siblings at the nearby mixed primary school for 400 children were infected with colicine type 7 kanamycin-resistant *Sh. sonnei*. In only one instance siblings, one at each school, excreted distinguishable strains, a boy having the kanamycin-resistant strain while his sister, who fell ill a week later, had a kanamycin-sensitive, colicine type 0 infection. During the Christmas holiday seven more children from the mixed school fell ill, also with a kanamycin-sensitive infection resistant only to sulphonamides.

In January this school too was visited and hand-washing and dipping and the prompt exclusion of suspects arranged, but plural cases continued to arise until late in February. A total of 19 cases and 9 excretors was recognized at the mixed school. Fifteen of these 28 infections were with kanamycin-sensitive, colicine type 0 strains and 13 with kanamycin-resistant, colicine type 7 strains. Nine of these 13 had a close contact at the boys' school. A single late case arose at the end of March, but this was probably unrelated since the *Sh. sonnei* strain isolated, although kanamycin-*sensitive* was of colicine type 7, and had other differences from either of the two outbreak strains.

METHODS

The first confirmed case of Sonne dysentery in any household was listed as the index case. Infected households discovered during the follow-up of contacts were also listed. Households were visited by a health inspector who recorded particulars of each member. Each person was asked to send a faecal sample to the laboratory before the start of treatment. Patients from whom shigellas were isolated were asked to send weekly specimens, starting three or more days after the end of any anti-bacterial treatment, until a negative result had been reported. The rest of the family were then re-examined. Most remained under surveillance until two or three negative specimens had been obtained from infected persons.

Faeces specimens were examined and *Sh. sonnei* identified by conventional methods (see below).

Household cross-infection rates, evidence of severe illness, bacteriological results of drug treatment and the duration of excretion of the two distinguishable strains of *Sh. sonnei* were compared, as far as the small numbers allowed.

RESULTS

Household infection

Information was available to study cross-infection in 46 households, in 31 of which the index case or excretor had a kanamycin-resistant *Sh. sonnei* infection. Altogether 65 % of households and 36 % of contacts were found infected. Table 1 shows that more households were found infected in the group of kanamycin-resistant infections, and that a higher proportion of adult contacts was infected by the kanamycin-resistant than by the kanamycin-sensitive strain. It also shows the usual higher susceptibility of children than of adults to infection.

Severity of illness

Nearly three-quarters of the ascertained *Sh. sonnei* infections among school-children were with symptoms and this was true for both kanamycin-sensitive and kanamycin-resistant strain infections. Naturally symptomless infections tend to escape diagnosis. One child with a kanamycin-resistant infection was admitted to hospital and family doctors in the district said that the kanamycin-resistant type of infection was more severe than the usual dysentery. Cellular stools were seen rather more often in kanamycin-resistant than in kanamycin-sensitive infections.

Treatment

Tetracycline, streptomycin, furazolidine and nalidixic acid were each used. Faeces were bacteriologically positive after treatment in about half the children regardless of the drug used.

Duration of infection

This was taken from the onset of diarrhoea to midway between the last positive and the first negative of a clearance series of faeces specimens. Seventeen children infected with the kanamycin-sensitive strain and 26 with the kanamycin-resistant strain were available for this comparison. No difference in the duration of infection was observed, the median being 24 days for both strains.

BACTERIOLOGY

The two school outbreaks were closely related epidemiologically and the characters by which strains were distinguished, colicinogeny and drug resistance, are known to be determinable by transmissible plasmids. We therefore investigated a sample of the strains in an attempt to find whether the difference between the *Sh. sonnei* found in the two schools could be explained simply by acquisition or loss of plasmids in a single bacterial host, or whether there really were two distinct epidemic strains.

Methods

Faeces were cultured on deoxycholate citrate agar before and after enrichment in selenite-F broth. A 30 μg. paper disk of kanamycin was placed upon each plate to enable rapid recognition of resistant strains. For sensitivity testing Oxoid 'Multodisks' were used on Oxoid D.S.T. agar with 4 % lysed horse blood. Kana-

Table 1. *Infection among household contacts according to* Sh. sonnei *strain and age group*

Shigella sonnei strain isolated	Households		Children		Adults 15+ years		Total	
	Exposed	Infected	Exposed	Infected	Exposed	Infected	Exposed	Infected
Kanamycin-sensitive	15	8 (53%)	33	18 (55%)	31	3 (9·7%)*	64	21 (32%)
Kanamycin-resistant	31	22 (71%)	56	27 (48%)	76	23 (40%)	132	50 (38%)

* $P < 0{\cdot}01$.

Table 2. *Characteristics of individual strains of* Sh. sonnei *studied*

Strain from patient no.	Pattern of drug resistance	Colicine type	Aspartic acid dependence	Characters transferred to *E. coli* K. 12
		Boys' school		
B1	A S K Su	7	–	S K Su col
B6	A S K Su	7	–	S K Su col
B11	A S K Su	7	–	S K Su col
B25a	A S K Su T	7	–	S K Su T col
B25b	A Su T	7	–	Su T col
		Mixed school		
M1	Su	0	+	Su
M6	Su	0	+	Su
M7	Su	0	+	Su
M20	A Su	7	–	Su col
M29	S Su	0	–	None

Note. B25a and b were variants of one isolate. B1, B6 and B11 were three strains studied among 40 of the same colicine type and resistance pattern. M1, M6 and M7 were three strains studied among 23 of the same colicine type and resistance pattern.

mycin (K), ampicillin (A), streptomycin (S), tetracycline (T), Chloramphenicol (C), sulphonamide (Su), nalidixic acid (Nal), and furazolidine (Fx) were tested.

Colicine testing was carried out by the method of Abbot & Graham (1961) and representative strains were sent to the Dysentery Reference Laboratory for confirmation.

Transfer of drug resistance and colicinogeny

Drug-resistant strains of *Sh. sonnei* were grown in mixed culture (Oxoid nutrient broth No. 2) with a nalidixic acid-resistant strain of *Escherichia coli* K 12, J 53–1, which requires proline and methionine for growth. The cultures were incubated overnight and subcultured on minimum salts agar (Clowes & Hayes, 1968) to which was added lactose, proline, methionine, nalidixic acid, and also the particular antibacterial drug for which transfer of resistance was under test. *Sh. sonnei* will *not* grow on this medium, and *E. coli* K 12 J 53–1 will grow *only* if it has acquired appropriate drug resistance. Colonies growing on these selective plates were purified and identified as *E. coli* K 12 J 53–1 by biochemical tests and their requirement for proline and methionine. They were tested for resistance to drugs listed above and for colicinogeny by using the set of *Sh. sonnei* colicine-typing indicators (Abbot & Graham, 1961).

Nutritional requirements

Strains of *Sh. sonnei* were tested for ability to grow on minimum salts agar with added glucose and nicotinic acid. Cultures unable to grow on this medium were tested on the same medium, further supplemented in turn with each known amino acid and a variety of vitamins, in order to define their nutritional requirements.

RESULTS

Drug resistance

Most strains from the boys' school were resistant to ampicillin (A) streptomycin (S), kanamycin (K) and sulphonamide (Su) and most strains from the mixed school were resistant only to Su. There were some exceptions.

Nutritional requirements

All strains tested showed the normal requirements of *Sh. sonnei* for nicotinic acid. Characteristic strains from the boys' school needed no other supplement and grew well on the minimum salts medium described. Typical strains from the mixed school, however, failed to grow on this medium unless aspartic acid was added and 300 μg./ml. was needed for optimal growth and no other amino acid would substitute for aspartic acid.

Colicinogeny

The characteristic boys' school strains were of colicine type 7, identified by colicinogenic activity against strain No. 17. Typical mixed school strains were of colicine type 0; that is, non-colicinogenic (Table 2). Late in the epidemic a boy excreted a colicine type 7 strain which was resistant to tetracycline (T) as well as to ASKSu, and from this isolate a variant arose spontaneously which was resistant

only to ASuT. Later still a strain of colicine type 7 which was resistant only to ASu was isolated from a girl at the mixed school.

Transfer

Characteristic boys' school strains of *Sh. sonnei* were able to transfer to *E. coli* K 12 resistance to SKSu and colicinogenic activity against indicator strain No. 17. Ampicillin resistance was never transferred. Tetracycline resistance, when it occurred, was transferable, as was sulphonamide resistance from every strain except the very last to be isolated in the outbreak (from M 29). This final strain had non-transmissible resistance to SSu, and unlike the other colicine type 0 strains its growth was independent of aspartic acid.

DISCUSSION

Two unusual strains of *Sh. sonnei* were isolated from cases of dysentery at two primary schools in one district during the same winter terms. Siblings attended both schools. The family doctors involved worked with the Public Health Department and the Public Health Laboratory to investigate and control the epidemic. Later the two strains of *Sh. sonnei* were compared epidemiologically and bacteriologically. A novel kanamycin-resistant colicine type 7 predominated in a boys' primary school, while most of the infections at a neighbouring mixed primary school were with a kanamycin-sensitive aspartic-acid-dependent colicine type 0. The kanamycin-resistant strain appeared to be more infective for adults, and was possibly more pathogenic than the concurrent kanamycin-sensitive strain. No difference was found in the duration of infection by the two strains.

The epidemiological markers differentiating the *Sh. sonnei* strains had practical importance. A strain of colicine type 7 was rife when the boys' school was inspected. Toilet facilities were thoroughly insanitary. Radical improvements were instituted during the autumn term and children who had been ill or absent were excluded until negative faeces cultures had been obtained from them. Nevertheless, dysentery recurred in the school in January, making it appear that control measures were ineffective. It was found, however, that at this stage most strains isolated from the boys' school were colicine type 0 and kanamycin-sensitive; that is, they were of the type prevailing at the mixed school. It seems probable that the control measures against the original outbreak had been effective within the boys' school, but that new introductions of *Sh. sonnei* by close contacts from the continuing outbreak at the mixed school were not prevented.

Our bacteriological studies indicate that the two epidemic strains of *Sh. sonnei* were distinct. Both were unusual, that originating at the boys' school in being kanamycin-resistant, and that arising at the mixed school in having a specific requirement for aspartic acid.

Colicine typing, described by Abbot & Shannon (1958) and Abbot & Graham (1961) is widely used for tracing *Sh. sonnei* epidemiologically, but for many years in Enfield a majority of strains have been non-colicinogenic, that is of type 0. Unusual antibiotic-resistance patterns may reveal a connexion between infections more quickly than colicine typing, which is commonly carried out in batches at

intervals. Davies, Farrant & Tomlinson (1968*b*) have shown that the colicine type and antibiotic resistance pattern of *Sh. sonnei* may change during the course of natural spread of infection in man. In the investigation described here our aim was to find whether it was possible by transfer of plasmids from the colicinogenic multiple-resistant *Sh. sonnei* prevailing at the boys' school, to convert that prevailing at the mixed school into an indistinguishable form.

We were able to transfer colicinogeny, determining colicine type 7, and also resistance to kanamycin, streptomycin and sulphonamides, from the 'boys' *Sh. sonnei* to *E. coli* K12, and thence to typical *Sh. sonnei* strains from the second school. But these transfers still left two 'labels' by which we could distinguish the strains, ampicillin resistance and aspartic acid dependence. The 'boys' strain was ampicillin-resistant, and that resistance was *not* transferable. Scrimgeour (1966) showed that strains of *Sh. sonnei* with a *high* level of ampicillin resistance (> 1000 g./ml.) could regularly transfer resistance, but that the lower level of ampicillin resistance (28–256 g./ml.) now commonly found in *Sh. sonnei* (Davies, Farrant & Uttley, 1970) was not transmissible. Scrimgeour pointed out the epidemiological value of this distinction.

The 'mixed school' strain was dependent upon aspartic acid for growth. Amino acid requirements are unusual in *Sh. sonnei* and when present are useful epidemiological markers (Davies *et al.* 1968*b*). Genetic information for amino acid independence can be carried on transmissible plasmids (Fredericq, 1969; Jacob & Adelberg, 1959), but we did not find aspartic acid dependence to be transferable between our strains of *Sh. sonnei* either directly or by way of *E. coli* K12.

We found that the colicine factor determining type 7 (Abbot & Graham, 1961) was carried on a transmissible plasmid, an observation not previously reported. However, in these outbreaks this colicine appeared to be as stable as was the non-transferable ampicillin resistance or aspartic acid dependence (Table 2). Patient M20 infected in the mixed school with a kanamycin-sensitive colicine type 7 *Sh. sonnei* was probably infected by a 'boys' strain, from which SK resistance had been lost. The atypical primary isolate from the boy (B25) was clearly an example of the acquisition of a transmissible factor determining tetracycline resistance. The subsequent spontaneous appearance in this culture of clones resistant only to ASuT showed that S and K resistance could be unstable. One isolate from a girl (M29) which arose a month after the outbreaks could not be allotted to either prevailing strain and we suggest that it was an unrelated sporadic infection. Using non-transmissible markers alone it was possible to allot all but this one of the strains studied to one or other of two parent lines. Although the first 'mixed school' colicine type 0 kanamycin-sensitive strain to be discovered came from a girl who developed dysentery just 8 days after her brother, who excreted the characteristic 'boys school' colicine type 7 kanamycin-resistant strain, the two strains had different stable epidemiological markers. We were unable to find bacteriological evidence for a common origin. Certainly after the first isolation of the 'mixed school' colicine type 0 strain there appeared to be two independent sources of infection in the double outbreak we have described, and we conclude that the initial differing infection in siblings is likely to have arisen by chance.

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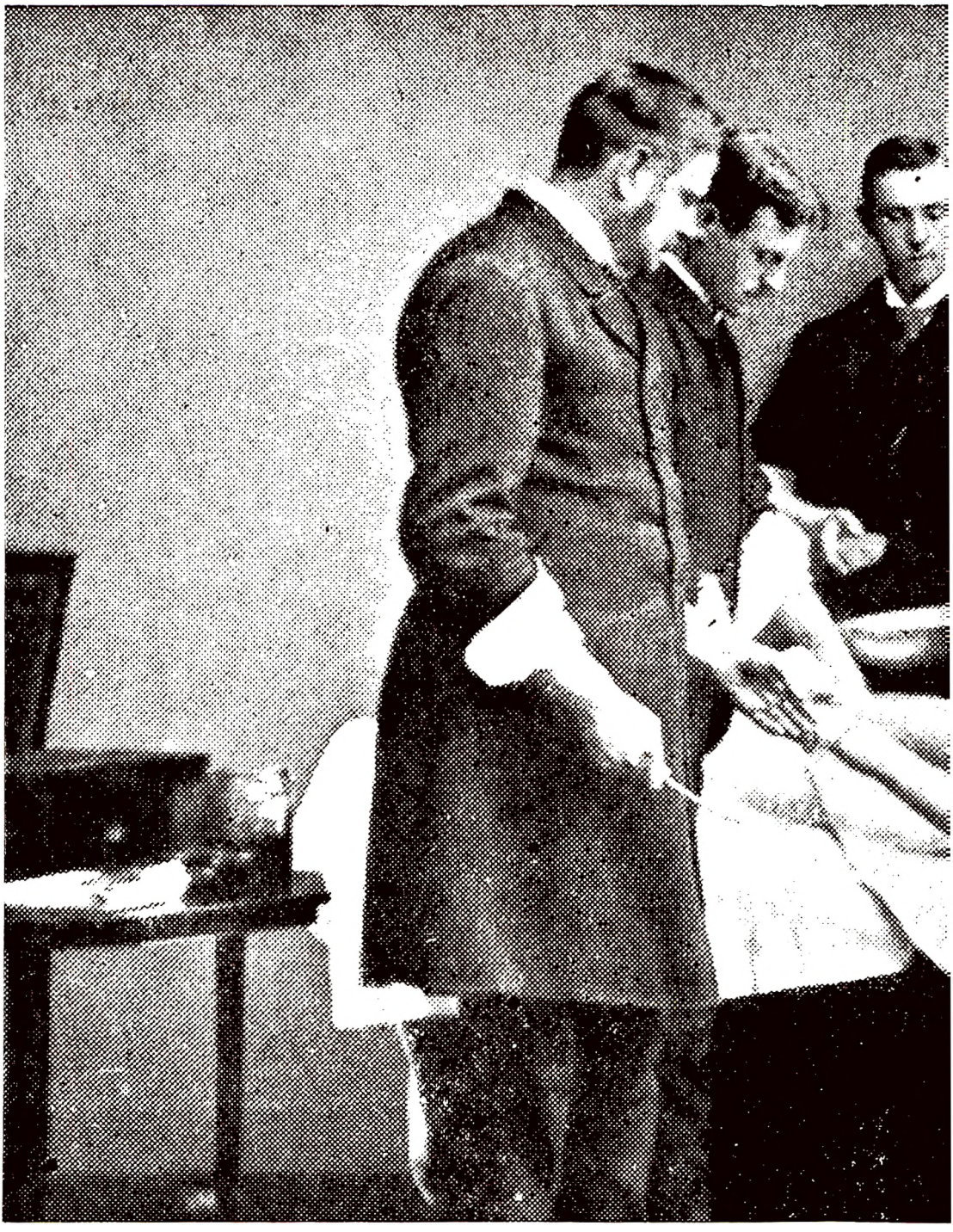

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